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Title	Molecular investigation of tick-borne protozoan parasites at the livestock- wildlife interface in Kenya and evaluation of a candidate anti-tick vaccine antigen [an abstract of entire text]
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Degree Grantor	北海道大学
Degree Name	博士(獣医学)
Dissertation Number	甲第11071号
Issue Date	2013-09-25
Doc URL	https://hdl.handle.net/2115/53836
Type	doctoral thesis
File Information	Naftaly_Githaka_summary.pdf



要 約

MOLECULAR INVESTIGATION OF TICK-BORNE PROTOZOAN PARASITES AT THE LIVESTOCK- WILDLIFE INTERFACE IN KENYA AND EVALUATION OF A CANDIDATE ANTI-TICK VACCINE ANTIGEN

(ケニア共和国の家畜および野生動物緩衝地帯における
ダニ媒介性原虫の分子生物学的解析と抗ダニワクチンの開
発)

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September 2013

AUTHOR'S DECLARATION

The studies presented in this thesis are my original work and have not been presented at any other University for the award of a degree. Parts of this thesis have been published in advance in the following publications:

1. Molecular detection and characterization of potentially new *Babesia* and *Theileria* species/variants in wild felids from Kenya.
Githaka N, Konnai S, Kariuki E, Kanduma E, Murata S, Ohashi K. *Acta Tropica*, 124: 71-78, 2012.
2. Extensive genotypic variations in field isolates of *Theileria* species infecting the giraffes (*Giraffa camelopardalis tippelskirchi* and *G. c. reticulata*) in Kenya.
Githaka N, Konnai S, Kariuki E, Skilton R, Kanduma E, Murata S, Ohashi K, *Parasitology International*, 62: 448-453, 2013.
3. Identification and sequence characterisation of new *Theileria* species from the waterbuck (*Kobus defassa*) in a *Theileria parva*-endemic area in Kenya.
Githaka N, Konnai S, Bishop R, Odongo D, Lekolool I, Kamau L, Gakuya F, Isezaki M, Murata S, Ohashi K, *Veterinary Parasitology*, in press.

DEDICATION

This thesis is dedicated to my family for always being there for me.

ACKNOWLEDGEMENTS

The completion of the studies detailed in this thesis was only possible due to the support of many people that I worked with. Firstly, I would like to say special thanks to my supervisors, Prof. Kazuhiko Ohashi, Associate Prof. Satoru Konnai, and Assistant Prof. Shiro Murata for offering me a place in their lab as a graduate student, and their guidance throughout these studies. I will always treasure this opportunity. I would also want to acknowledge Prof. Chihiro Sugimoto for introducing me to Hokkaido University through the GCOE training program that led to the current studentship, and for reviewing this thesis. Thanks also to Prof. Ken Katakura for his helpful suggestions on this thesis. I am very thankful to the Japanese government for the MEXT scholarship that supported my stay in Japan and the current studies.

I owe a lot of gratitude to past and present members of the Laboratory of Infectious Diseases for their generosity in teaching me how to do things the Japanese way. In particular, I am indebted to Dr. Masayoshi Isezaki for his generous technical guidance in the laboratory, and to fellow students, past and present, for making my stay here memorable. In the same vein, I would like to acknowledge the superb discussions and working moments shared with the members of the two Brazilian collaborating groups whom I worked with at different time points led by Associate Prof. Itabajara da Silva Vaz Junior. Once again, thank you Drs. Luis, Leo, Adriana, Jorge, Helga, and Associate Prof. Carlos Logullo.

I received immense support from colleagues in Kenya during sample collection and subsequent phases of this thesis. In that regard, I am especially grateful to Dr. Edward Kariuki of Kenya Wildlife Service Veterinary department for assistance with wildlife

samples. Thanks to Dr. David Odongo for insightful discussions on ECF and providing us with the cattle samples from Marula. Special thanks to Drs. Richard Bishop and Robert Skilton of International Livestock Research Institute (ILRI) and Bioscience for east and central Africa (BecA), respectively, for their collaboration on various aspects of my studies. The skills and understanding of parasites and tick vectors acquired under your mentorship at ILRI have been invaluable in undertaking the present studies.

Last but not least, I would like to appreciate my parents, my siblings, and relatives for always praying for me during these tough years. In particular, I dedicate this work to my grandmother, cûcû Wangeci, for the years she have spent on her knees praying for my success in education. May God bless you and give you a long life. Thank you Almighty God for bringing me this far, Lord you are tremendous to me.

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ABBREVIATIONS

BLAST	-	Basic local alignment search tool
ECF	-	East Coast fever
ITS	-	Internal transcribed spacer
IUCN	-	International Union for the Conservation of Nature
KWS	-	Kenya Wildlife Service
PCR	-	Polymerase chain reaction
RLB	-	Reverse line blot
RNAi	-	RNA interference
RACE	-	Rapid amplification of cDNA ends
SDS-PAGE	-	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
18S rRNA	-	18S ribosomal RNA
SSU rRNA	-	Small subunit ribosomal RNA
TBDs	-	Tick-borne diseases
V4	-	Variable region 4

PREFACE

Ticks are haematophagous obligate arthropods that parasitize every class of vertebrates in almost every region of the world. They have been recognized as human and animal parasites for thousands of years, having been described by ancient Greek writers, including Homer and Aristotle (Sonenshine, 1991). They transmit a great variety of pathogenic microorganisms; protozoa, rickettsia, bacteria, viruses and fungi. Eight hundred and seventy one species or subspecies of ticks have been recorded (Sonenshine, 1991; Camicas et al., 1998).

Ticks and mites are currently grouped with members of the subclass *Acari*, which is the largest subclass in the class *Arachnida*. There are three families of ticks: (1) *Ixodidae* or “hard ticks” so called because of their sclerotized dorsal plate, and is the most important family both in numeric terms and in medical importance, (2) *Argasidae* and (3) *Nutelliellidae*. The *Ixodidae* family contains more than 650 species in four subfamilies and thirteen genera. In the commonly used classification of ticks, the family *Ixodidae* comprises of two major groups, the *Prostriata* and the *Metastricata*. Two subfamilies are recognized in the *Argasidae*: the *Argasinae* and the *Omithodorinae* (Klompen and Oliver, 1993). The *Nutelliellidae* family contains only one species in one genus. The lone species *Nuttalliella namaqua* parasitizes hyraxes in South Africa, and is of minor veterinary or medical importance. In many parts of Africa, multiple tick species of veterinary and medical importance are found, including numerous species of the genus *Rhipicephalus*. Consequently, tick-borne diseases (TBDs) are a major impediment to social-economic development of the continent where many people depend on livestock for their livelihoods. In Japan, ticks in the genus *Ixodes* such as *Ixodes persulcatus* and *Ixodes ovatus* are of medical relevance (Murase et al., 2011; Konnai et al., 2008). Ixodid ticks are particularly competent vectors of pathogens due to their relatively longer feeding periods (several days) during which they remain firmly attached to thier host

with painless bites that may go unnoticed for lengthy periods of time (Sonenshine, 1991).

The genera *Theileria* and *Babesia*, collectively referred to as piroplasms, are haemoprotozoan parasites of the phylum Apicomplexa that are transmitted by ticks to wild and domestic animals (Bishop et al., 2004; Penzhorn, 2006). Although a majority of piroplasms are non-pathogenic, infecting their host benignly, others are highly pathogenic in both domestic and game animals causing piroplasmoses such as East Coast fever (ECF), tropical theileriosis and babesiosis (Penzhorn, 2011; Bishop et al., 2004). Although the life cycles of both *Theileