Occurrence of *Babesia rossi* in domestic dogs, black-backed jackals and African wild dogs in South Africa

by

Shabangu N

Submitted in fulfilment of the requirements for the degree of

Magister Scientiae in Veterinary Science, Department of Veterinary Tropical Diseases

Faculty of Veterinary Science

University of Pretoria

Supervisor: Prof Matjila P.T.

Co-supervisors: Prof Oosthuizen M.C. and Prof Penzhorn B. L

October 2018

Declaration

I hereby declare that this dissertation, which I submit for the fulfilment of the degree Master of Science in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, to be my own work and has not been submitted by me for degree purposes at any institution.

The Animal Ethics Committee of the University of Pretoria approved this study (reference number V031-17). Permission to conduct research in terms of the Section 20 of the Animal Diseases Act (1984) was granted by the Department of Agriculture, Forestry and Fisheries, South Africa (reference number: 12/11/1/1/6).

……………………….

Shabangu Ntji

October 2018

Acknowledgements

This dissertation benefited from many individuals, to whom I owe sincere gratitude. My greatest gratitude goes to Prof Matjila P.T., for being a great mentor and supervisor who guided me from the very first days of my experience in academia. I am grateful for his endless support and great insight into this project.

My sincere gratitude also goes to my co-supervisors, Prof Penzhorn B.L. and Prof Oosthuizen M.C., for their endless mentorship, support and contribution to this project. I am grateful to have been co-supervised by these "meticulous academic giants".

Ms Vorster I.: I am grateful for your endless efforts, patience and support, introducing me to various molecular biology techniques and your valuable encouragement during my laboratory work.

Dr van Schalkwyk L. and Mr Harrison-White R.: thank you for providing blood specimens from African wild dogs and black-backed jackals, respectively.

I would like to acknowledge the funding institutions that made this project possible: AgriSETA for research support grant, as well as the National Research Foundation (NRF) Scarce Skills scholarship. The World Association for the Advancement of Veterinary Parasitology African Foundation (WAAVP AF) partial scholarship and the Thermo-Scientific travel grant.

To my colleagues and friends at the DVTD, the Vector and Vector-borne Diseases Research Group thank you for your endless support and contribution to my growth.

To my dearest daughter, Kgaogelo Khathutshelo, you have been of great strength and the mighty phoenix to my endless efforts to make our lives better. Through your endurance, I survived. My father Mr P.P. Shabangu, your life philosophy has carried me daddy, "life is a tantalising mirage", the relentless pursuit of self-discovery; I am here because you lived and thank you for an honest life. My dearest mother "ngwana waka" Mosima, ke a leboga. Bokoko Ms Salphy Mogale, thank you for being a mother to my daughter while I was away studying. To my brother, Adv. Justice Shabangu. My sisters, Sesi Maria, Sesi Mamma le Sesi Nakedi Shabangu, thank you for your support. Ditlogo tsaka, Thabo Shabangu, Lebogang Shabangu, Thabang Shabangu. Mahlogonolo, "rakgolo" Thato and Thabang Shabangu and "my koko" Lethabo Mma mahlola, you have all been a waving reminder of peace and genuine love.

To Mr Mbambala E. and Mrs Mbambala A., thank you for your support during my internship at UNISA.

Finally, yet importantly, the giants that kept my sanity, waving hope through literature and story-telling: Dr Chinua Achebe, Dr Ben Okri, Prof Pumla Dineo Gcola and Dr Cindy Trimm. Thank you for keeping me sane in the challenges of my life and prospects of the future of my generation.

My greatest gratitude also goes to my mentors: Mr Shabangu M.E. and Ms Joemat R., for your contribution to my growth. To live a life of purpose and contribute selflessly to others. In addition, the young man I look up to "Mbele" Shabangu S., you are my role model.

God Almighty the author and finisher of my life, you have been faithful, and all glory goes to you, for the angels you have set on my path, "Thank you Father"

- *"Purpose is to enchant the human mind to a sense of its true kingdom"*

Ben Okri

"Rest in peace mama Winnie Nomzamo Madikezela-Mandela"

"We are the ones we have been waiting for"

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Symbols and Abbreviations

%	:	Percentage
µm	:	Micrometer
°C	:	Degree Celsius
µl	:	Microlitre
BLAST	:	Basic Local Alignment Search Tool
*BrEMA*1	:	*Babesia rossi* erythrocyte membrane antigen 1
DNA	:	Deoxyribonucleic acid
DVTD	:	Department of Veterinary Tropical Diseases
EDTA	:	Ethylenediaminetetraacetic acid
Fig	:	Figure
HDSS	:	Health and demographic surveillance system
IFAT	:	Indirect fluorescent antibody test
KNP	:	Kruger National Park
M	:	Molarity
MSc	:	Master of Science
MEGA	:	Molecular Evolutionary Genetics Analysis
ml	:	Millilitre
$NaHCO_3$	:	Sodium bicarbonate
NZG	:	National Zoological Gardens
OVAH	:	Onderstepoort Veterinary Academic Hospital

PCR	:	Polymerase chain reaction
pmol	:	Picomole
RLB	:	Reverse Line Blot
RNA	:	Ribonucleic acid
rRNA	:	Ribosomal RNA
SA	:	South Africa
SDS	:	Sodium dodecyl sulphate
SNP	:	Single nucleotide polymorphism
SSPE	:	Sodium chloride-sodium phosphate
UDG	:	Uracil DNA Glycosylase
UP	:	University of Pretoria

Dissertation summary

Occurrence of *Babesia rossi* in domestic dogs, black-backed jackals and African wild dogs in South Africa

Candidate: Shabangu Ntji

Supervisor: Prof Matjila P.T.

Co-supervisors: Prof Oosthuizen M.C. and Prof Penzhorn B.L.

Department: Veterinary Tropical Diseases

Degree: MSc in Veterinary Science

Numerous studies have reported on the occurrence of *Babesia rossi* in domestic dogs in South Africa, but information on the occurrence of *B. rossi* in free-ranging indigenous canids, e.g. African wild dogs and black-backed jackals, is meagre. In addition, it is also not known whether the *B. rossi* that occurs in subclinical indigenous wild canids is genetically the same as the one that occurs in susceptible domestic dogs. This study aimed at investigating the occurrence of *B. rossi* in apparently healthy free-ranging black-backed jackals (n = 77) from Mogales Gate Biodiversity Centre and a population of captive black-backed jackals (n = 25) from S.A. Lombard Nature Reserve. In addition, an apparently healthy free-ranging population of African wild dogs (n = 52) from Kruger National Park (KNP) and a few captive African wild dogs (n = 5) from the National Zoological Gardens (NZG) were investigated. Domestic dogs (n = 75) that were suspected to be suffering from babesiosis at the outpatient clinic of the Onderstepoort Veterinary Academic Hospital also formed part of the study. Results of the Reverse Line Blot hybridization assay revealed *B. rossi* to be present in 66 domestic dogs (88%), in 5 (10%) free-ranging African wild dogs and 22 (29%) free-ranging black-backed jackals, and 7 (28%) captive black-backed jackals.

Phylogenetic analysis of the near full-length 18S rRNA gene sequence confirmed the occurrence of *B. rossi* in black-backed jackals and domestic dogs. This study confirms that *B. rossi* occurs freely in domestic dogs and black-backed jackals, and is therefore not host-specific. Elucidation of *B. rossi* in African wild dogs through molecular characterisation of the

18S rRNA gene was not successful; on the contrary, sequence analysis confirmed *Hepatozoon canis* DNA in *B. rossi*-positive specimens, affirming mixed infections. RLB also detected mixed infection in domestic dogs and black-backed jackals. Simultaneous detections with the *Theileria* / *Babesia*-group specific, *Babesia* genus-specific 1, *Babesia* genus-specific 2 and *Babesia rossi* probe were detected in individual domestic dog specimens. In black-backed jackal specimens, the highest multiple detections were with the *Ehrlichia* / *Anaplasma* group-specific probe together with the *Babesia* genus-specific 1 probe. In African wild dog spe cimens simultaneous detection on individual specimen were observed with the *Babesia* genus 1 and *Babesia* genus 2 probes. This study was able to indicate that wild canids and domestic dogs share similar tick-borne parasites of importance. This highlights the importance of tick control, especially in domestic dogs as they are more susceptible to these tick-borne pathogens that are less problematic to wild canids.

CHAPTER 1

1. General introduction

1.1. Rationale

Canine babesiosis caused by *Babesia rossi* has been reported to cause morbidity and mortality in domestic dogs in South Africa (Schoeman, 2009). Indigenous wild canids, such as black-backed jackals (*Canis mesomelas*) and African wild dogs (*Lycaon pictus*), were postulated to be natural reservoir hosts of *B. rossi*, since *B. rossi* and its proven vector *Haemaphysalis elliptica* only occur in sub-Saharan Africa (Penzhorn, 2011). *Babesia rossi* DNA has been confirmed in free-ranging and captive black-backed jackal populations through molecular characterisation of the 18S rRNA gene (Penzhorn et al., 2017). Regardless of the virulent nature of *B. rossi* previously reported in domestic dogs suffering from babesiosis (Matjila et al., 2009), sub-inoculation of blood from dogs into indigenous canids such as African wild dogs and black-backed jackals, results in subclinical infection (Van Heerden et al., 1995).

Co-evolution with the tick vector and the pathogen probably contributed to indigenous wild canids coping better with *B. rossi* infections (Penzhorn, 2011). On the contrary, a breed of domestic dogs known as the Africanis type is not native to sub-Saharan Africa origin, its introduction is postulated to have been during the Pleistocene era, although several introductions are speculated and possible interbreeding with *Canis lupus lupister* (Mitchell, 2015). Domestic dogs presented at the Onderstepoort Veterinary Academic Hospital suffering from babesiosis induced by *B. rossi* are not of African origin. Most are breeds imported into South Africa from Europe, Asia and America (Penzhorn, 2011). Repeated tick exposure and vaccination gradually provide immunity to cope better with infection. However, this cannot be achieved without thorough understanding of the genetics of *B. rossi*. This study addresses the problem by investigating the occurrence and diversity of haemoparasites in domestic dogs, black-backed jackals and African wild dogs, with special focus on *B. rossi*, and further accessing the relationships between the *B. rossi* that is found in subclinical indigenous canids and susceptible domestic dogs. Future studies would unravel the parasite evolution by accessing the *B. rossi* wild type spill-over and spill-back patterns in domestic dogs, black-backed jackals and African wild dogs.

Other heamoparasites that sometimes play a role as important parasites of canids include parasites from the genus Hepatozoon. Hepatozoonosis has been reported in domestic dogs, black-backed jackals and African wild dogs in South Africa (Basson et al., 1971; Christopher, 1907; Matjila et al., 2008a; Porter, 1918; Wenyon, 1911). Infection occurs during an ingestion of an infected tick .The known tick vector for *H. canis* is *R. sanguineus*, which also transmits *E. canis* and *B. vogeli* in S.A (Baneth et al., 2001; McCully et al., 1975; Matjila et al., 2008a). *Hepatozoon canis* has no clinical significance in canine hosts although co infection of *H. canis* and *Babesia* contributes to a clinical syndrome in immune compromised hosts (McCully et al., 1975). It is evident that tick vectors harbours multiple pathogens, which results in multiple infections of host species. A wide range of tick-borne pathogens circulates in canine hosts in South Africa (Matjila et al., 2008b). A study conducted on ixodid ticks infesting on dogs correlates with infection reports from pathogens such as *Ehrilichia canis*, *Theileria* spp. dog and *Anaplasma phagocytophilum* (Horak, 1995; Matjila et al., 2008b; Kolo et al., 2016).

This study is very important in providing updated surveillance of haemoparasites in susceptible domestic dog hosts and subclinical indigenous canids, and further elucidate on the *B. rossi* found, which will provide understanding of disease pathogenesis, and inspire future ideas on possible means of control.

1.2. Objectives of the study

In view of the above, the main objectives of this study were to investigate (i) the occurrence and diversity of tick-borne haemoparasites present in domestic dogs, black-backed jackals and African wild dogs, and (ii) the molecular characterization of *B. rossi* using 18S rRNA gene sequence and phylogenetic analysis.

CHAPTER 2

2. Literature review

2.1. Introduction

Babesia is classified as a protozoan piroplasm parasite of the phylum Apicomplexa, family Babesiidae (Neitz, 1956). The first record of *Babesia* was made by Victor Babes (Babes, 1888) from infected erythrocytes of cattle in Romania. This parasite is currently known as *Babesia bovis.* Five years later *Babesia bigemina* was discovered in Texas, USA (Smith and Kilbourne, 1893). Historically, babesias of canines were traditionally grouped as either "small" or "large", known as the *Babesia gibsoni* and *Babesia canis* groups, respectively (Uilenberg, 2006).

The main focus of this study is on one of the "large" babesias of canines, *B. rossi,* which was described initially in Kenya, East Africa (Nuttall, 1910). Other large babesias described from dogs are *Babesia canis* and *Babesia vogeli,* which are similar morphologically, but differ on the basis of pathogenicity, geographical distribution and vector specificity (Carret et al., 1999).

Canine babesiosis in South Africa is caused by *B. vogeli* and *B. rossi*. Infection rates were reported to be 10% in private clinics; although the highest occurrence observed occurred in dogs at the outpatients clinic of the Onderstepoort Veterinary Academic Hospital (OVAH) in Gauteng Province, South Africa (Collett, 2000, Shakespeare, 1995). In the year 2000, dog owners spent approximately ZAR 20 million on treatment of canine babesiosis (Collett, 2000). Canine babesiosis remains a burden to domestic dogs. Further research is needed to understand the biology of the pathogen from its reservoir hosts such as wild canids and their susceptible counterparts, *viz.* domestic dogs, in order to address the control of canine babesiosis in the country.

2.2. Life cycle of *Babesia* species

Babesia spp. are primarily transmitted by engorging ticks. The parasite life cycle occurs in the tick vector and the vertebrate host (Fig. 1)

2.2.1. The development in the vertebrate host

The infectious phase to vertebrates starts when an infected tick attaches to a host. When the tick engorges, sporozoites deposited into the blood stream of the host with the saliva of the tick directly infect the erythrocytes (Chauvin et al., 2009). Within the erythrocytes, sporozoites develop into actively feeding and growing trophozoites. These become meronts, which undergo division to form daughter cells or merozoites. The merozoites are released and infect other erythrocytes. Destruction of erythrocytes leads to anaemia as well as free haemoglobin in the blood stream. The host's immune system responds by activating cells and producing antibodies (de Vos & Callow, 1987, Gray, 2008, Uilenberg, 2006). The distinction between large and small babesias that infect vertebrates is on the size of the merozoites. Merozoites of large babesias are larger than the erythrocyte radius, while small babesias have merozoites that are smaller than the erythrocyte radius.

In a host infected with *Babesia*, the cellular immune response is more important than the humoral immune response, as demonstrated by infection with *B. bovis* (Zintl et al., 2005). During acute and chronic infection, stimulation of Interlukin 8 (IL8) and Interlukin 12 (IL12) facilitates the production of Natural killer cells (NK), Interferon gamma (IFNγ) and nitric oxide (NO), which lead to destruction of the infected erythrocytes (Garcia et al., 2004, Goff et al., 2006, Shoda et al., 2000). Comparison of immune response to *B. bovis* in calves and adult cattle, respectively, revealed that the innate immune response by calves is facilitated by the early production of IFNγ and IL12 before Interlukin 10 (IL10); when IL10, IL12 and IFNγ are produced the T helper cell type 1 (Th1) response is activated, leading to a delayed response of NO production (Goff et al., 2001).

2.2.2. The development in the tick vector

Infection starts when engorging ticks ingest sporozoites with host blood. In the tick piroplasms develop into gametocytes, which undergo fusion, resulting in a zygote which eventually develops into kinetes (Mehlhorn and Schein, 1985). Kinetes penetrate the gut wall and enter the haemocoel where they migrate to various organs including the salivary glands and ovaries. In transovarial transmission, the resulting larvae are infected and potentially pass their infection to the next generation, unlike in Theileria where the infected adult does not pass the infection to the next generation (Uilenberg, 2006). Ticks owe their success as *Babesia* hosts to transovarial transmission, which is not the case in Apicomplexans such as *Plasmodium* which

are only preserved through horizontal transmissions (Chauvin et al., 2009). This enabled the ticks to co-evolve with the *Babesia* parasite, and it has developed mechanisms to preserve its fitness as demonstrated with *B. bovis* (Yokoyama et al., 2002). Theoretically speaking *Babesia* parasites are better evolved to lower virulence with their hosts (Lipsitch et al., 1995). Generalist ticks have a potential to spread *Babesia* to potentially new vertebrate hosts. Generalist ticks such as *Rhipicephalus microplus*, *Rhipicephalus annulata* and *Ixodes ricinus* have a wide host range, equivocally widen the spread of the pathogens their transmit such as *B. bovis*, *B. bigemina* and *B. divergens* and due to this successful expansion, new host ranges are eminent, which in turn would lead to divergence of Babesias to new species (Chauvin et al., 2009). Tick behavioural patterns influence the spread of disease and infects new hosts which contributes to the biology of pathogens.

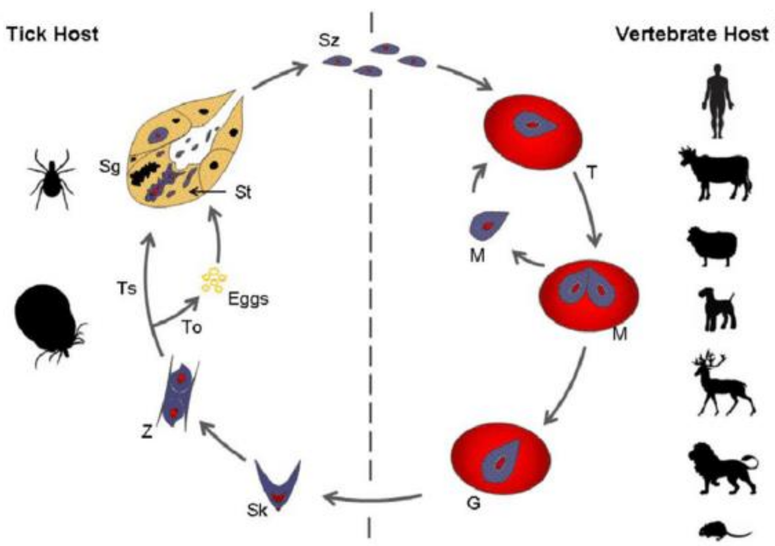

Figure 1: General schematic representation of *Babesia* life cycle in vector and hosts adopted from (Mehlhorn and Schein, 1985). *Babesia* sporozoites are represented by (Sz), which are injected into the bloodstream of a verterbrate host with saliva, during the blood meal of an infected tick. After invading the erythrocytes they differentiate into trophozoites (T), which undergo merogony (M) forming two or four merozoites. Merozoites exit the erythrocytes and invade new erythrocytes, continuing the replicate cycle in the host. A few merozoites stop dividing and form gamonts (G). Gamogony and sporogony takes place in the tick, when gamonts are taken up by a tick feeding on an infected host they divide in the gut into gametes also known as Strahlenkorper (SK) that fuse forming a diploid zygote (Z), which will undergo meiosis giving rise to motile tick organelles including salivary glands. Permission to reproduce this lifecycle was granted by Elsevier (Appendix 3)

2.3. *Babesia* of domestic dogs and wild canids in South Africa

Babesiosis of domestic dogs is frequently reported in South Africa. There are well-documented findings of infections caused by *B. rossi* in domestic dogs across the country, in hospitals and private clinics (Matjila et al., 2008b). There were reports of *B. rossi* parasite detections in 65% of in sick dogs admitted at the Onderstepoort Veterinary Academic Hospital (OVAH). A survey of apparently healthy dogs in Mpumalanga Province revealed a *B. rossi* detection rate in 97% of dogs. Sites closer to the coastal areas reported infection rates of 6% in KwaZulu-Natal, 22% and 4% in Eastern Cape and Western Cape, respectively (Matjila et al., 2008b).

Babesia vogeli has been reported in South Africa, although its prevalence is much lower than that of *B. rossi* (Matjila et al., 2004). Tick vectors of *B. rossi* and *B. vogeli* namely *Haemaphysalis elliptica* and *Rhipicephalus sanguineus* respectively ; were the most abundant ticks on dogs presented at the OVAH (Horak, 1995). Co-infections of *B. rossi* and *Ehrlichia canis* commonly occur in domestic dogs (Matjila et al., 2008c). Although *E. canis* and *B. vogeli* are transmitted by the same vector, *Rhipicephalus sanguineus*, co-infection with *B. rossi* and *B. vogeli* in domestic dogs was less frequent, than co-infection of *B. rossi* and *E. canis* (Matjila et al., 2004, Matjila et al., 2008b). This would imply that more *R. sanguineus* ticks are infected with *E. canis* than with *B. vogeli*.

Habitat fragmentation, habitat loss and diseases contribute to population declines of wildlife. When the Kruger National Park African wild dog population decreased sharply between 1990 and 1993, diseases induced by parasites were incriminated as 10% cause of the decline (Van Heerden, 1995). Subclinical infection with *B. rossi* and presence of its vector *H. elliptica* were reported in African wild dogs in the KNP (Van Heerden, 1995). Although there has not been any records of mortalities due to canine babesiosis, possible contact between free-ranging African wild dogs with other canine hosts of *B. rossi* such as domestic dogs in close proximity to the KNP occurs, van Heerden et al (1995), recommended constant vaccination of domestic dogs that live in close vicinity to KNP. An incidental finding of an African wild dog pup that died of babesiosis was reported from an enclosure in the Johannesburg Zoological Gardens (Colly and Nesbit, 1992). The National Zoological Gardens was said to have historically experienced an outbreak of babesiosis in 1976, but there had not been any records of fatal babesiosis in black-backed jackals (*Canis mesomelas*) (Colly and Nesbit, 1992). Based on molecular characterization and analysis of the 18S rRNA gene, black-backed jackals were postulated to be natural reservoirs host of *B. rossi* (Penzhorn et al., 2017). Blood of a domestic

dog suffering from severe babesiosis sub-inoculated into black-backed jackals resulted in the development of parasitaemia without clinical signs. However, sub-inoculation of blood from sub-clinically infected black-backed jackals caused severe babesiosis in domestic dogs (Neitz and Steyn, 1947, Van Heerden, 1980).

Canine babesiosis in clinics and hospitals across South Africa is usually reported to occur in recently introduced breed of domestic dogs (Penzhorn, 2011). Canine babesiosis mainly affects dogs in the urban areas more than dogs in the rural areas irrespective of high tick burdens and infestations in rural South Africa, where tick control is barely practised. It is postulated that Africanis-type dogs probably cope better with *B. rossi* infection due to natural selection over centuries or millennia (Penzhorn, 2011).

Although *Hepatozoon canis* is frequently seen on blood smears of dogs, clinical hepatozoonosis is not regarded as of major importance in South Africa. There are high infection rates of *H. canis* amongst populations of wild canids, with 93% occurrence reported in African wild dogs in 1995 (Van Heerden, 1995). *Hepatozoon canis* has also been reported from black-backed jackals in South Africa (Penzhorn et al., 2018).

2.4. "Large" *Babesia* species and their tick vectors

Babesia spp. are grouped as either large or small. Small babesias (trophozoites are 1.0-2.5µm) include species such as *Babesia gibsoni*, *B. microti* and *B. rodhaini.* These babesias are said to be closely related to *Theileria* species based on phylogenetic analysis of the nuclear small subunit-ribosomal RNA (Homer et al., 2000). This similarity is substantiated by the morphological characteristic of invasion, as these species together with the *Theileria* species are not transovarially transmitted in ticks.

Large *Babesia* spp. (trophozoites are 2.5-5.0 µm) include species such as *B. bigemina, B. major, B. motasi, B. caballi* and *B. canis*. The focus of this discussion will be the *B. canis* group, namely *B. canis, B. rossi* and *B. vogeli.*

Babesia canis which causes canine babesiosis of dogs in Europe (Cacciò et al., 2002) is transmitted by the tick vector *Dermacentor reticulatus* which is endemic in southern Europe (Estrada-Peña et al., 2004). Various reports have indicated that this vector is also found in north-western European countries such as Switzerland, Germany, Belgium and the Netherlands (Losson et al., 1999 , Nijhof et al., 2007, Zahler and Gothe, 1997). This species of *Babesia* has

been described in domestic dogs and wild canids, viz., coyotes (*Canis latrans*), wolves (*Canis lupus*), golden jackals (*Canis aureus*) and foxes (*Vulpes vulpes*) (Kakoma and Mehlhorn, 1994, Kuttler, 1988). A report of *B. canis* from Nigeria is suspect, since *D. reticulatus,* the only confirmed vector of *B. canis,* is presumed absent in Nigeria. One could speculate that this incidental finding could be a result of infection with *B. rossi* or *B. vogeli* (Gherman and Mihalca, 2017).

Babesia vogeli has a global distribution. It is transmitted by the cosmopolitan tick vector *R. sanguineus* (Cacciò et al., 2002, Matjila et al., 2004). The distribution of the vector is reported in Africa, America, Asia, Australia and Europe (Uilenberg, 2006). This species of *Babesia* has been reported from cats in Thailand (Simking et al., 2010). The pathogen is less pathogenic to dogs (Cacciò et al., 2002). The initial discovery of this pathogen made in South Africa was from dogs that were subclinically infected at the Onderstepoort Academic Hospital (OVAH), Bloemfontein, Durban, East London and Johannesburg (Matjila et al., 2004). *Babesia vogeli* is also recorded in Namibia, Sudan and Nigeria (Adamu et al., 2014, Oyamada et al., 2005, Penzhorn et al., 2016).

Babesia rossi, which is restricted to sub-Saharan Africa, was initially described from side-striped jackals (*Canis adustus*) in East Africa (Nutall, 1910). The tick vector responsible for transmission of this parasite is *H. elliptica,* which was previously synonymised with *Haemaphysalis leachi* (Apanaskevich et al., 2007). *Babesia rossi* has a wide distribution in South Africa and it occurs in almost all the provinces (Jacobson, 2006, Lobetti, 1998, Matjila et al., 2008c, Reyers et al., 1998). *Babesia rossi* has been reported from African wild dogs in the KNP (Van Heerden, 1995). Black-backed jackals were also reported to harbour *B. rossi* in Mogales Gate Biodiversity Centre and S.A. Lombard nature reserve (Penzhorn et al., 2017).

Wild canids were reported to be natural reservoir hosts of babesias (Penzhorn, 2011, Penzhorn et al., 2017). They play a role in the life cycle of the pathogens. *Babesia rossi* isolates from South Africa were historically reported to be highly pathogenic as compared to strains of *B. canis* in Italy as well as isolates of the same species in other African countries (Adamu et al., 2014, Nuttall and Hadwen, 1909). In Nigeria, the *B. rossi* isolates are said to be less virulent than the South African isolates. Furthermore, the difference in virulence is associated with specific genotypes (Adamu et al., 2014, Malatji, 2011, Matjila et al., 2009). It was reported that extinction of indigenous canids in the wild contributed to elimination of the wild type strain of *B. rossi* in Nigerian populations, since the pathogen is only maintained in the domestic dog

populations. This is in contrast to South Africa where there are still viable populations of wild canids that may contribute to the life cycle of this pathogen (Adamu et al., 2014). In every natural population without pathogen pollution, natural selection would possibly render the loss of virulence of the South African strain of *B. rossi*, similar to the situation in Nigeria (Daszak et al., 2000; Adamu et al., 2014)

2.5. *Babesia* genotyping

Babesia rossi has not been intensively studied in terms of genomics and proteomics. This pathogen is associated with poor prognosis in infected dogs because of the complicated clinical signs attributed to it (Matjila et al., 2009). Co-infection could also be a contributing factor since the hosts can become infected with multiple pathogens contributing to a clinical syndrome (Matjila et al., 2008b).

Various authors have studied the phylogeny of *Babesia* based on the 18S rRNA gene, which has proved to be a useful tool in the classification of various canine *Babesia* piroplasms to species level (Kjemtrup and Conrad, 2000, Matjila et al., 2008c). The classification of small *Babesias* from canines based on complete sequence analysis of the 18S rRNA gene revealed that *Babesia* species from Asia, Midwestern United States, California and Spain were three genotypically distinct *Babesia* species (Kjemtrup and Conrad, 2000). Criado-Formelio et al. (2003) studied the full sequence analysis of the 18S rRNA gene of the babesias, which revealed that the *B. microti* group, which includes *B. rodhaini, B. felis*, *B. leo* and *B. microti*-type, formed a monophyletic group. The second group included *Theileria*-like spp. from western USA, which was comprised of *B. conradae*. The third group was mainly made up of *Theileria* species that infect bovines. The fourth group was the large babesias (Kjemtrup and Conrad, 2006): *B. canis*, *B. rossi*, *B. vogeli*, *B. bigemina*, *B. divergens, B. odocoilei.* The fifth monophyletic group was babesias from ungulates, e.g., *B. bicornis.* The 18S rRNA gene has added important information in understanding the taxonomic position of many small and large piroplasm parasites, including those that affect canine hosts.

Biomarkers such as Glycosyl-phosphatidylinositol (GPI) anchors merozoite surface antigen gene as well as the *B. rossi* erythrocyte membrane antigen (BrEMA 1) gene are used for profiling of *Babesia* parasites (Carcy et al., 2006, Matjila et al., 2009). GPI-anchor merozoite surface antigen gene is said to aid in invasion of the host immune system as it serves in transporting proteins that function in cell adhesion, cell wall synthesis and cell surface

protection (Carcy et al., 2006). *Babesia rossi* Erythrocytes Membrane Antigen 1 gene, although not intensively studied, is postulated to be a potential marker for South African isolates of *B. rossi,* as it is associated with virulence and is unique to *B. rossi* (Matjila et al., 2009). When modelling such genes for genotyping, the information of the parasite survival, as well as fragments that are unique to the specific pathogen can aid in guiding which genes can be targeted for controlling the pathogen and generating vaccines.

2.6. Diagnostic methods for canine *Babesia* parasites

The isolation of infected erythrocytes with Percoll gradient can be used to enhance the recovery and detection of parasitized erythrocytes (Comazzi et al., 1999). Conventional methods of *Babesia* identification and classification such as microscopic examinations of Giemsa-stained blood smears show inconsistency, especially in cases of variations in levels of parasitaemia of chronically infected hosts. The indirect fluorescent antibody test (IFAT) and the enzyme-linked immunosorbent assay (ELISA) are sensitive and moderately specific tools for detection of antibodies to *Babesia* parasites in dogs as compared to stained blood smear investigation (Furuta et al., 2009, Yamane et al., 1993). The disadvantages of the methods of antibody detections for screening is that antibodies cannot always be detected in long-term carriers such as reservoir hosts despite the presence of parasitaemia (Kim et al., 2007).

Molecular diagnostic tools are reliable and widely used for the detection and characterization of blood parasite infection in the host and vectors (Birkenheuer et al., 2004, Inokuma et al., 2003). Advances in molecular methods such as Polymerase Chain Reaction (PCR) and Reverse Line Blot (RLB) hybridization assays are invaluable in detecting multiple pathogens of various species as well as identification of subspecies or species variants (Nagore et al., 2004a, Nagore et al., 2004b). These techniques make it possible to detect pathogens in wild animals or reservoir hosts whereby both mixed species and mixed genus infections may be present (Oosthuizen et al., 2008, Penzhorn et al., 2017).

Real-time PCR is another method that employs fluorescent labels to enable the continuous monitoring of amplicon formation in the reaction (Monis et al., 2005). It is useful in pathogen detection (Francino et al., 2006), gene expression and regulation (Follo et al., 2006) and allelic discriminations. This technique can be applied in *Babesia* parasite research.

Different chemistries are used with the technique, depending on the objective of the specific project and affordability. SYBR Green real-time PCR assay was previously used for discrimination of *B. rossi* genotypes, of BrEMA 1 gene (Malatji, 2011). This method is reliable, sensitive and specific. Taqman probe assay is also used in this technique. It is considered more reliable, sensitive and specific, since it targets a specific single-stranded segment of a gene with the use of oligonucleotide probes. During amplification the parasite load concurrently quantifies within the sample (Kim et al., 2007). Automated sequencing and phylogenetic sequence analysis have also made it possible to understand the evolutionary relationships of species from genes (Stevens et al., 2001).

CHAPTER 3

3. Materials and methods

3.1. Experimental design and methodology

A schematic presentation of the laboratory processes and analysis are shown in Figure 2. Details of the process are explained below.

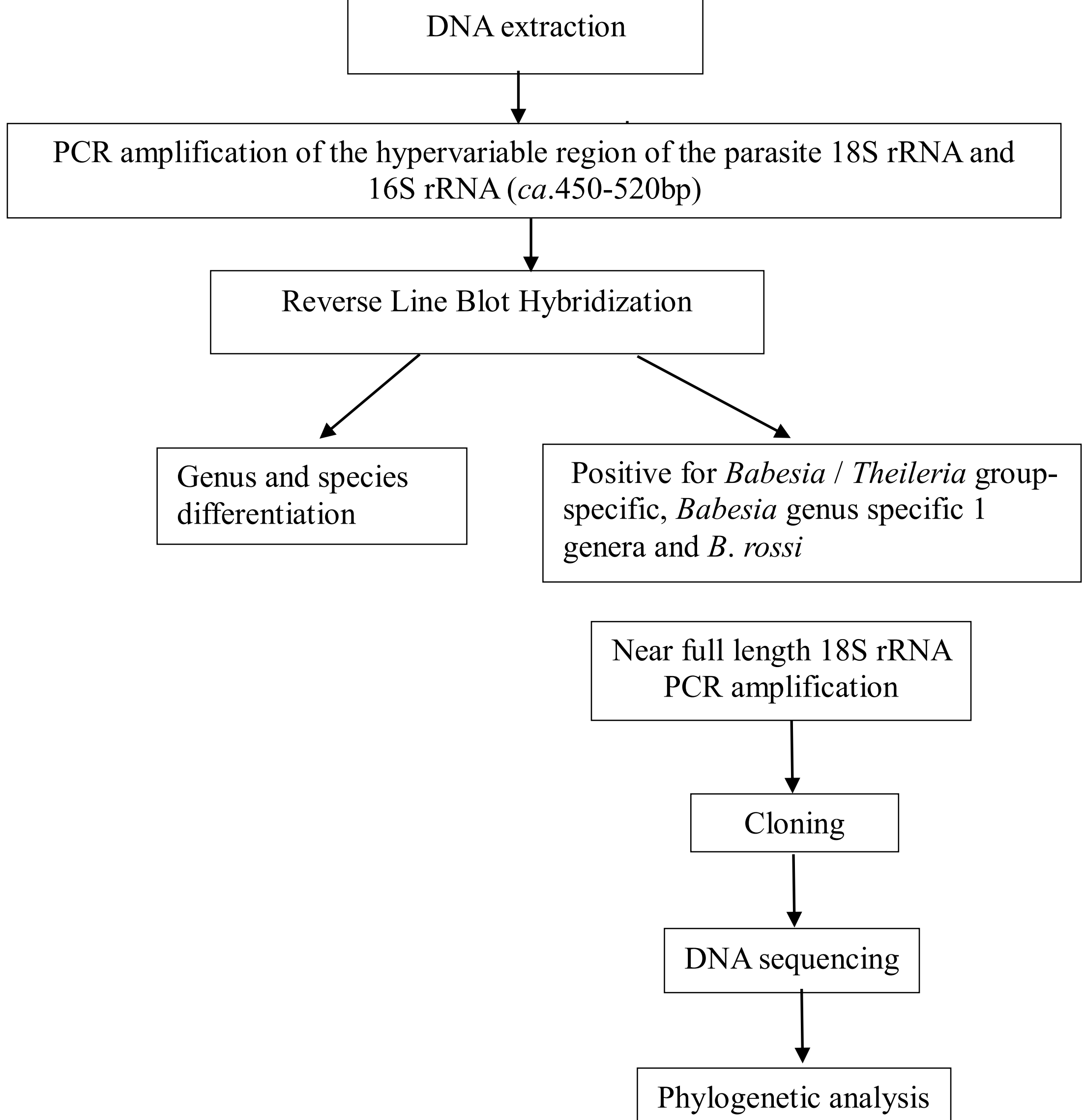

Figure 2: Workflow of laboratory processes and analysis

3.2. Ethical approval

The Animal Ethics Committee of the University of Pretoria approved this study (reference number v031-17). Permission to conduct this research in terms of Section 20 of the Animal Disease Act (1984) was granted by the Department of Agriculture, Forestry and Fisheries, South Africa (reference number: 12/11/1/1/6).

3.3. Sample Collection

Domestic dog blood specimens (n = 75) were initially collected in a previous study (Matjila et al., 2008b). These specimens were collected over a six-year period (2000 - 2006) from domestic dogs at the outpatient clinic of Onderstepoort Veterinary Academic Hospital (OVAH) (25 6478°S, 28 1806 °E), Faculty of Veterinary Science, University of Pretoria, South Africa. Blood was drawn directly from the cephalic vein and stored in EDTA-containing vacutainer tubes (Matjila et al., 2008c). About 200 µl of blood was aliquoted into 1.5 ml Eppendorf tubes and stored at -20°C until DNA was extracted.

Blood specimens from free-ranging black-backed jackals (n = 77) and captive black-backed jackals (n = 25) were collected by Mr R. Harrison-White, an associate of the North West Park and Tourism Board, as part of an ongoing study of radio-collared black-backed jackals. Specimen collection of captive black-backed jackals was done at S.A. Lombard Nature Reserve, 17 km northwest of Bloemhof (27 6263 °S, 25 5800°E), and the free-ranging black-backed jackal specimens were collected at the Mogales Gate Biodiversity Centre (25 9807°S, 27 6425°E), at the border between North West province and Gauteng province, in South Africa. Blood was collected in 2012 and 2014, respectively. Black-backed jackals were immobilized by intramuscular injection of a combination of Tiletamine and Zolazepam (Zoletil®, Virbac Animal Health, Halfway House, South Africa). Blood was collected into ethylenediamine-tetra-acetic acid (EDTA) vacutainer tubes from the cephalic vein of the jackals (Penzhorn et al., 2017)

African wild dog blood specimens were collected from a free-ranging population in the Kruger National Park (KNP), South Africa (23 594985°S, 31 385098°E). Dr L. van Schalkwyk, a state veterinarian at Skukuza, coordinated sampling. Blood specimens (n = 52) were collected into vacutainer R tubes (Franklin Lakes, USA) from cephalic veins of apparently healthy African wild dogs. The specimens were kept refrigerated until transferred to the Department of

Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, South Africa. Additionally, 6 blood specimens were received from the National Zoological Gardens, in Pretoria, South Africa (25 441079°S, 28 111260°E) for routine screening of a captive population of apparently healthy African wild dogs.

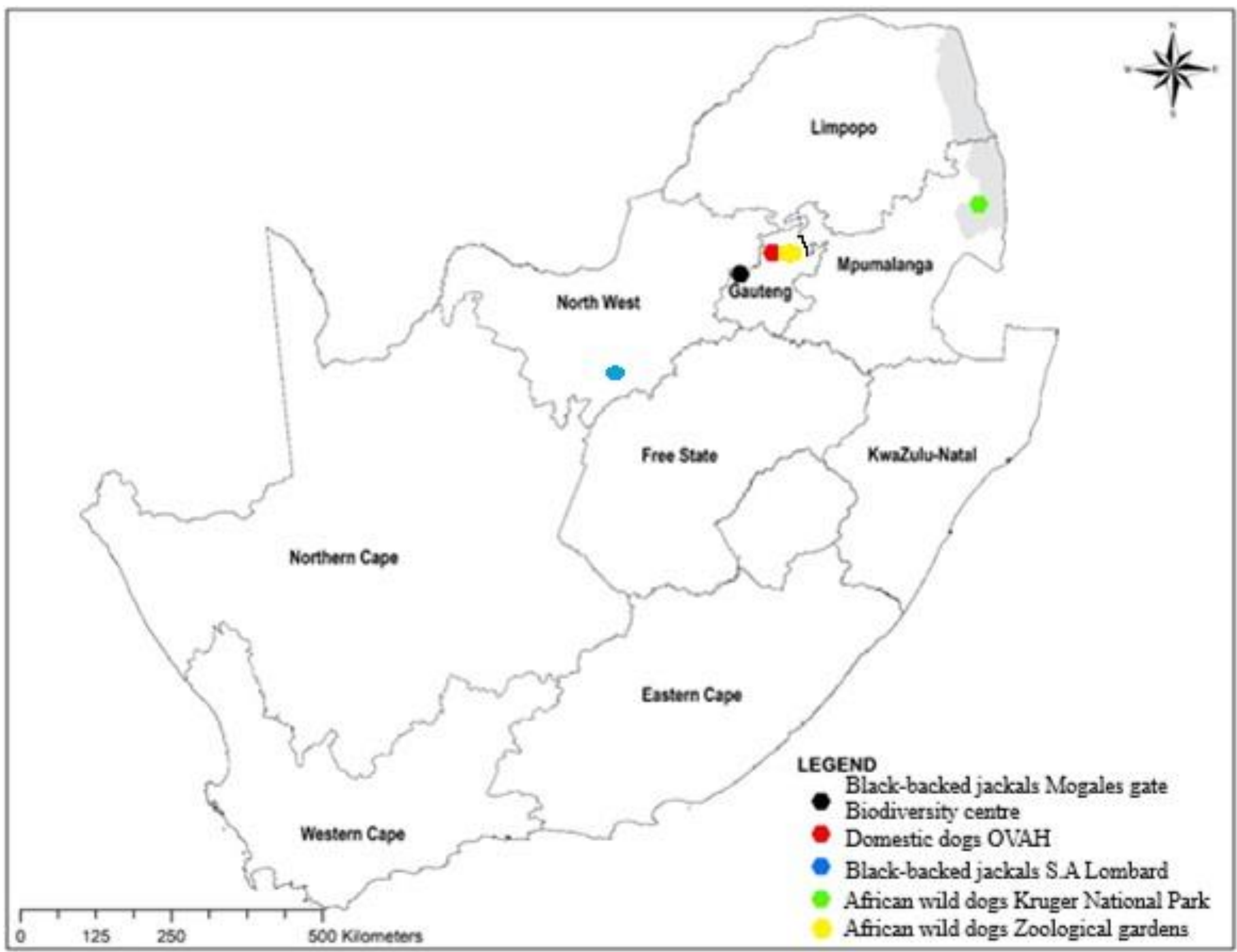

Figure 3: Map of South Africa, showing the locations where samples were collected across three provinces: black-backed jackal specimens at Mogales Gate Biodiversity Centre (indicated by black marker), domestic dog specimens at Onderstepoort Veterinary Academic Hospital (indicated by red marker), black-backed jackal specimens at S.A. Lombard Nature Reserve

(indicated by a blue marker), African wild dog specimens at Kruger National Park (indicated by a lime marker) and the National Zoological Gardens (indicated by a yellow marker)

3.3.1. Reverse Line Blot hybridization assay

The Reverse Line Blot (RLB) hybridization assay was conducted as previously described by Gubbels et al. (1999). *Babesia bovis* and *Anaplasma centrale* PCR amplicons derived from blood vaccines were used as positive control to ensure that all genus and species-specific probes were correctly bound to the RLB membrane and that they were functional.

3.4. DNA extraction

Genomic DNA was extracted from 200 µl of EDTA anticoagulated whole blood (stored at -20°C) using the QIAamp®DNA mini kit (Qiagen, Southern Cross, Biotechnology Pty Ltd, Hilden, Germany), following the manufacturer's instructions: 20µl of Proteinase K was added to 200µl of whole blood, and 200µl of buffer AL was also added to ensure effective lysis. The mixture was incubated at 56ºC for 10 minutes. After incubation, 200µl of ethanol (96-100%) was added, and then the mixture was applied to a 2ml QiAamp mini-spin column. Washing buffer AW1 was added for the initial wash phase followed by buffer AW2. The resulting DNA was eluted using buffer AE and preserved at -20ºC until further use.

3.5. Polymerase Chain Reaction (PCR) in preparation for the Reverse Line Blot (RLB) hybridization assay

Theileria and *Babesia* genus-specific primers RLB-F (5'-GAC ACA GGG TAG TGA CAA G-3') and RLB-R (biotin-5'-CTA AGA ATT TCA CCT CTG ACA GT-3') were used to amplify the V4 hypervariable region (~ 450-520 bp) of the parasite 18S rRNA gene as previously described (Gubbels et al., 1999, Matjila et al., 2004). Similarly, *Ehrlichia* and *Anaplasma* genus-specific primers PCR Ehr-F (5'-GGA ATT CAG AGT TGG ATC MTG GYT CAG-3') and Ehr-R (biotin 5'-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT-3'), were used to amplify the V1 variable region (~ 460-520 bp) of the parasite 16S rRNA gene (Bekker et al., 2002, Nijhof et al., 2005). A 25 µl PCR reaction mixture was prepared, comprising of final concentration of 1X of platinum® Quantitative PCR SuperMix-UDG (LTC Tech SA, Johannesburg, SA), 0.32 µM of each primer, 5 µl of genomic DNA and 7 µl of ultra-pure water. DNA extracted from *Babesia bovis* and *Anaplasma centrale* blood vaccines (Onderstepoort Biological Products, Pretoria, South Africa) was used as a positive control, and

ultra-pure water as a negative control. A touchdown PCR thermocycler program (Table. 1) was applied for amplification under stringent conditions according to the methods described by (Nijhof et al., 2005). The Gene Amp®PCR system 9700 (Applied Biosystems, South Africa) was used for amplification. After amplification, 2 µl of the PCR products were loaded onto a 2% agarose gel containing 10 mg/ml of ethidium bromide and ran at 400 A, 120 V for 25 minutes to verify amplification.

Table 1: Thermocycler program for *Ehrlichia* / *Anaplasma* and *Babesia* / *Theileria* touchdown PCR

No of cycles	Duration	Temperature in °C	
1	2 min	42°C	Activation of UDG
1	10 min	94°C	Inactivation of UDG & activation of *Taq.*
2	20 min	94°C	Denaturation
	30 sec	67°C	Annealing
	30 sec	72°C	Elongation
2	20 min	94°C	Denaturation
	30 sec	65°C	Annealing
	30 sec	72°C	Elongation
2	20 sec	94°C	Denaturation
	30 sec	63°C	Annealing
	30 sec	72°C	Elongation
2	20 sec	94°C	Denaturation
	30 sec	61°C	Annealing
	30 sec	72°C	Elongation
2	20 sec	94°C	Elongation
	30 sec	59°C	Annealing
	30 sec	72°C	Elongation
40	20 sec	94°C	Denaturation
	30 sec	57°C	Annealing
	30 sec	72°C	Elongation
2	10 min	65°C	Final elongation

3.5.1. Reverse Line Blot hybridization assay

The Reverse Line Blot (RLB) hybridization assay was conducted as previously described by Gubbels et al. (1999). *Babesia bovis* and *Anaplasma centrale* PCR amplicons derived from DNA extracted from blood vaccines of *B. bovis* and *Anaplasma centrale* were used as positive control to ensure that all genus and species-specific probes were correctly bound to the RLB membrane and that they were functional.

3.5.2. Preparation of the membrane

The genus and species-specific oligonucleotides (Table 2) were diluted in 142 µl of 0.5 M $NaHCO_3$ (pH 8.4). The Biodyne®C membrane (Pall Biosupport, Ann Arbor, USA) was marked for direction of probe application and incubated for 10 minutes in 10 ml freshly prepared 16% EDTA buffer at room temperature. It was then washed for 2 minutes with distilled water. The species-specific oligonucleotides probes were linked to the membrane by loading them on the MN45 miniblotter apparatus (Immunetics, Cambridge, UK), followed by 2 minutes incubation at room temperature. The probe solution was aspirated in the same order as applied. Then the membrane was further inactivated by incubation in 100 ml of 100 mM NaOH solution for 8 minutes at room temperature, followed by washing with 100 ml of 2X SSPE / 0.1% sodium dodecyl sulphate (SDS) solution for 5 minutes at 60°C.

3.5.3. Reverse Line Blot hybridization

The membrane was incubated at room temperature for 5 minutes under gentle shaking (Heidolph Duomax 1030) with 50 ml of 2X SSPE / 0.1 SDS buffer. The amplified PCR products of *Theileria / Babesia* and *Ehrlichia / Anaplasma* for each specimen, were pooled, and further diluted to an end volume of 180 µl using the 2X SSPE / 0.1 SDS buffer. The solution was denatured for 10 minutes at 99.9°C in a thermal cycler machine, Gene Amp®PCR system 9700 (Applied Biosystems, South Africa), and cooled on ice immediately for 2 minutes. The membrane was placed on the MN45 miniblotter apparatus with slots perpendicular to line patterns of the applied probes. PCR products were loaded onto the membrane, with empty slots filled with 2X SSPE / 0.1% SDS buffer to avoid cross flow. The MN45 mini blotter apparatus was incubated for 60 minutes on a horizontal surface at 42°C for hybridization to occur. The products were removed by aspiration in the same order they were loaded on the membrane. The membrane was washed twice in preheated 2X SSPE / 0.5% SDS for 10 minutes at 50°C in the incubator (Labcon) with a shaking platform. After washing, the membrane was further

incubated with 10 ml of preheated 2X SSPE / 0.5% SDS and 12.5 µl streptavidin-POD (peroxidase-labelled) conjugate (1.25 U) for 30 minutes at 42°C under gentle shaking (Labnet Rocker 25). The membrane was then washed twice with preheated 2X SSPE / 0.5% SDS for 10 minutes at 42°C incubation under gentle shaking. Further washed twice at room temperature for 5 minutes under gentle shaking (Heidolph Duomax 1030). After washing, the membrane was incubated for 1 minute in 6 ml of chemiluminescent detection fluid (ECL) to develop a probe-PCR amplicon streptavidin (DNA Thunder™, Perkin Elmer, Separation Scientific, South Africa) complex to enable visualization on the X-ray film (X-OMAT™Blue XB-1, Kodak, Separation Scientific, South Africa). The ECL was removed from the membrane, placed between two overhead sheets, and inserted in the exposure cassette. The X-ray film was exposed for a maximum of 4 seconds, then developed in the liquid developer and fixer, and rinsed with tap water respectively for detection of hybridized PCR products in the dark room. The film was placed in a grid and each sample lane correlated with the DNA probes used.

After use, the PCR products were stripped off the membrane with two washes for 30 minutes each time at 80°C in 1% SDS solution. The membrane was further rinsed in 20 mM EDTA (pH 8.0) for 15 minutes and then stored in fresh 20 mM EDTA solution for re-use (Gubbels et al., 1999).

Table 2: The list of oligonucleotide probes and their sequences used for detecting pathogen DNA with PCR-RLB hybridization. R=A / G, W=A / T are the symbols used to indicate degenerate positions

Oligonucleotide Probes	Sequences (5'-3')	Reference
Ehrlichia / *Anaplasma* genus-specific	GGG GGA AAG ATT TAT CGC TA	Bekker et al., 2002
Anaplasma bovis	GTA GCT TGC TAT GRG AAC A	Bekker et al., 2002
Anaplasma centrale	TCG AAC GGA CCA TAC GC	RLB Manual, Isogen
Anaplasma marginale	GAC CGT ATA CGC AGC TTG	Bekker et al., 2002
Anaplasma sp. Omatjenne	CGG ATT TTT ATC ATA GCT TGC	Bekker et al., 2002
Anaplasma phagocytophilum	TTG CTA TAA AGA ATA ATT AGT GG	Bekker et al., 2002
Ehrlichia canis	TCT GGC TAT AGG AAA TTG TTA	Bekker et al., 2002
Ehrlichia chaffeensis	ACC TTT TGG TTA TAA ATA ATT GTT	RLB Manual, Isogen
Ehrlichia ruminantium	AGT ATC TGT TAG TGG CAG	RLB Manual, Isogen
Theileria / *Babesia* genus-specific	TTA TGG TTA ATA GGA RCR GTT G	Gubbels et al., 1999
Theileria genus- specific 1	ATT AGA GTG CTC AAA GCA GGC	Nijhof (Unpublished)
Theileria annae	CCG AAC GTA ATT TTA TTG ATT TG	Matjila et al., 2008a
Theileria annulata	CCT CTG GGG TCT GTG CA	Georges et al., 2001
Theileria buffeli	GGC TTA TTT CGG WTT GAT TTT	Gubbels et al., 1999
Theileria bicornis	GCG TTG TGG CTT TTT TCT G	Nijhof et al., 2003
Theileria sp. (Buffalo)	CAG ACG GAG TTT ACT TTG T	Oura et al., 2004

Theileria equi	TTC GTT GAC TGC GYT TGG	Butler et al., 2008
Theileria sp. (Kudu)	CTG CAT TGT TTC TTT CCT TTG	Nijhof et al., 2005
Theileria mutans	CTT GCG TCT CCG AAT GTT	Gubbels et al., 1999
Theileria ovis	TTG CTT TTG CTC CTT TAC GAG	Altay et al., 2007
Theileria parva	GGA CGG AGT TCG CTT TG	Nijhof et al., 2003
Theileria sp. (Sable)	GCT GCA TTG CCT TTT CTC C	Nijhof et al., 2005)
Theileria separata	GGT CGT GGT TTT CCT CGT	Schnittger et al., 2004
Theileria taurotragi	TCT TGG CAC GTG GCT TTT	Gubbels et al., 1999
Theileria velifera	CCT ATT CTC CTT TAC GAG T	Gubbels et al., 1999
Babesia genus-specific 1	ATT AGA GTG TTT CAA GCA GAC	Nijhof (Unpublished)
Babesia genus-specific 2	ACT AGA GTG TTT CAA ACA GGC	Nijhof (Unpublished)
Babesia bicornis	TTG GTA AAT CGC CTT GGT C	Nijhof et al., 2003
Babesia bigemina	CGT TTT TTC CCT TTT GTT GG	Gubbels et al., 1999
Babesia bovis	CAG GTT TCG CCT GTA TAA TTG AG	Gubbels et al., 1999
Babesia caballi	GTG TTT ATC GCA GAC TTT TGT	Butler et al., 2008
Babesia canis	TGC GTT GAC CGT TTG AC	Matjila et al., 2004
Babesia gibsoni	CGT TTT TTC TTT GTT GG	Nijhof et al., 2003
Babesia felis	TTA TGC GTT TTC CGA CTG GC	Bosman et al., 2007
Babesia leo	ATC TTG TTG CCT TGC AGC T	Bosman et al., 2007
Babesia major	TCC GAC TTT GGT TGG TGT	Georges et al., 2001
Babesia microti	GRC TTG GCA TCW TCT GGA	Nijhof et al., 2003

Babesia rossi	CGG TTT GTT GCC TTT GTG	Matjila et al., 2004
Babesia sp. (Sable)	GCT GCA TTG CCT TTT CTC C	Oosthuizen et al., 2008
Babesia vogeli	AGC GTG TTC GAG TTT GCC	Matjila et al., 2004

3.6. Near full length 18S rRNA gene amplification

The near full-length *B. rossi* 18S rRNA gene from randomly selected domestic dog, African wild dog and black-backed jackal specimens was amplified using primers Nbab-1F (5'-AAG CCA TGC ATG TCT AAG TAT AAG CTT TT-3') and TB-R (5'-AAT AAT TCA CCG GAT CAC TCG-3') (Matjila et al., 2008a, Oosthuizen et al., 2008). Twelve specimens that showed simultaneous positive signal with *Babesia* / *Theileria* group specific, *Babesia* genus specific 1 and *B. rossi* probes in domestic dogs (5), black-backed jackals (4) and African wild dogs (2) specimens were selected for sequencing. However, an attempt to sequence 6 specimens per host was unsuccessful, the resulting sequences were too short to assemble, even when including internal region.

Five separate reactions were prepared per specimen. Phusion Flash High-Fidelity *Taq* master mix was used according to the manufacturer's instructions (Thermo Scientific™ South Africa, Pty Ltd). The 25 µl reaction comprised of 1X Phusion Flash High-Fidelity master mix, 0.2 µM of Nbab-1F and TB-R primers, respectively and 7.5 µl of nuclease-free water per reaction and 5 µl of extracted DNA per specimen. *B. rossi* plasmid DNA, isolated from a confirmed *B. rossi* positive specimen (RE 16 / 016) was used as a positive control and nuclease-free water was used as a negative control. The Gene Amp®PCR system 9700 (Applied Biosystems, South Africa) was used for amplification. The amplification was done under stringent conditions as follows: the initial denaturation was performed at 94°C for 2 minutes and 35 cycles of 94°C for 30 seconds, 58°C for 1 minute, 72°C for 5 minutes and final extension at 72°C for 15 minutes, the products were further preserved at 4°C.

PCR amplicons were further visualized on a 2% agarose gel electrophoresis. A volume of 5 µl of amplicon and 2 µl of loading dye were mixed and loaded on the gel, a 100 bp plus gene ruler (Thermo Scientific™, LTC Tech South Africa Pty Ltd) was used. The gel was immersed in TAE buffer and subjected to 120 V and 400 A of current for 35 minutes.

3.6.1. PCR product purification

Amplicons of all five reactions per specimen were pooled to avoid *Taq* polymerase-induced errors and cleaned using High pure PCR product purification kit (Roche Diagnostic, Mannheim, Germany) following the manufacturer's instructions: Amplified PCR product of

the adjusted volume of 100µl per specimen was mixed with 500µl of binding buffer. The solution was centrifuged, before it was transferred to a collection tube and washed twice. The first wash was done with 500µl of the wash buffer, and the second wash with 200µl. The purified PCR product was eluted using 50µl of the elution buffer and preserved at -20ºC until further analysis. The PCR product purification was carried out to remove un-used primers, nucleotides, enzymes and other impurities that might interfere with subsequent analysis. The purified PCR products were eluted in 50 µl of elution buffer and stored at -20°C until further analysis.

3.6.2. Cloning of the near full length 18S rRNA gene

Cloning was done using the CloneJET PCR cloning kit (Thermo Scientific^{TM}, LTC Tech South Africa Pty Ltd) following the manufacturer's instructions with modifications in incubation period and temperature during the ligation reaction. The vector used was the pJET1.2/blunt, with the T4 ligase and 2X reaction buffer. The 10 µl reaction mix was comprised of 5 µl of 2X reaction buffer, 3 µl of purified PCR product, 1 µl of pJET cloning vector and 1 µl of T4 ligase. Ligation was achieved through incubation at 4°C overnight. The *E. coli* JM109 competent cells (JM109 High Efficiency competent cells, Promega, USA) were used for transformation. Transformation involved thawing of the JM109 competent cells on ice, with 2 µl of ligation reaction, 50 µl of JM109 competent cells, this was followed by incubation on ice for 2 minutes and heat shocking at 42°C for 45 seconds using a thermocycler (GeneAmp®PCR system 9700 Applied Biosystems, South Africa). The reaction was transferred to a 2 ml tube of SOC medium (Thermo Scientific^{TM}, LTC Tech South Africa Pty Ltd) and further incubated at 37°C for 1.5 hours under shaking conditions. After incubation the reaction was centrifuged for 1 minute at 8 000 rpm, 150 µl of the supernatant was removed and the pellet re-suspended and plated out onto ImMedia AMP Agar plates (Invitrogen, USA) in 100 µl and 50 µl respectively. The plates were incubated overnight at 37°C.

Colonies were randomly selected and tested for correct insert by PCR using the Nbab-1F and TB-R primers. Colonies that had the right DNA insert were placed into ImMedia AMP liquid broth (Invitrogen, USA) and incubated at 37°C overnight in a shaking incubator (at 150 rpm). Following the incubation step, the recombinant plasmid was isolated using High Pure Plasmid Isolation Kit (Roche Diagnostics, Germany) following manufacturer's instructions. The DNA insert was amplified using Phusion flash high fidelity master mix (Thermo ScientificTM, LTC Tech South Africa Pty Ltd).

3.6.3. DNA sequencing and phylogenetic analysis

Sequencing was performed using the ABI Big Dye™ Terminator cycle sequencing ready reaction kit (PE Applied Biosystems), with a reaction mix of 350 ng plasmid DNA and 3.2 pmol of the vector primers pJET1.2 forward (5'-CGA CTC ACT ATA GGG AGA GCG GC-3' and pJET1.2 reverse (5'-AAG AAC ATC GAT TTT CCA TGG CAG-3'). An ABI3100 genetic analyzer located at Inqaba Biotec (South Africa) sequencing facility was used to analyze the purified products.

The generated sequences were assembled and edited using CLC genomics work bench version 7.5.1 (CLC Bio, MA, Boston, USA) and further identified by comparison with the GenBank database by homology searches made at the National Center for Biotechnology Information (NCBI) through BLASTn (Altschul et al., 1990). A multiple sequence alignment was performed using Multiple Alignment with Fast Fourier Transform (MAFFT) [version 7] program (Katoh and Standley, 2013). The alignment was manually truncated to size of the smallest sequence *ca* 1500 bp , using Bio-Edit version 7.2.5 (Hall, 1999). Phylogenetic trees were constructed with Maximum likelihood methods as implemented in the Molecular Evolutionary Genetics Analysis version 7 (MEGA 7) software package (Tamura et al., 2013). The evolutionary history was inferred using Maximum likelihood based on the Kimura 2 parameter model for trees generated from *B. rossi* sequences, and Kimura 2 parameter model was used for construction of trees from the *H. canis* sequences (Kimura, 1980; Kimura et al., 2016). In each method bootstrap analysis was done for the percentage of replicate trees in which the associated taxa clustered together in 1000 replicates (Felsenstein, 1985). The genetic distances between the sequences were estimated by determining the number of base differences between the sequences using MEGA 7 (Kimura., 1980; Kimura et al., 2016)). The GenBank accession numbers of reference sequences used to construct the phylogenetic trees are shown in Table 3.

Table 3: Names and GenBank accession numbers of 18S rRNA gene sequences of related genera used in the phylogenetic trees

Accession number	Taxonomic classification	Location	Hosts	References
L19079	*B. rossi*	South Africa	Dog	Allsopp et al., 1994
DQ111760	*B. rossi*	Sudan	Dog	Oyamada et al., 2005
KY463434	*B. rossi*	South Africa	Black-backed jackal	Penzhorn et al., 2017
KY463431	*B. rossi*	South Africa	Black-backed jackal	Penzhorn et al., 2017
KY463430	*B. rossi*	South Africa	Black-backed jackal	Penzhorn et al., 2017
MH143395	*Babesia* sp.	China	dogs	Wang et al., (Unpublished)
AB935167	*Babesia* sp.	Japan	Racoon	Jinnai et al., 2009
JQ861964	*Babesia* sp.	Kenya	Wild felids	Githaka et al., 2012
MH504116	*Babesia.* sp.	Turkey	Hedgehog	Orkun et al.,2019
AY072926	*B. canis*	Europe	Dog	Cacciò et al ., 2002
AY072925	*B. vogeli*	Europe	Dog	Cacciò et al ., 2002
AF205636	*B. gibsoni*	USA	Dog	He et al., 2017

AY693840	*B. microti* Gray	USA	Human	Slemenda et al., 2004 (Unpublished)
GQ411405	*B. lengau*	South Africa	Cheetah	Bosman et al., 2010
M87565	*B. rodhaini*	Australia	Dog	Ellis et al., 1992
AF244912	*B. felis*	South Africa	Lion	Penzhorn et al., 2001
AF244911	*B. leo*	South Africa	Lion	Penzhorn et al., 2001
KF724377	*B. venatorum*	China	Human	Sun et al., 2014
U16370	*B. divergence*	USA	Cattle	Holman, 1994
U16369	*B. odocoilei*	USA	White-tailed deer	Holman et al., 2000
AF158702	*B. conradae*	USA	Dog	Kjemtrup et al., 2000
AF158700	*B. duncani*	USA	N/A	Kjemtrup et al., 2000
X68523	*Toxoplasma gondii*	USA	N/A	Ding (unpublished)
M64244	*Sarcocystis muris*	N/A	Mice	Gajadhar et al., 1991
U03069	*N. caninum*	Sweden	Dog	Holmdahl et al., 1994
KJ499510	*H*. sp. JMP fox	North Africa	Pale fox	Maia et al., 2014
KJ499493	*H*. sp. JPM-2014	North Africa	Pale fox	Maia et al., 2014

KU893122	*H. canis* fox 5-2	Czech Republic	Red fox	Mitkova et al., 2016
KU893118	*H. canis* fox 1-2	Czech Republic	Red fox	Mitkova et al., 2016
KU893127	*H. canis* dog4	Czech Republic	Dog	Mitkova et al., 2016
AY471615	*Hepatozoon sp.*	Spain	Fox	Criado-fornelio et al., 2006
JX531911	*Hepatozoon* sp.	Portugal	Lizard	Maia et al., 2012
KY392885	*H, canis*	Brazil	White eared opossum	Silva et al., 2016
KX776369	*Hepatozoon.* sp.	Brazil	Wild carnivores	Sousa et al.,2016
AB771512	*H. felis*	Japan	Cat	Okamura, unpublished
AY461378	*H. canis* Spain2	Spain	Dog	Criado-fornelio et al., 2006
AY628681	*H. felis* Spain 2	Spain	Cat	Criado-fornelio et al., 2006
MG919987	*H. canis*	South Africa	Black-backed jackal	Penzhorn et al., 2017
MG919981	*H. canis*	South Africa	Black-backed jackal	Penzhorn et al., 2017
DQ111754	*H. canis*	Sudan	Dog	Oyamada et al., 2005
AY150067	*H. canis*	Spain	Fox	Criado-fornelio et al., 2006
AY620232	*H. felis* Spain 1	Spain	Cat	Criado-fornelio et al., 2006
AF176836	*H. americanum*	USA	Dog	Mathew et al., 2000

KC127679	*H. canis*	Brazil	Fox	Almeida et al., 2013
HQ224957	*Dactylosoma ranarum*	France	Frog	Barta et al., 2012
HQ224961	*Hemolivia mariae*	South Australia	Reptile	Barta et al., 2012

CHAPTER 4

4. Results

4.1. Reverse Line Blot PCR amplification

The V4 hypervariable region of the parasite 18S rRNA gene was successfully amplified from specimens using the *Theileria* and *Babesia* genus-specific primers as evident by a ~400 bp amplicon seen on an agarose gel (Fig 4). The V1 hypervariable region of the parasite 16S rRNA gene using the *Ehrlichia* and *Anaplasma* group specific primers amplification was not visible on the gel from parasite DNA of blood specimens from domestic dogs.

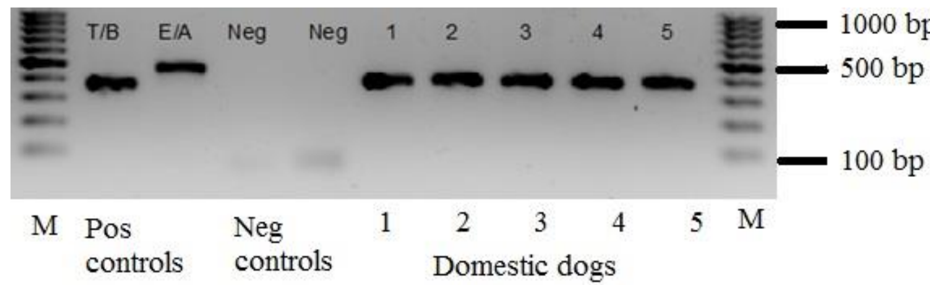

Figure 4: Gel image representing amplified PCR products from extracted DNA of blood specimens from domestic dogs. The primers used targeted a fragment length of approximately 450-520 bp of the 16S rRNA gene / 18S rRNA genes. Lane M: 100 bp plus gene ruler (Thermo Scientific^{TM}, LTC Tech South Africa (Pty) Ltd), lanes "Pos controls" represent the *Babesia bovis* and *Anaplasma centrale* (positive control), ultra-pure water was used as a negative control represented by Lanes "Neg controls". PCR amplicons of the V4 hypervariable region of the 18S rRNA gene are shown in lane 1-5: sample ID: BC 186, BC 187, BC 188, BC 189 and BC 191

4.2. Reverse Line Blot hybridization results

The RLB results were obtained from 77 free-ranging and 25 captive black-backed jackals, as well as 52 free-ranging and six captive African wild dogs (Table 4). Furthermore, 75 domestic dogs that were suspected to be sick from babesiosis were screened. Of the 77 free-ranging black-backed jackals, 69 (90%) hybridized with the *Babesia* genus-specific 1 probe, and 22

(29%) hybridized with *B. rossi* species-specific probe. Of the 25 specimens from captive black-backed jackals, 10 (40%) hybridized with the *Babesia* genus 1 probe, and seven (28%) hybridized with *B. rossi*. Of the 52 free-ranging African wild dogs 44 (85%) hybridized with *Babesia* genus-specific 1 probe, and five specimens (10%) reacted positive with the *B. rossi* probe. None of the specimens from six captive African wild dogs hybridized to any of the probes on RLB. Of the 75 domestic dogs, 67 (89%) hybridized with the *Babesia* genus-specific 1 probe, and 66 (88%) were detected positive for *B. rossi*. Negative detections were observed in some of the specimens. In free-ranging black-backed jackals, three (4%) and 10 specimens (40%) of the captive black-backed jackals were negative for all *Theileria* and *Babesia* haemoparasites respectively. In free-ranging African wild dogs, seven specimens (14%) and six specimens (100%) of the captive African wild dogs were negative for all *Theileria* and *Babesia* haemoparasites respectively. Six specimens (8%) of domestic dogs were also negative all *Theileria* and *Babesia* haemoparasites.

Mixed infections of *Ehrlichia* / *Anaplasma* group specific with *Babesia* genus-specific 1 and *B. rossi* were detected in free-ranging black-backed jackals, and captive black-backed jackals. In free-ranging African wild dogs, mixed infections of *Ehrlichia* / *Anaplasma* group with *Babesia* genus-specific 1 were also detected, and there were no mixed infections detection in captive African wild dogs. Mixed infections of *Theileria* sp. (Sable) with *Babesia* genus-specific 1, *Babesia* genus-specific 2 and *B. rossi* were detected in domestic dogs. Furthermore, mixed infections of *Babesia vogeli* with *Babesia* genus-specific 1, *Babesia* genus-specific 2 and *B. rossi* were also detected in domestic dogs. A representation of the RLB membrane showing genus and species-specific detections is shown in Fig. 6.

Table 4: The occurrence of haemoparasites in African wild dogs, black-backed jackals and domestic dogs as determined by the Reverse Line Blot hybridization assay

	African wild dogs (n = 52) KNP	**African wild dogs (n = 6) NZG**	**Black-backed jackals (n = 25) SALNR**	**Black-backed jackals (n = 77) MGBC**	**Domestic dogs (n= 75) OVAH**
***Theileria / Babesia* genus-specific**	4 (8%)	0 (0%)	7 (28%)	22 (29%)	69 (92%)
***Babesia* genus-specific 1**	44 (85%)	0 (0%)	10 (40%)	69 (90%)	67 (89%)
***Babesia* genus-specific 2**	19 (37%)	0 (0%)	2 (8%)	27 (35%)	0 (0%)
Babesia rossi	5 (10%)	0 (0%)	7 (28%)	22 (29%)	66 (88%)
Babesia vogeli	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (1%)
***Theileria* sp. (Sable)**	0 (0%)	0 (0%)	0 (0%)	0 (0%)	10 (13%)
***Ehrlichia / Anaplasma* genus-specific**	3 (6%)	0 (0%)	10 (40%)	39 (51%)	0 (0%)

Negative / below detection limit	7 (14%)	6 (100%)	10 (40%)	3 (4%)	6 (8%)

KNP : Kruger National Park

NZG : National Zoological Gardens

SALNR : S.A. Lombard Nature Reserve

OVAH : Onderstepoort Veterinary Academic Hospital

MGBC : Mogales Gate Biodiversity Centre

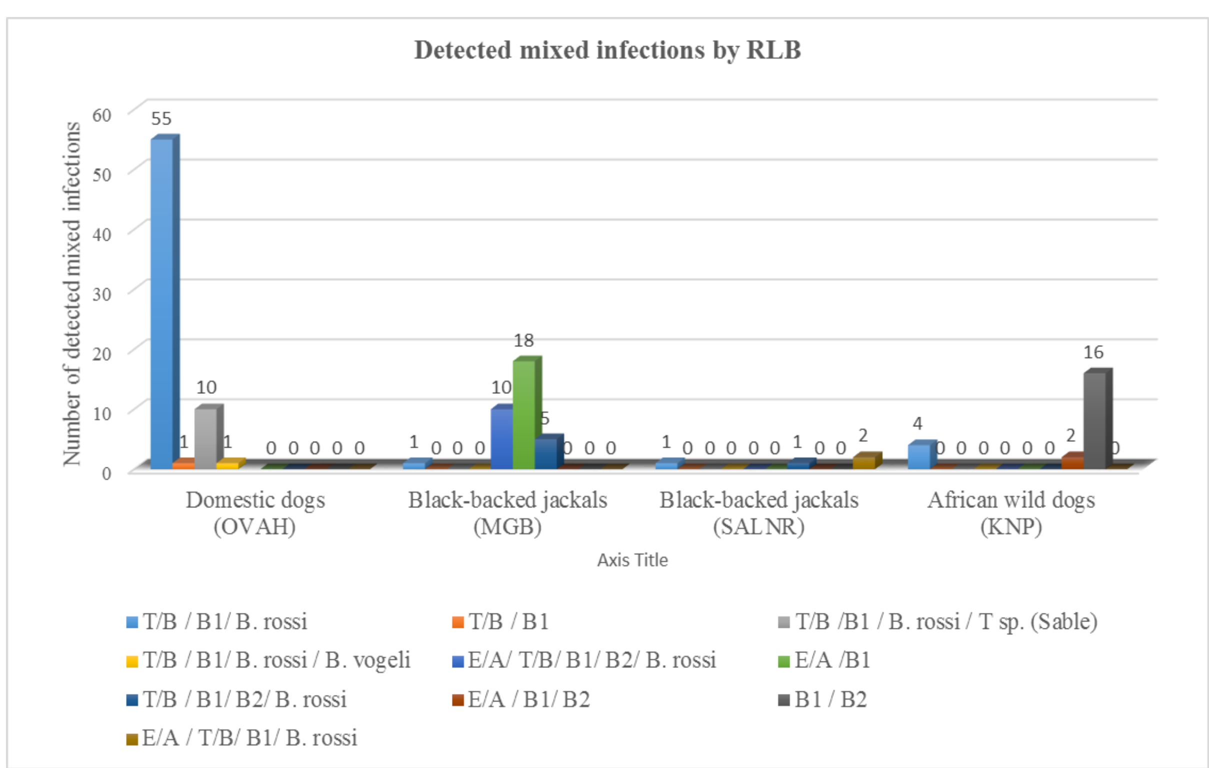

Figure 5: The number of detected mixed infections per specimens of African wild dogs, black-backed jackals and domestic dogs as determined by the Reverse Line Blot hybridization assay (RLB)

T/B : *Theileria* and *Babesia* group specific

E/A : *Ehrlichia* and *Anaplasma* group specific

B1 : *Babesia* genus specific 1

B2 : *Babesia* genus specific 2

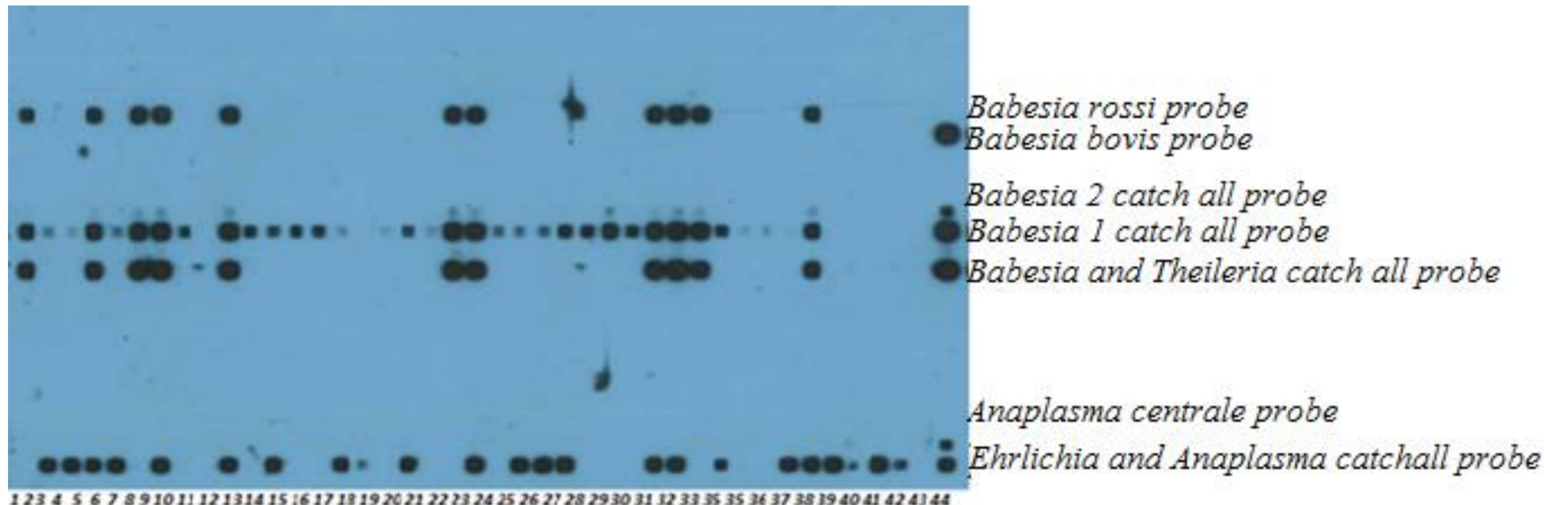

Figure 6: X-ray film image of a representative RLB membrane showing genus- and species-specific oligonucleotide probes applied in horizontal rows, and the PCR products from test samples and controls applied in vertical rows. Lanes 1-42 indicate PCR products from black-backed jackals, lane 43 indicates the negative control and lane 44 indicates the positive control. The black spots on the X-ray image represent positive signals and the areas without spots indicate negative detection

4.3. 18S rRNA gene sequence analysis

The near full-length 18S rRNA gene (~ 1 600 bp) of *B. rossi* was successfully amplified from six domestic dogs and three free-ranging black-backed jackals. An additional black-backed jackal specimen that signalled positive for *B. rossi* on RLB was successfully sequenced although the sequencing results revealed *H. canis* tobe present. Initially, five specimens were selected for sequencing in African wild dog due to their positive *B. rossi* signals on RLB, and only two were successfully sequenced. An attempt to generate near full-length sequences for the three additional specimens of African wild dog was unsuccessful due to failure to generate long reads, with the inclusion of internal primers i.e. Nbab-1F, TB-R and RLB-F. Moreover, their BLAST search matched with *Hepatozoon canis*. Finally, 25 recombinant sequences were generated. From which 11 sequences were derived from six domestic dogs with 3 clones each from specimen BC 223, and 2 clones each from specimen BC 289; BC 238; BC 194 and 1 clone each from specimens BC 186 and BC 261. Ten sequences were derived from four black-backed jackals with 3 clones each from specimens RLB 1485 and RLB 1516, and 2 clones each from specimens RLB 1507 and RLB 1493. Four sequences were derived from 2 African wild dogs specimens, with 3 clones from RE 52 and 1 clone from RE 40. Although more contigs were generated for each specimen, failure to assemble into long reads due to sequence quality lead to few sequences being presented in the study.

BLASTn homology searches revealed that the obtained 11 recombinant sequences from domestic dogs and 10 recombinant sequences from black-backed jackals were identical (100%) to published *B. rossi* sequences previously described in South African black-backed jackals (accession numbers KY463430-KY463434) (Penzhorn et al., 2017), and further showed 99% sequence identity to *B. canis* (presumably *B. rossi*) from a South African dog (L19079) (Allsopp et al., 1994) and *B. rossi* from eastern Sudan (DQ111760) (Oyamada et al., 2005).

BLASTn homology searches further revealed that 1 partial recombinant sequence from a black-backed jackal, as well as 4 recombinant sequences obtained from two African wild dogs had 100 % sequence identity to *Hepatozoon* spp. in wild pale foxes (*Vulpes pallida*) from North Africa (KJ499493, KJ499502-KJ499507) (Maia et al., 2014) and 99% sequence similarity to *Hepatozoon* sp. previously described from black-backed jackals in South Africa (MG919973-MG919987) (Penzhorn et al., 2018). Furthermore, it had 99% sequence similarity to various

H. canis sequences previously described from dogs from various countries, as well as 97% sequence identity to *H. felis* (AY620232, AY628681) (Criado-Fornelio et al., 2006). Interestingly, these specimens all tested positive for *B. rossi* DNA using the RLB assay, indicating mixed infections

Table 5: RLB hybridization assay, cloning and sequencing results of domestic dogs, black-backed jackal and African wild dog specimens selected for 18S rRNA gene sequence analysis.

Sample no	Place of Origin	RLB results	Clone No	Sequence length (bp)	Phylogenetic grouping
Domestic dog					
BC 194	OVAH[1]	*T / B* group-specific[2], *Babesia* genus-specific 1	D1C1	1454	*B. rossi*
			D1C3	1569	*B. rossi*
BC 186	OVAH	*T / B* group-specific, *Babesia* genus-specific 1, ***B. rossi***	D2C1	1569	*B. rossi*
BC 238	OVAH	*T / B* group-specific, *Babesia* genus-specific 1, ***B. rossi***	D11C1	1569	*B. rossi*
			D11C2	1569	*B. rossi*
BC 223	OVAH	*T / B* group-specific, *Babesia* genus-specific 1, ***B. rossi***	D12C2	1514	*B. rossi*
			D12C3	1569	*B. rossi*
			D12C4	1581	*B. rossi*
BC 289	OVAH	*T / B* group-specific, *Babesia* genus-specific 1, ***B. rossi,*** *Theileria* sp. (sable)	D13C1	1569	*B. rossi*
			D13C3	1569	*B. rossi*
BC 261	OVAH	*T / B* group-specific, *Babesia* genus-specific 1, ***B. rossi,*** *Theileria* sp. (sable)	D14C2	1550	*B. rossi*
Black-backed jackal					
RLB 1493			J1C1	451	*Hepatozoon* sp.

	Mogales Gate Biodiversity Centre	*T / B* genus-specific, *Babesia 1* genus-specific, *Babesia 2* genus-specific, ***B. rossi***	J1C2	451	*Hepatozoon* sp.
RLB 1485	Mogales Gate Biodiversity Centre	*E / A* genus-specific[3], *T/B* genus-specific, *Babesia 1* genus-specific, *Babesia 2* genus-specific, ***B. rossi***	J2C1	1396	*B. rossi*
			J2C2	1063	*B. rossi*
			J2C3	963	*B. rossi*
RLB 1516	Mogales Gate Biodiversity Centre	*T / B* genus-specific, *Babesia 1* genus-specific, *Babesia 2* genus-specific, ***B. rossi***	J7C1	1063	*B. rossi*
			J7C2	999	*B. rossi*
			J7C3	789	*B. rossi*
RLB 1507	Mogales Gate Biodiversity Centre	*T / B* genus-specific, *Babesia 1* genus-specific	J11C4	1419	*B. rossi*
			J11C5	1459	*B. rossi*
African wild dog					
RE40	Kruger National Park	*T / B* genus-specific, *Babesia 1* genus-specific, ***B. rossi***	RE40C9	1664	*Hepatozoon* sp.
RE52	Kruger National Park	*T / B* genus-specific, *Babesia 1* genus-specific, ***B. rossi***	RE52C1	1661	*Hepatozoon* sp.
			RE52C9	1656	*Hepatozoon* sp.
			RE52C1	1674	*Hepatozoon* sp.

[1] OVAH = Onderstepoort Veterinary Academic Hospital

[2] *T / B* group-specific = *Theileria / Babesia* genus-specific probe positive

[3] *E / A* group-specific = *Ehrlichia / Anaplasma* genus-specific probe positive

Estimated evolutionary divergence between the observed *B. rossi* gene sequences and published *B. rossi* 18S rRNA gene sequences was compared by determining the number of base differences per near full-length 18S rRNA gene sequence (Table 6). All positions containing gaps and missing data were eliminated. There were 1282 positions in the final dataset. The obtained recombinant sequences were identical to that of published *B. rossi* sequences previously described from South African black-backed jackals (Penzhorn et al., 2017) and *B. rossi* described form a South African dog (L19079) (Allsopp et al., 1994), while it differed by 3 base pairs from *B. rossi* (DQ111760) from eastern Sudan (Oyamada et al., 2005). The observed sequence similarities were subsequently confirmed by phylogenetic analyses. Maximum likelihood confirmed the relationship between the obtained sequences and related *Babesia* sequences previously deposited in Genbank. There was no significant difference in the topology of the phylogenetic tree or in the bootstrap values found. A representative tree obtained by the Maximum Likelihood method is shown in Figure 7. The obtained sequences formed a monophyletic group with the published *B. rossi* sequences, which in turn formed a monophyletic group with *B. canis* and *B. vogeli.*

Similarly, the estimated evolutionary divergence between the obtained *Hepatozoon* 18S rRNA gene sequences and published *Hepatozoon* sequences was subsequently compared (Table 7). All positions containing gaps and missing data were eliminated. There were 512 positions in the final dataset. The obtained sequences were identical to *Hepatozoon* spp. previously described in wild pale foxes from North Africa (Maia et al., 2014). Our generated sequences further differed by 2 base pairs from *H. canis* previously described in Spain (AY150067) (Criado-fornelio et al., 2006) , and by 2 base pairs from *Hepatozoon* spp previously described from black-backed jackals in South Africa (Penzhorn et al., 2018). The observed sequence similarities were then also confirmed by phylogenetic analyses. Sequence homology was also observed amongst certain strains such as the *H. canis* strain from Brazil (AY471615) and a strain from Spain *H. canis* (AY150067). Maximum likelihood analyses was used and no significant difference in the topology of the phylogenetic trees or in the bootstrap values was found. A representative tree obtained by the Maximum Likelihood method is shown in Figure 8. The obtained sequences formed a monophyletic group with the published *Hepatozoon* spp. sequences described from pale foxes and in turn also with *H. canis* and the *Hepatozoon* spp. previously described from black-backed jackals from South Africa.

Table 6: A representative of estimated evolutionary divergence between *Babesia rossi* sequences and previously published sequences of *B. rossi.* The sequences were derived in blood samples from domestic dog (D2C3) and black-backed jackal (J11C5).

	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
*B.*spp (JQ861964)																											
B. spp (MH504116)	29																										
B. spp (MH143395)	28	18																									
B. rossi (KY463430)	24	14	6																								
Domestic dog D2C3*	24	14	6	0																							
Black-backed jackal J11C5*	24	14	6	0	0																						
B. rossi (KY463434)	24	14	6	0	0	0																					
B. ross i (KY463431)	24	14	6	0	0	0	0																				
B. rossi (L19079)	24	14	6	0	0	0	0	0																			
B. rossi (DQ111760)	27	15	9	3	3	3	3	3	3																		
B. canis (AY022926)	64	58	56	52	52	52	52	52	52	55																	
B. vogeli (AY072925)	64	58	61	57	57	57	57	57	57	59	22																
B . sp (AB935167)	66	64	67	62	62	62	62	62	62	61	55	58															
B. gibsoni (AF205636)	72	71	72	69	69	69	69	69	69	70	63	66	42														
B. venatorum (KF724377) A	87	84	86	81	81	81	81	81	81	80	68	67	45	52													
B. divergens (U16370) AM	74	75	76	72	72	72	72	72	72	71	72	69	46	49	21												
B. odocoilei (U16369) AM	75	74	77	73	73	73	73	73	73	72	65	68	42	44	21	19											
B. microti Gray (AY693840)	148	148	152	150	150	150	150	150	150	147	142	142	132	133	133	131	129										
B. felis (AF244912)	139	140	141	139	139	139	139	139	139	139	138	137	123	127	129	127	119	43									
B. leo (AF244911) AM	142	143	148	146	146	146	146	146	146	143	142	141	128	132	128	128	125	34	30								
B. rodhaini (M87565) AM	145	149	153	151	151	151	151	151	151	149	141	139	136	139	138	136	133	47	50	42							
B. lengau (GQ411405)	129	125	124	122	122	122	122	122	122	124	128	128	119	119	110	109	111	96	101	104	107						
B. conradae (AF153700) AM	133	131	135	131	131	131	131	131	131	133	128	128	119	123	117	116	114	99	97	100	102	35					
B. duncani WA1 (AF158700	128	126	128	124	124	124	124	124	124	126	128	125	115	121	110	108	109	100	96	102	108	25	29				
Toxoplasma gondii (X68523	173	177	178	175	175	175	175	175	175	176	173	169	167	171	167	162	168	159	156	153	162	149	149	144			
N. caninum (U03069)	173	177	178	175	175	175	175	175	175	176	173	169	167	171	167	162	168	159	156	153	162	149	149	144	1		
Sarcocystis muris (M64244)	177	182	183	180	180	180	180	180	180	181	173	172	174	174	170	165	171	156	152	155	161	153	146	145	30	31	

The number of base differences from pairwise analyses of 27 sequences are shown. There were 1282 positions in the dataset. Evolutionary analyses were conducted in MEGA 7(Kimura, 1980)

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Table 7: A representative of estimated evolutionary divergence between *Hepatozoon canis* sequence and previously published sequences of *H. canis*. The sequence was derived in blood sample from African wild dog (RE52C1) in Kruger National Park.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
H. sp. JMP fox (KJ499510)																							
H. sp. JMP 2014 (KJ499493)	0																						
African wild dog RE52C1*	0	0																					
H. canis Spain2 (AY461378)	1	1	1																				
H. canis Curupira4 (AY471615)	1	1	1	2																			
H. canis Spain1 (AY150067)	1	1	1	2	0																		
H. canis (KY392885)	1	1	1	2	0	0																	
H. canis (MG919981)	2	2	2	1	1	1	1																
H. canis Dog-26 (DQ111754)	2	2	2	1	1	1	1	0															
H. canis (MG9119981)	2	2	2	1	1	1	1	0	0														
H. canis fox 5-2 (KU893122)	4	4	4	3	3	3	3	2	2	2													
H. canis 1-2 (KU893118)	4	4	4	3	3	3	3	2	2	2	0												
H. canis dog4 (KU893127)	4	4	4	3	3	3	3	2	2	2	0	0											
H. felis Spain1 (AY620232)	19	19	19	20	20	20	20	21	21	21	21	21	21										
H. canis Spain2 (AY628681)	19	19	19	20	20	20	20	21	21	21	21	21	21	0									
H. canis (AB771512)	15	15	15	16	16	16	16	17	17	17	17	17	17	7	7								
H. canis (KX776369)	21	21	21	22	22	22	22	23	23	23	23	23	23	14	14	10							
H. sp. (JX531911)	21	21	21	22	22	22	22	23	23	23	23	23	23	12	12	9	17						
H. sp. (JX531912)	21	21	21	22	22	22	22	23	23	23	23	23	23	12	12	9	17	0					
D. ranarum (HQ224957)	42	42	42	41	41	41	41	42	42	42	42	42	42	37	37	34	40	37	37				
Hemolivia mariae (HQ224961)	43	43	43	42	42	42	42	43	43	43	43	43	43	38	38	35	41	38	38	1			
H. americanum (AF176836)	27	27	27	28	28	28	28	29	29	29	30	30	30	18	18	16	25	23	23	45	46		
H. sp. (KC127679)	24	24	24	25	25	25	25	26	26	26	27	27	27	15	15	13	22	19	19	42	43	9	

The number of base differences from pairwise analyses of 23 sequences are shown. There were 512 positions in the data set. Evolutionary analyses were conducted in MEGA 7 (Kimura, 1980)

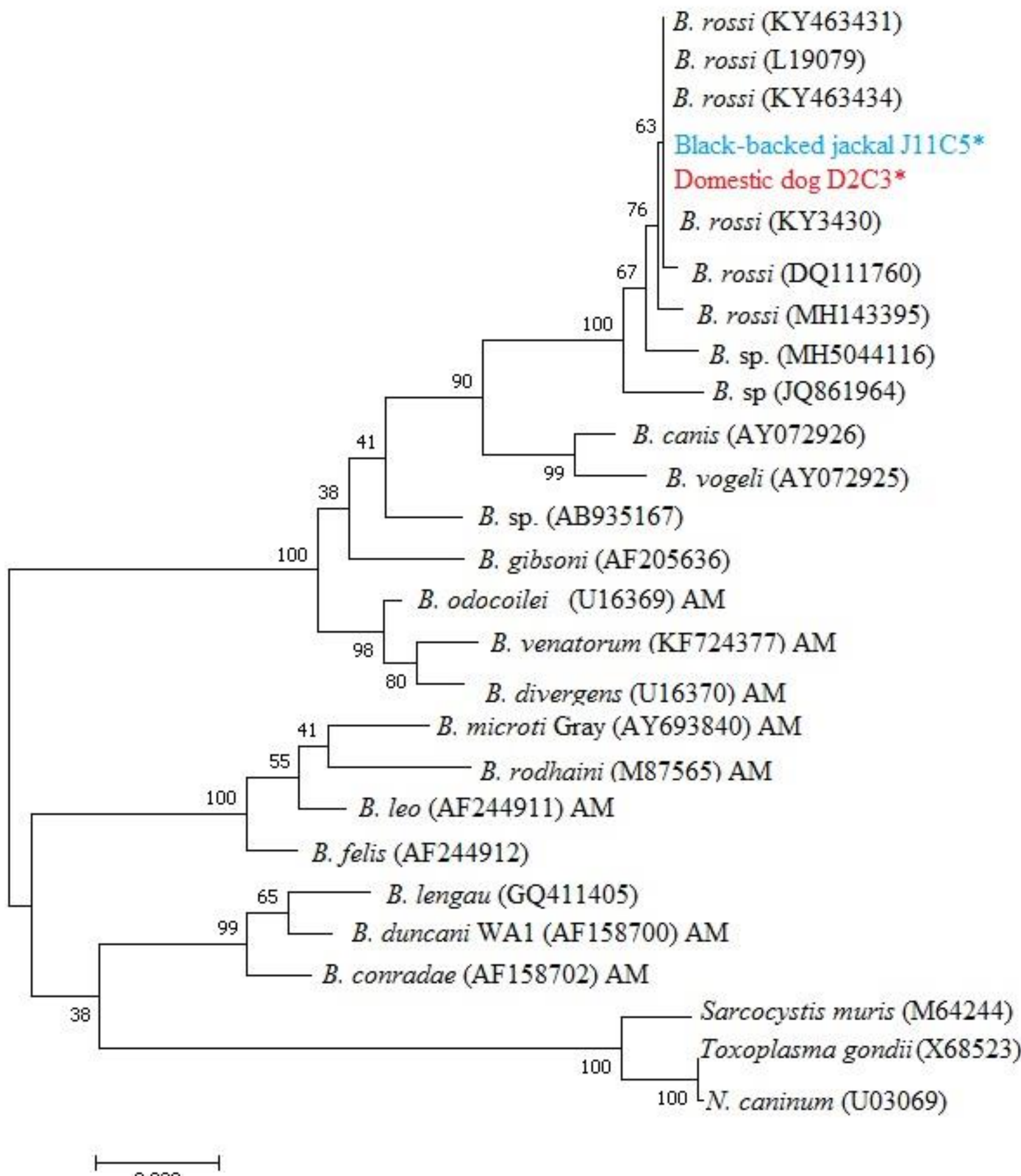

Figure 7: Maximum Likelihood tree, with the Kimura two-parameter distance calculation showing the phylogenetic relationship of the obtained parasite 18S rRNA sequences to related *Babesia* species. Relationships are presented as an unrooted tree with branch lengths being proportional to the estimated genetic distance between the strains. The scale bar represents the % nucleotide difference. The analysis involved 27 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were 1282 positions in the final dataset. *Sarcocystis muris, Neospora caninum* and *Toxoplasma gondii* were used as outgroup. The Genbank accession numbers are indicated in parentheses. **Note**: As sequences obtained in

this study were identical to each other, we have included only one representative sequence per host.

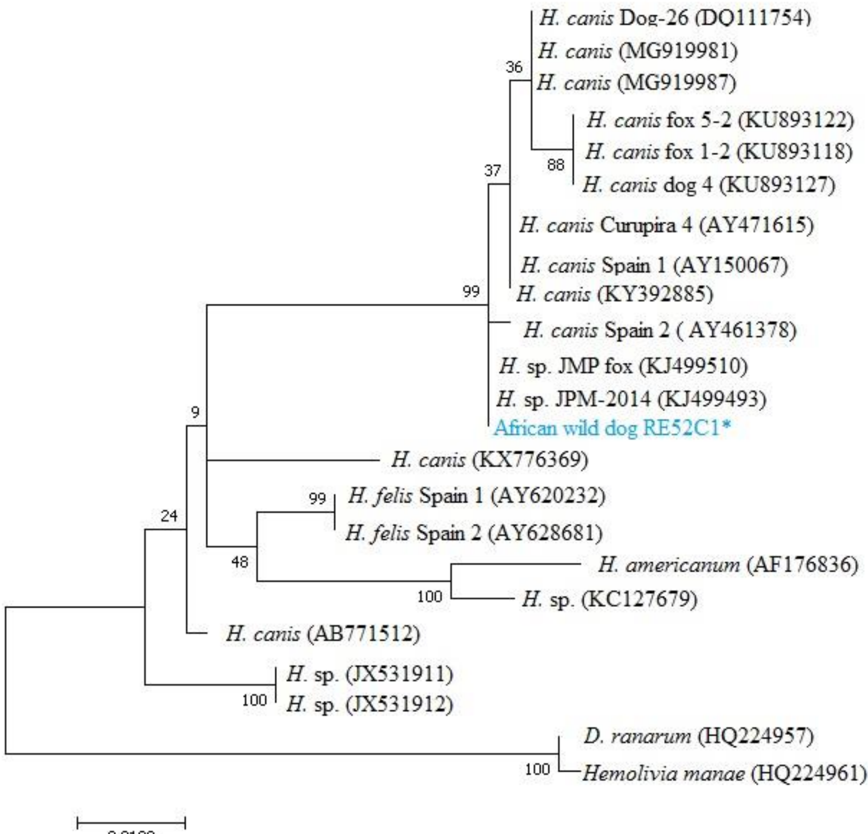

Figure 8: Maximum Likelihood tree, with the Kimura two-parameter distance calculation showing the phylogenetic relationship of the obtained parasite 18S rRNA sequences to related *Hepatozoon* species. Relationships are presented as an unrooted tree with branch lengths being proportional to the estimated genetic distance between the strains. The scale bar represents the % nucleotide difference. The analysis involved 23 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were 512 positions in the final dataset. *Dactylosoma ranarum* and *Hemolivia mariae* were used as outgroup. The Genbank accession numbers are indicated in parentheses. Note: As sequences obtained in this study were identical to each other we have included only one representative sequence.

CHAPTER 5

5. Discussion and Conclusion

5.1. General discussion

5.1.1. Haemoparasite detection

The initial method used for haemoparasite detection was PCR followed by the Reverse Line Blot hybridisation assay. Simultaneous amplification of the V4 and V1 hypervariable region of *Theileria* / *Babesia* and *Ehrlichia* / *Anaplasma* 18S rRNA and 16S rRNA genes was done using PCR under stringent conditions. Haemoparasites present in the blood specimens of domestic dogs, black-backed jackals and African wild dogs were identified using genus- and species-specific probes. The *B. rossi* and incidental *Hepatozoon* spp. positive specimens were further characterised molecularly, through gene sequence and phylogenetic analysis.

5.1.1.1. Haemoparasites detection in domestic dogs

Reverse Line Blot hybridisation assay was used for simultaneous detection of haemoparasites of *Babesia*, *Theileria*, *Ehrlichia* and *Anaplasma* in domestic dog specimens, which correlate with a study that reported on these haemoparasites in domestic dogs at the outpatient clinic of OVAH (Matjila et al., 2008b). The RLB results indicated that *Babesia* species infections were common amongst sick domestic dogs with 89% occurrence level and 88% of the specimens positive for *B. rossi*. This high prevalence correlates with a previous report of 72% of *B. rossi* in domestic dogs at the OVAH (Matjila et al., 2008b). *Babesia rossi* has been frequently reported in dogs at OVAH, therefore, as only sick dogs were sampled, the true prevalence of *B. rossi* in the local domestic dog population remains unknown.

It has also been reported that *Babesia*-infected dogs at OVAH were heavily infested with *Haemaphysalis elliptica*, which is the only known vector for *B. rossi* in the country (Horak, 1995). *Babesia rossi* owes its success to the large distribution pattern of the tick vector as well as the presence of competent hosts within its circles.

Another described *Babesia* parasite of dogs in the country is *B. vogeli*, which was detected in only one specimen (1%) in this study. Matjila et al. (2008c) reported similar findings of *B. vogeli* (0.9%) detections in domestic dogs at OVAH. Based on the current knowledge there is generally a low prevalence of *B. vogeli* in dogs presented at OVAH as compared to *B. rossi*. The possible reasons for this could be that the known vector for *B. vogeli*, *Rhipicephalus sanguineus,* is not frequently found on *Babesia*-infected dogs as compared to *H. elliptica* at OVAH (Horak, 1995). *R. sanguineus* transmits *B. vogeli* (Uilenberg et al., 1989) and *Ehrlichia canis* (Groves et al., 1975).

The highest co-infection was observed with simultaneous detections with the genus *Theileria* / *Babesia* group-specific, *Babesia* genus- specific 1 and *Babesia* genus specific 2 probes with 55% detection level in domestic dogs (Fig. 5). In South Africa, the geographic distributions of *R. sanguineus* and *H. elliptica* overlap (Horak, 1995). Infections resulting from *H. canis*, *E. canis, B. vogeli* occurs in dog populations. In this study co-infections of *B. rossi* and *B. vogeli* was reported in 1% of the sampled dogs, similarly, there was a previous report of co-infections of *B. rossi* and *E. canis* (2%) in sampled dogs at OVAH (Matjila et al., 2008b), despite the co-existence of *H. elliptica* and *R. sanguineus* on *Babesia*-infected dogs (Horak, 1995). The RLB results did not indicate the presence of any Ehrlichias in the domestic dogs, the results support the idea that *R. sanguineus* was not a problem for dogs in this sample set.

The high prevalence of *B. rossi* in this study was expected since only dogs that were suffering from babesiosis were investigated. Although previous reports of *B. rossi* in OVAH has always been high as compared to other tick-borne parasites since infected dogs have a high chance of developing canine babesiosis and of being presented at clinics. RLB detections of *Theileria* spp. (Sable) was observed in 13% of domestic dogs in this study. This finding is similar to a study by Matjila et al. (2008a) which reported on *Theileria* spp. in specimens from OVAH and Pietermaritzburg. Although generally the prevalence of *Theileria* is lower than that of *Babesia* in domestic dogs at OVAH.

5.1.1.2. Haemoparasite detection in black -backed jackals

Babesia species infect black-backed jackals, as detected using the RLB in this study. In free-ranging and captive black-backed jackals, *Babesia* spp. occurred in 90% and 40% of the sampled jackals, respectively. These findings correlate with a study conducted on free-ranging and captive black-backed jackals from the same locations, which reported 87% and 38%

Babesia spp. detections on RLB, respectively (Penzhorn et al., 2017). *Babesia rossi* detections amounted to 29% of specimens from free-ranging black-backed jackals and 28% of the captive black-backed jackals. In the previous study detection rates of 29.7% and 37.5% respectively, were reported in free-ranging and captive black-backed jackals using RLB (Penzhorn et al., 2017). This implies that the occurrence of *B. rossi* in black-backed jackal is lower than that of domestic dogs even though its tick vector is common in the country. Although one could argue that this low prevalence might be an indication of strict parasite control measures in domestic dogs surrounding farm areas to prevent tick transfer to wild canids (Van Heerden et al., 1996).

The *Babesia* spp. detections with the *Babesia* genus-specific 1 probe in domestic dogs was an indication of *B. rossi*. Discrepant results were observed with the *Babesia* genus-specific 1 probe in African wild dogs and black-backed jackals specimens. In domestic dogs there appeared a direct correlation pattern of detections with the *Babesia* genus-specific 1 probe (89%) with the actual finding of *B. rossi* (88%) was observed on RLB. In African wild dogs 85% of the specimens reacted positive with *Babesia* genus-specific 1 probe with 10% of the specimens testing positive for *B. rossi,* a similar pattern was observed in free ranging and captive black-backed jackals *viz* 90%; 40% with their respective *B. rossi* detections as 29% and 28%. A suspected cross-reaction of the *Babesia* genus-specific 1 probe was tested, it was revealed that *H. canis* which is absent on RLB is possibly cross reacting with *Babesia* genus-specific 1 probe. A sequence alignment of the *Babesia* genus-specific 1 probe with previously published *H. canis* sequences and the obtained sequences that revealed identity to *H. canis*, matched with 2 nucleotide differences which would not have prohibited binding. A previous related study that investigated the prevalence of *B. rossi* in free ranging black-backed jackals reported similar findings of high detection with *Babesia* genus-specific 1 probe (77%) and relatively low detections with the *B. rossi* probe (27%) on RLB (Penzhorn et al., 2017). We therefore recommend that the *H. canis* probe should be included on RLB especially when testing wild life specimens because of the high occurrence rate of *H. canis* in black-backed jackals and African wild dogs to improve diagnosis. Although possibilities of low parasitaemia of *B. rossi* in the specimens, as well as the presence of a novel or a variant species of *Babesia* cannot be overruled.

Despite the high momentum of surveillance studies of babesias of canines in the country, black-backed jackals have received little attention. Research has focused mainly on sub-inoculation studies of *B. rossi* in black-backed jackals, and their role as natural reservoir hosts of the

parasite (Nuttall, 1910, Penzhorn, 2006, Penzhorn et al., 2017, Van Heerden, 1980). Information on the genetics of the parasites in black-backed jackals would underpin important discoveries of the parasite biology and address the risks posed to domestic dogs. It was speculated that the role that wild canids play in the life cycle of *B. rossi* might be a contributing factor to the virulent nature of the South African isolates (Adamu et al., 2014). The speculation arose from lack of wild canids in the wild in Nigeria, resulting in the life cycle of *B. rossi* being maintained in domestic dogs, which was believed to be a contributing factor to the non-virulent nature of *B. rossi* isolates in Nigeria (Adamu et al., 2014).

Detections with *Ehrlichia* / *Anaplasma* group-specific probe were observed in black-backed jackals in this study. From the free-ranging black-backed jackals it was 51% and from the captive black-backed jackals 40% of specimens reacted with *Ehrlichia* / *Anaplasma* group-specific probe only, without any species detection. This could suggest the presence of a novel species or a variant of a species. Since the focus of the study was more on *B. rossi*, processing of *Ehrlichia / Anaplasma* genus-specific results were not followed up further through molecular characterisation. However, a recent study reported a 56% specimen detections of *Ehrlichia* / *Anaplasma* genus specific in free-ranging black-backed jackals and a 69% specimen detections in captive black-backed jackals, with no species detections (Penzhorn et al., 2018). Molecular characterisation of the 16S rRNA gene revealed sequences most closely related to *Anaplasma* spp. in South African Dog, various uncultured *Anaplasma* spp., as well as various *Anaplasma phagocytophilum* genotypes present in free-ranging and captive black-backed jackals in South Africa (Penzhorn et al., 2018).

Mixed infections of *B. rossi* and *Ehrlichia* / *Anaplasma* were observed in this study, albeit at low incidences of 17% and 20% in free-ranging and captive black-backed jackals, respectively. Co-infections between *B. rossi* and *E. canis* is well documented in domestic dogs (Matjila, 2008b). But there is a single study that reported on co-infections of *B. rossi* with *Ehrlichia* and *Anaplasma* genus in black-backed jackals (Penzhorn et al., 2018). Due to the overlapping populations of *H. elliptica* and *R. sanguineus* reported in South Africa (Horak, 1995), it is possible to encounter co-infections of the two parasites in black-backed jackals.

5.1.1.3. Haemoparasite detection in African wild dogs

There are more studies conducted on the occurrence of *B. rossi* in African wild dogs in South Africa than on black-backed jackals, because African wild dogs are an endangered species

(Matjila et al., 2008a, Penzhorn, 2006, Van Heerden, 1980). This study reported 85% specimen detections of *Babesia* spp. in free-ranging African wild dogs, with 10% of specimens positive for *B. rossi*. No infections were detected in any of the captive African wild dog specimens screened for haemoparasites. Findings on African wild dogs from a captive centre (De Wildt Cheetah and Wildlife Centre) revealed a 5% occurrence of *B. rossi* (Matjila et al., 2008a). The low levels of occurrence of *B. rossi* in African wild dogs are common. The first report of *B. rossi* in African wild dogs from the Kruger National Park (KNP) was during an assessment of possible causes of population declines over 10 years ago. Findings revealed that piroplasmids could not be incriminated as the cause of deaths as they only occurred in 10% of sampled dogs. Preclusion of domestic dogs from African wild dog habitats, was suggested for infection prevention since vaccination and tick control measures are not practised in KNP (Van Heerden, 1995). Surprisingly, molecular characterisation of the 18S rRNA gene sequences obtained from African wild dogs revealed *Hepatozoon canis* to be present in specimens detected positive for *B. rossi* by RLB. Mixed infection of *B. rossi* and *H. canis* have been reported in African wild dogs (Matjila, et al., 2008c). Low parasitaemia in specimens of *B. rossi* which supposedly resulted in low DNA concentrations with mixed infections of *H. canis* and *B. rossi* might have led to failure to generate *B. rossi* gene sequences.

A high prevalence of *Hepatozoon* gametocytes (90%) has been reported from blood smears of African wild dogs in the KNP (Van Heerden, 1995). Two wild dogs from De Wildt Cheetah and Wildlife Centre were screened positive for *H. canis*, and the level of *H. canis* was low in specimens (Matjila et al., 2008b). This was also the case with wild dogs sampled in Serengeti National Park in Tanzania (Peirce et al., 1995). The current study confirmed the presence of *H. canis* in free-ranging African wild dogs in the KNP. However, probably owing to the small sample size of African wild dogs from KNP, *H. canis* was not well represented. These results affirm the possibilities of a mixed infection of *B. rossi* and *H. canis* present in the African wild dog specimens. Although it is important to note that *H. canis* has no clinical significance in SA as opposed to *Hepatozoon americanum*, described in dogs in the USA (Baneth et al., 2003).

Although the V4 hypervariable region of the 18S rRNA is considered fairly conserved in all members of *Theileria* and *Babesia*, RLB probe cross reactivity has been reported in field isolates resulting from single nucleotide polymorphisms (SNPs) , and these variations are considered fixed in geographically isolated populations (Mans et al., 2015). Therefore, the *B. rossi* probe was checked for cross-reactivity with *H. canis* to validate the detection of *B. rossi*

in the specimens by RLB. A sequence alignment of the obtained African wild dog sequence, and the published *B. rossi* and *H. canis* sequences, showed that the *B. rossi* probe bound (without nucleotide mismatches or gaps) with the published *B. rossi* sequences and the obtained *B. rossi* sequence from a domestic dog. The African wild dog and a black-backed jackal sequences revealed sequence identity to published *H. canis* sequences, which revealed 17 mismatches to the *B. rossi* probe, inhibiting any possibility of binding. This proved that the *B. rossi* probe is not cross-reacting with *H. canis* DNA. The *B. rossi* detections in African wild dog specimens were not false positives. It is possible that *H. canis* outcompeted *B. rossi* with higher parasitaemia, hence all the amplicons resulted in *H. canis* sequences. *Babesia*- 18S rRNA primers could have been used for sequencing to improve specificity, on the contrary universal 18S rRNA (TB-R and Nbab-1F) primers were used for amplifying 18S rRNA gene of all members of *Theileria, Babesia* as well as *Hepatozoon* spp. RLB assay needs to include probes for common pathogens such as *H. canis* to improve diagnostics.

5.1.2. Phylogenetic analysis

5.1.2.1. Domestic dogs and black-backed jackals

Of the obtained domestic dog and black-backed jackal sequences, comparison of estimated evolutionary divergence revealed sequence identity to *B. rossi* sequences previously described in South Africa and a similarity to a *B. rossi* strain from Sudan. All the sequences obtained in this study were identical, therefore one represented sequence per host derived from specimens D2C3 of domestic dog and J11C5 of black-backed jackals were similar to the previously described *B. rossi* sequences (L19079, KY463430- KY463434) in South Africa (Allsopp et al., 1994, Penzhorn et al., 2017). The lack of difference in nucleotides between the obtained sequences and the *B. rossi* described sequences is an indication of the sequence homology in the country, which is often the case since the geographical location of the tick vector, hosts and environmental conditions are similar. Variations within *B. rossi* exist in isolates from similar or different geographical locations. In this study, three nucleotide differences were observed with a *B. rossi* isolate from Sudan (Oyamada et al., 2005), which shows that the *B. rossi* that occurs in SA is different from isolates in other African countries. Bhoora et al. (2009) showed sequence heterogeneity of the V4 hypervariable region of the 18S rRNA gene of *B. caballi* in South African horses, despite the conserved nature of the 18S rRNA gene. Variations within sequences were observed in specimens collected from the same geographical area (Bhoora et al., 2009). Based on the findings of this study, there could be similar variations of *B. rossi*

isolates from black-backed jackals and domestic dogs, regardless of the different clinical signs induced by *B. rossi* in the two canid hosts. Our results have shown that the *B. rossi* that is found in black-backed jackals and domestic dogs formed a monophyletic group with the described *B. rossi* from South Africa and a *B. rossi* isolate from Sudan. Therefore, the isolates that infect domestic dogs and black-backed jackals appear to be similar based on 18S rRNA analysis, despite the different clinical response by the hosts. Ebert (1998) suggested that it is common for pathogens to have clinical significance in recent niche hosts, which is the case in domestic dogs. This was affirmed by Matjila et al., (2009) who reported that specific genotypes of *B. rossi*, such as Genotype 19 and Genotype 31 based on the BREMA 1 gene, have clinical significance in domestic dogs. To elucidate on the pathogenesis of *B. rossi*, BREMA1 should be investigated in indigenous canid populations.

5.1.2.2. African wild dogs

The *H. canis* observed in African wild dogs appears to be the same as the *H. canis* described in foxes from North Africa. Comparison of estimated evolutionary divergence revealed no nucleotide difference between the obtained African wild dog sequence representative (RE52C1) and the described *H. canis* (KJ 499510; KJ499493). Furthermore, phylogenetic analysis of the V4 hypervariable region of the 18S rRNA gene confirmed the relationship whereby a monophyletic group was formed with described sequences. *Hepatozoon canis* was reported in black-backed jackals earlier this year, although the results revealed that the presence of subtypes of *H. canis* varies within the black-backed jackal population (Penzhorn et al., 2018). The frequency of *H. canis* in the KNP is not well represented in this study owing to the small specimen size used for molecular characterisation. The low prevalence of *B. rossi* in African wild dogs from KNP is possibly due to low surveillance studies, mainly because African wild dogs are sub-clinically infected with *B. rossi*,

5.1.3. Conclusion

This study investigated the occurrence of *B. rossi* in sick domestic dogs, apparently healthy free-ranging and captive black-backed jackals and African wild dogs. Based on findings of this study, *B. rossi* occurs naturally in domestic dogs and black-backed jackals. There were various unsuccessful attempts of analysing *B. rossi* in African wild dogs. The RLB signals of *B. rossi* was an indication of the presence of *B. rossi* in African wild dogs, albeit at very low concentrations. A *B. rossi* Taqman probe real time PCR assay would have been used to

elucidate the presence of *B. rossi* in the specimens. Based on the sequence similarities of the *B. rossi* in domestic dogs and black-backed jackals, it appears the same *B. rossi* is occurring in the two hosts. Although further elucidations are recommended using a *B. rossi* specific gene such as BREMA 1 gene. The current general assumption is that *B. rossi* is not host specific. Due to the conserved nature of the *B. rossi* 18S rRNA gene in domestic dogs and black-backed jackals possible genotype differences in the two hosts could not be revealed. The general recommendation is that the 18S rRNA gene marker should be used concurrently with a more diverse gene marker such as ITS1 / ITS 2 genes to reveal more of the parasite phylogenetics.This study was able to indicate that wild canids and domestic dogs share similar tick-borne parasites of importance. This highlights the importance of tick control, especially in domestic dogs as they are more susceptible to these tick-borne pathogens that are less problematic to wild canids.

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APPENDICES

Appendix 1: Table showing the detection of haemoparasites from blood specimens of domestic dogs at the outpatient clinic of OVAH, free-ranging black-backed jackals from Mogales Gate Biodiversity Centre and captive black-backed jackals from S.A. Lombard Nature Reserve, free-ranging African wild dogs from Kruger National Park and captive African wild dogs from the National Zoological Gardens.

Domestic dogs samples batch # 1.Membrane: Vorster 3.16: 26/Oct/2016	batch # 1: membrane: Vorster 3.16. 26/Oct/2016		z								
Lab number	Sample ID	*Ehrlichia / Anaplasma*	*Anaplasma .centrale*	*Theileria / Babesia*	*Babesia 1 genus*	*Babesia 2 genus*	*Babesia bovis*	*Babesia rossi*	*Theileria* sp (Sable)	*Babesia vogeli*	Location
RLB1/16	BC169	0	0	1	1	0	0	1	0	0	OVAH
RLB2/16	BC170	0	0	0	0	0	0	0	0	0	OVAH
RLB3/16	BC171	0	0	1	1	0	0	1	0	0	OVAH
RLB4/16	BC175	0	0	1	1	0	0	1	0	0	OVAH
RLB5/16	BC176	0	0	1	1	0	0	1	0	0	OVAH
RLB6/16	Bc179	0	0	1	1	0	0	1	0	0	OVAH
RLB7/16	Bc180	0	0	1	1	0	0	1	0	0	OVAH
RLB8/16	Bc181	0	0	0	0	0	0	0	0	0	OVAH
RLB9/16	Bc182	0	0	1	1	0	0	1	0	0	OVAH
RLB10/16	Bc183	0	0	0	0	0	0	0	0	0	OVAH
RLB11/16	Bc186	0	0	1	1	0	0	1	0	0	OVAH

RLB 12/16	Bc187	0	0	1	1	0	0	1	0	0	OVAH
RLB13/16	Bc188	0	0	1	1	0	0	1	0	0	OVAH
RLB14/16	Bc189	0	0	1	1	0	0	1	0	0	OVAH
RLB15/16	Bc190	0	0	1	0	0	0	0	0	0	OVAH
RLB14/16	Bc191	0	0	1	1	0	0	1	0	0	OVAH
RLB18/16	Bc 193	0	0	1	0	0	0	0	0	0	OVAH
RLB19/16	Bc 194	0	0	1	1	0	0	0	0	0	OVAH
RLB20/16	Bc195	0	0	0	0	0	0	0	0	0	OVAH
RLB21/16	Bc197	0	0	1	1	0	0	1	0	0	OVAH
RLB22/16	Bc198	0	0	0	0	0	0	0	0	0	OVAH
RLb23/16	Bc200	0	0	1	1	0	0	1	0	0	OVAH
RLB24/16	Bc201	0	0	1	1	0	0	1	0	0	OVAH
RLB25/16	Bc203	0	0	1	1	0	0	1	0	0	OVAH
RLB26/16	Bc205	0	0	1	1	0	0	1	0	0	OVAH
RLB27/16	Bc206	0	0	1	1	0	0	1	0	0	OVAH
RLB28/16	Bc207	0	0	1	1	0	0	1	0	0	OVAH
RLB29/16	Bc208	0	0	1	1	0	0	1	0	0	OVAH
RLB30/16	Bc209	0	0	1	1	0	0	1	0	0	OVAH
RLB31/16	Bc210	0	0	1	1	0	0	1	0	0	OVAH
RLB32/16	Bc211	0	0	1	1	0	0	1	0	0	OVAH
RLB33/16	Bc213	0	0	1	1	0	0	1	0	0	OVAH
RLB34/16	Bc243	0	0	1	1	0	0	1	0	0	OVAH
RLB35/16	Bc217	0	0	0	0	0	0	0	0	0	OVAH
RLB36/16	Bc219	0	0	1	1	0	0	1	0	0	OVAH
RLB37/16	Bc221	0	0	1	1	0	0	1	0	0	OVAH
RLB38/16	Bc222	0	0	1	1	0	0	1	0	0	OVAH
Neg		0	0	0	0	0	0	0	0	0	
E/A & T/B		1	1	1	1	1	1	0	0	0	

Domestic dogs Batch # 2 , Membrane : Vorster 3.16:
28/Oct/2016

Lab number	Sample ID	*Ehrlichia / Anaplasma*	*Anaplasma centrale*	*Theileria / Babesia*	*Babesia* 1 genus	*Babesia* 2 genus	*Babesia bovis*	*Babesia rossi*	*Theileria* sp.(Sable)	*Babesia vogeli*	Location
RLB 39/16	Bc271	0	0	1	1	0	0	1	0	0	OVAH
RLB 40/16	Bc273	0	0	1	1	0	0	1	0	0	OVAH
RLB 41/16	Bc277	0	0	1	1	0	0	1	0	0	OVAH
RLB 42/16	Bc278	0	0	1	1	0	0	1	0	0	OVAH
RLB 43/16	Bc283	0	0	1	1	0	0	1	1	0	OVAH
RLB 44/16	Bc286	0	0	1	1	0	0	1	1	0	OVAH
RLB 45/16	Bc288	0	0	1	1	0	0	1	0	0	OVAH
RLB46/16	Bc289	0	0	1	1	0	0	1	1	0	OVAH
RLB47/16	Bc290	0	0	1	1	0	0	1	1	0	OVAH
RLB48/16	Bc291	0	0	1	1	0	0	1	1	0	OVAH
RLB50/16	Bc292	0	0	1	1	0	0	1	0	0	OVAH
RLB51/16	Bc293	0	0	1	1	0	0	1	1	0	OVAH
RLB52/16	Bc261	0	0	1	1	0	0	1	1	0	OVAH
RLB53/16	Bc294	0	0	1	1	0	0	1	0	0	OVAH
RLB54/16	Bc296	0	0	1	1	0	0	1	1	0	OVAH
RLB55/16	Bc301	0	0	1	1	0	0	1	0	0	OVAH
RLB56/16	Bc304	0	0	1	1	0	0	1	0	0	OVAH
RLB57/16	Bc305	0	0	1	1	0	0	1	1	0	OVAH
RLB58/16	Bc306	0	0	1	1	0	0	1	0	0	OVAH
RLB59/16	Bc307	0	0	1	1	0	0	1	1	0	OVAH
RLB60/16	Bc308	0	0	1	1	0	0	1	0	0	OVAH
RLB61/16	Bc309	0	0	1	1	0	0	1	0	0	OVAH
RLB62/16	Bc310	0	0	1	1	0	0	1	0	0	OVAH
RLB63/16	Bc311	0	0	1	1	0	0	1	0	0	OVAH

RLB64/16	Bc302	0	0	1	1	0	0	1	0	0	OVAH
RLB65/16	Bc316	0	0	1	1	0	0	1	0	0	OVAH
RLB70/16	Bc315	0	0	1	1	0	0	1	0	0	OVAH
RLB71/16	Bc314	0	0	1	1	0	0	1	0	0	OVAH
RLB72/16	Bc276	0	0	1	1	0	0	1	0	0	OVAH
RLB73/16	Bc223	0	0	1	1	0	0	1	0	0	OVAH
RLB74/16	Bc224	0	0	1	1	0	0	1	0	1	OVAH
RLB75/16	Bc225	0	0	1	1	0	0	1	0	0	OVAH
RLB76/16	Bc227	0	0	1	1	0	0	1	0	0	OVAH
RLB77/16	Bc228	0	0	1	1	0	0	1	0	0	OVAH
RLB78/16	Bc231	0	0	1	1	0	0	1	0	0	OVAH
RLB79/16	Bc233	0	0	1	1	0	0	1	0	0	OVAH
RLB80/16	Bc237	0	0	1	1	0	0	1	0	0	OVAH
RLB81/16	Bc238	0	0	1	1	0	0	1	0	0	OVAH
E/A&T/B +control		1	1	1	1	1	1	0	0	0	
Neg control		0	0	0	0	0	0	0	0	0	

Black-backed jackals

Batch # 1:10/ 06/ 2017

Lab Number	Sample ID	Canine probes detected							Location
		Ehrlichia / Anaplasma	*Anaplasma centrale*	*Theileria / Babesia*	*Babesia* 1 genus	*Babesia* 2 genus	*Babesia bovis*	*Babesia rossi*	
RLB43/16	positive control	1	1	1	1	1	1	0	
RLB44/16	negative control	0	0	0	0	0	0	0	
RLB 1/16	No 44	1	0	0	0	0	0	0	S.A Lombard nature reserve
RLB 2/16	No 40	1	0	0	0	0	0	0	S.A Lombard nature reserve
RLB 3/16	No35	0	0	0	0	0	0	0	S.A Lombard nature reserve
RLB4/16	No51	1	0	0	0	0	0	0	S.A Lombard nature reserve
RLB5/16	No46	1	0	1	1	1	0	1	S.A Lombard nature reserve
RLB6/16	M15	1	0	0	0	0	0	0	Mogales Gate Biodiversity Center
RLB7/16	M81	0	0	0	1	0	0	0	Mogales Gate Biodiversity Center
RLB8/16	M80	0	0	0	1	0	0	0	Mogales Gate Biodiversity Center
RLB9/16	M78	1	0	0	1	0	0	0	Mogales Gate Biodiversity Center
RLB10/16	M77	0	0	1	1	1	0	1	Mogales Gate Biodiversity Center
RLB11/16	M76	1	0	1	1	1		1	Mogales Gate Biodiversity Center
RLB12/16	M46	1	0	1	1	1	0	1	Mogales Gate Biodiversity Center
RLB13/16	M85	0	0	0	1	0	0	0	Mogales Gate Biodiversity Center
RLB14/16	M74	0	0	0	1	1	0	0	Mogales Gate Biodiversity Center
RLB15/16	M82	0	0	0	1	0	0	0	Mogales Gate Biodiversity Center
RLB16/16	M84	1	0	0	1	0	0	0	Mogales Gate Biodiversity Center
RLB17/16	M2	1	0	0	1	0	0	0	Mogales Gate Biodiversity Center
RLB18/16	M27	1	0	0	1	0	0	0	Mogales Gate Biodiversity Center
RLB19/16	M90	0	0	0	1	0	0	0	Mogales Gate Biodiversity Center
RLB20/16	M66	1	0	1	1	1	0	1	Mogales Gate Biodiversity Center

RLB21/16	M51	0	0	1	1	1	0	1	Mogales Gate Biodiversity Center
RLB22/16	M39	0	0	0	1	0	0	0	Mogales Gate Biodiversity Center
RLB23/16	M33	1	0	0	1	0	0	0	Mogales Gate Biodiversity Center
RLB24/16	M35	0	0	0	1	0	0	0	Mogales Gate Biodiversity Center
RLB25/16	1583/14	1	0	0	0	0	0	0	Mogales Gate Biodiversity Center
RLB26/16	M88	1	0	0	1	0	0	0	Mogales Gate Biodiversity Center
RLB27/16	M90	0	0	0	1	0	0	0	Mogales Gate Biodiversity Center
RLB28/16	M87	0	0	0	1	0	0	0	Mogales Gate Biodiversity Center
RLB29/16	M91	1	0	0	1	0	0	0	Mogales Gate Biodiversity Center
RLB30/16	M92	0	0	0	1	0	0	0	Mogales Gate Biodiversity Center
RLB31/16	M75	1	0	1	1	1	0	1	Mogales Gate Biodiversity Center
RLB32/16	M59	0	0	0	0	1	0	0	Mogales Gate Biodiversity Center
RLB33/16	M53	0	0	0	1	0	0	0	Mogales Gate Biodiversity Center
RLB34/16	M47	1	0	1	1	1	0	1	Mogales Gate Biodiversity Center
RLB35/16	M37	0	0	1	1	1	0	1	Mogales Gate Biodiversity Center
RLB36/16	M58	1	0	0	1	0	0	0	Mogales Gate Biodiversity Center
RLB37/16	M65	1	0	1	1	0	0	1	Mogales Gate Biodiversity Center
RLB38/16	M22	1	0	0	1	0	0	0	Mogales Gate Biodiversity Center
RLB39/16	M26	1	0	0	1	0	0	0	Mogales Gate Biodiversity Center
RLB40/16	M67	0	0	1	1	0	0	1	Mogales Gate Biodiversity Center

Black-backed jackals
Batch # 2 (14/07/16)

				Canine probes detected					
Lab number	Old lab number	*Ehrlichia* / *Anaplasma*	*Anaplasma centrale*	*Theileria* / *Babesia* genus	*Babesia* 1 genus	*Babesia* 2 genus	*Babesia bovis*	*Babesia rossi*	Location
RLB41/16	RLB1482	1	0	0	0	0	0	0	Mogales Gate Biodiversity Centre
RLB42/16	RLB1483	0	0	0	1	1	0	0	Mogales Gate Biodiversity Centre

RLB43/16	RLB1484	1	0	0	1	0	0	0	Mogales Gate Biodiversity Centre
RLB44/16	RLB1485	1	0	1	1	1	0	1	Mogales Gate Biodiversity Centre
RLB45/16	RLB1487	1	0	1	1	1	0	1	Mogales Gate Biodiversity Centre
RLB46/16	RLB1489	1	0	0	1	0	0	0	Mogales Gate Biodiversity Centre
RLB47/16	RLB1492	1	0	0	1	0	0	0	Mogales Gate Biodiversity Centre
RLB48/16	RLB1493	0	0	1	1	1	0	1	Mogales Gate Biodiversity Centre
RLB49/16	RLB1494	1	0	0	1	1	0	0	Mogales Gate Biodiversity Centre
RLB50/16	RLB1495	1	0	0	1	0	0	0	Mogales Gate Biodiversity Centre
RLB51/16	RLB1496	1	0	0	1	0	0	0	Mogales Gate Biodiversity Centre
RLB52/16	RLB1498	0	0	0	1	0	0	0	Mogales Gate Biodiversity Centre
RLB53/16	RLB1500	0	0	1	1	1	0	1	Mogales Gate Biodiversity Centre
RLB54/16	RLB1502	0	0	0	1	0	0	0	Mogales Gate Biodiversity Centre
RLB55/16	RLB1505	1	0	1	1	1	0	1	Mogales Gate Biodiversity Centre
RLB56/16	RLB1506	0	0	1	1	1	0	1	Mogales Gate Biodiversity Centre
RLB57/16	RLB1507	1	0	0	1	0	0	0	Mogales Gate Biodiversity Centre
RLB58/16	RLB1508	0	0	0	1	1	0	0	Mogales Gate Biodiversity Centre
RLB59/16	RLB1509	1	0	1	1	1	0	1	Mogales Gate Biodiversity Centre
RLB60/16	RLB1512	0	0	0	1	0	0	0	Mogales Gate Biodiversity Centre
RLB61/16	RLB1514	1	0	1	1	1	0	1	Mogales Gate Biodiversity Centre
RLB62/16	RLB1516	0	0	1	1	1	0	1	Mogales Gate Biodiversity Centre
RLB63/16	RLB1518	0	0	0	0	0	0	0	Mogales Gate Biodiversity Centre
RLB64/16	RLB1520	0	0	0	0	0	0	0	Mogales Gate Biodiversity Centre
RLB65/16	RLB1521	1	0	0	1	0	0	0	Mogales Gate Biodiversity Centre
RLB66/16	RLB1524	0	0	0	0	0	0	0	Mogales Gate Biodiversity Centre
RLB67/16	RLB1525	0	0	0	1	0	0	0	Mogales Gate Biodiversity Centre
RLB68/16	RLB 1526	0	0	0	1	0	0	0	Mogales Gate Biodiversity Centre
RLB69/16	RLB1527	1	0	0	1	0	0	0	Mogales Gate Biodiversity Centre
RLB70/16	RLB1528	0	0	0	1	0	0	0	Mogales Gate Biodiversity Centre
RLB71/16	RLB1531	1	0	0	1	0	0	0	Mogales Gate Biodiversity Centre
RLB72/16	RLB1532	0	0	0	1	1	0	0	Mogales Gate Biodiversity Centre

RLB73/16	RLB1533	1	0	0	1	1	0	0	Mogales Gate Biodiversity Centre
RLB74/16	RLB1534	0	0	0	1	0	0	0	Mogales Gate Biodiversity Centre
RLB75/16	RLB1535	0	0	1	1	1	0	1	Mogales Gate Biodiversity Centre
RLB76/16	RLB1536	1	0	1	1	1	0	1	Mogales Gate Biodiversity Centre
RLB77/16	RLB1537	0	0	0	1	0	0	0	Mogales Gate Biodiversity Centre
RLB78/16	RLB1543	1	0	1	1	1	0	1	Mogales Gate Biodiversity Centre
RLB79/16	RLB1547	1	0	0	1	0	0	0	Mogales Gate Biodiversity Centre
RLB80/16	RLB1550	1	0	0	1	0	0	0	Mogales Gate Biodiversity Centre
RLB81/16	Negative control	0	0	0	0	0	0	0	
RLB82/16	Positive control	1	1	0	1	1	1	0	

Black-backed jackals
3RD batch 27/07/2016

Lab number	Sample ID	Ehrlichia / Anaplasma genus	Anaplasma centrale	*Theileria / Babesia*	*Babesia* 1 genus	*Babesia* 2 genus	*Babesia bovis*	*Babesia rossi*	Location
RLB 83/16	44/12	1	0	1	1	0	0	1	S.A Lombard
RLB84/16	45/12	0	0	0	1	0	0	0	S.A Lombard
RLB85/16	46/12	0	0	0	0	0	0	0	S.A Lombard
RLB 86/16	47/12	0	0	0	0	0	0	0	S.A Lombard
RLB87/16	48/12	1	0	1	1	0	0	1	S.A Lombard
RLB88/16	49/12	0	0	1	1	0	0	1	S.A Lombard
RLB89/16	50/12	0	0	0	1	0	0	0	S.A Lombard
RLB90/16	51/12	0	0	0	0	0	0	0	S.A Lombard
RLB91/16	52/12	0	0	1	1	1	0	1	S.A Lombard
RLB92/16	53/12	0	0	0	0	0	0	0	S.A Lombard
RLB93/16	54/12	1	0	0	0	0	0	0	S.A Lombard
RLB94/16	55/12	0	0	0	0	0	0	0	S.A Lombard
RLB95/16	56/12	1	0	1	1	0	0	1	S.A Lombard

RLB96/16	57/12	0	0	0	0	0	0	0	S.A Lombard
RLB97/16	334/12	0	0	0	0	0	0	0	S.A Lombard
RLB98/16	336/12	0	0	0	0	0	0	0	S.A Lombard
RLB99/16	337/12	0	0	0	0	0	0	0	S.A Lombard
RLB100/16	348/12	1	0	1	1	0	0	1	S.A Lombard
RLB101/16	1551/12	0	0	0	1	0	0	0	S.A Lombard
RLB102/16	1553/12	1	0	0	0	0	0	0	S.A Lombard
RLB103/16	1557/14	0	0	0	1	0	0	0	Mogales Gate Biodiversity Center
RLB104/16	1488/14	1	0	0	0	0	0	0	Mogales Gate Biodiversity Center
RLB105/16	Negative control	0	0	0	0	0	0	0	
RLB107/16	Positive control	1	1	1	1	1	1	0	

Wild dogs samples RLB results

Lab number	Sample ID	*Ehrlichia / Anaplasma* genus	*Anaplasma central*	*Theileria/ Babesia* genus	*Babesia* 1 genus	*Babesia* 2 genus	*Babesia bovis*	*Babesia rossi*	Location
Pos control		1	0	1	1	0	1	0	
Neg control		0	0	0	0	0	0	0	
RE 1		0	0	0	1	1	0	0	KNP
Re 2		0	0	0	1	1	0	0	KNP
Re 3		1	0	0	1	1	0	0	KNP
Re 4		0	0	0	1	1	0	0	KNP
Re 5		0	0	0	0	0	0	0	KNP
Re 6		0	0	0	1	1	0	0	KNP
Re 7		0	0	0	1	1	0	0	KNP
Re 8		0	0	0	1	1	0	0	KNP
Re 9		0	0	0	1	1	0	0	KNP
Re 10		0	0	0	1	1	0	0	KNP

Re 11	1	0	0	1	1	0	0	KNP
Re 12	0	0	0	1	1	0	0	KNP
Re 13	0	0	0	1	0	0	0	KNP
Re 14	0	0	0	1	1	0	0	KNP
Re 15	0	0	0	0	0	0	0	KNP
Re 16	0	0	0	1	1	0	0	KNP
Re 18	0	0	0	1	0	0	0	KNP
Re 19	0	0	0	1	1	0	0	KNP
Re 20	1	0	0	1	1	0	0	KNP
Re 21	0	0	0	1	1	0	0	KNP
Re 22	0	0	0	1	0	0	0	KNP
Re 23	0	0	0	1	1	0	0	KNP
Re 24	0	0	0	1	1	0	0	KNP
Re 25	0	0	0	1	0	0	0	KNP
Re 26	0	0	0	1	1	0	0	KNP
Re 27	0	0	0	0	0	0	0	KNP
RE 28	0	0	1	1	0	0	1	KNP
RE 29	0	0	0	1	0	0	0	KNP
Re 30	0	0	0	0	0	0	0	KNP
RE31	0	0	0	1	0	0	0	KNP
RE32	0	0	0	1	0	0	0	KNP
RE33	0	0	0	1	0	0	0	KNP
RE34	0	0	0	1	0	0	0	KNP
Re35	0	0	0	1	0	0	0	KNP
RE36	0	0	0	1	0	0	0	KNP
Re37	0	0	0	1	0	0	0	KNP
RE38	0	0	0	1	0	0	0	KNP
Re39	0	0	0	1	0	0	0	KNP
Re40	0	0	1	1	0	0	1	KNP
Re41	0	0	0	0	0	0	0	KNP

RE42	0	0	0	0	0	0	1	KNP
Re43	0	0	0	0	0	0	0	KNP
Re44	0	0	1	1	0	0	1	KNP
Re45	0	0	0	1	0	0	0	KNP
Re46	0	0	0	1	0	0	0	KNP
Re47	0	0	0	1	0	0	0	KNP
Re48	0	0	0	1	0	0	0	KNP
Re49	0	0	0	1	0	0	0	KNP
Re50	0	0	0	1	0	0	0	KNP
Re51	0	0	0	1	0	0	0	KNP
Re52	0	0	1	1	0	0	1	KNP
Re53	0	0	0	0	0	0	0	KNP
RE 17/192	0	0	0	0	0	0	0	NZG
RE 17/193	0	0	0	0	0	0	0	NZG
RE 17/194	0	0	0	0	0	0	0	NZG
RE 17/195	0	0	0	0	0	0	0	NZG
RE 17/196	0	0	0	0	0	0	0	NZG
RE 17/197	0	0	0	0	0	0	0	NZG
Pos control	1	1	1	1	1	1	0	
Neg control	0	0	0	0	0	0	0	

Appendix 2 : Permission to conduct research in terms of seaction 20 of the Animal Disease ACT, 1984 , (ACT No. 35 of 1984), for the Research project Occurrence of *Babesia rossi* in domestic dogs, black-backed jackals and African wild dogs in South Africa.

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/6

Professor Barend Penzhorn

Department of Tropical Diseases

University of Pretoria

Email: banie.penzhorn@up.ac.za

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Dear Professor Barend Penzhorn

Your fax / memo / letter/ Email dated 10 November 2016, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions :

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. The study must be conducted in compliance with the Veterinary and Para-Veterinary Professions Act 1982 (Act No. 19 of 82);
4. Samples must be packaged and transported in accordance with International Air Transport Association (IATA) requirements and/or the National *Road Traffic Act*, 1996 (*Act* No. 93 of 1996);

- 1 -

5. This permit is only valid for sampling in the areas of Mogale's Gate, Hekpoort, Gauteng province that have supplied a State Veterinary letter of no restriction to the researcher. No sampling may take place in any other area or province without written permission from the Director: Animal Health;
6. Study must be conducted in compliance with all the relevant permissions from the local nature conservation authorities;
7. No part of the study may continue until the relevant, valid ethical approval has been obtained from the relevant authority;
8. All black backed jackels EDTA blood samples must travel under the cover of Red Cross Permit issued by the State Veterinarian of the area of origin and in full compliance with all the conditions of such a permit;
9. Samples taken from dead black backed jackels must test negitive for rabies prior to testing for Babesia rossi, Theileria, Ehrlichia and Anaplasma.

Title of research/study: Prevalence and genetic diversity of Babesia rossie infecting free-ranging black backed jackels (Canis mesomelas)

Researcher (s): Professor Barend Penzhorn

Institution: Department of Tropical Diseases, University of Pretoria

Our ref Number: 12/11/1/1/6

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2016-11-21

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/6

Professor Barend Penzhorn

Department of Tropical Diseases

University of Pretoria

RE: DISPENSATION ON SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "PREVALENCE AND GENETIC DIVERSITY OF BABESIA ROSSIE INFECTING FREE-RANGING BLACK BACKED JACKELS (CANIS MESOMELAS)"

A dispensation is hereby granted on Point 2 of the Section 20 approval that was issued for the above mentioned study (attached):

i) Extracted DNA obtained from black backed jackel blood samples must be stored at the access controlled Department of Tropical Diseases, University of Pretoria

ii) Stored samples may not be outsourced without prior written approval from DAFF.

iii) Should samples be used for further research, written approval from the Director of Animal Health must be obtained prior to start of project

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2016-11-21

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ANIMAL USE AND CARE COMMITTEE: APPLICATION FOR APPROVAL

A. PROJECT DETAILS

Project Title	Targeted vaccination and health survey of the Kruger National Park African wild dog population south of the Olifants River		
Researcher	Dr Louis van Schalkwyk	SANParks Reference No.	013/16

B. SCIENTIFIC REVIEW STATEMENT

(Every application should be supported by a declaration that it has undergone prior scientific review through at least one of the SANParks Research Nodes.)

This research protocol has been reviewed by the Savanna and / or Arid Research Centres SANParks and has been judged to be of national importance, designed in accordance with accepted scientific practices and norms and is in the opinion of the reviewers likely to be successful in achieving its objective.

Name: [illegible] Designation: [illegible] Signature: [signature] Date: 15/7/2016

As per SANParks Research Approval process

Note: In accordance with the South African National Standard (SANS 10386-2008) "The Care and Use of Animals for Scientific Purposes", an animal is regarded as being "live, sentient non-human vertebrate, including eggs, foetuses and embryos, that is, fish, amphibians, reptiles, birds and mammals, including domestic animals, purpose-bred animals, farm animals, wildlife and higher invertebrates such as advanced members from the Cephalopoda and Decapoda".

This form should be submitted with the SANParks standard Research Project Application, and (where relevant) the following supporting documents: CVs of practitioners in support of competence to handle or treat animals, notices of approval of other ethics committees, diagrams or references illustrating the equipment and/or techniques to be applied.

For Administrative Purposes			
Submission Date	14th July, 2016	APPROVED	DISAPPROVED
AUCC approval / Disapproval Date	15/7/2016	Signature [signature]	
Reason for Decision	The study fully meets ethical standards		

643 Leyds Street
MUCKLENEUK
0002

P.O. Box 787
PRETORIA
0001

Tel: 012 426-5000

central reservations: 012 428 9111
reservations@sanparks.org
www.sanparks.org

Appendix 3: Permission to use the *Babesia* life cycle image from an article.

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Dear Ms. Ntji Shabangu,

Thank you for placing your order through Copyright Clearance Center's RightsLink® service.

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Appendix 4: Animal Ethics Approval certificate issued by the University of Pretoria to conduct research.

UNIVERSITEIT VAN PRETORIA
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Animal Ethics Committee

PROJECT TITLE	**Occurrence of *Babesia rossi* in domestic dogs, black-backed jackals and African wild dogs in South Africa.**
PROJECT NUMBER	**V031-17 (Amendment 1)**
RESEARCHER/PRINCIPAL INVESTIGATOR	**N Shabangu**

STUDENT NUMBER (where applicable)	**U_16316186**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL BLOOD SAMPLES	**Dogs**	**African Wild-dogs**	**Black-backed jackals**
NUMBER OF SAMPLES	**80**	**80**	**82**
Approval period to use animals for research/testing purposes			**November 2018- November 2019**
SUPERVISOR	**Prof. PT Matjila**		

KINDLY NOTE:

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

APPROVED	Date 5 November 2018
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15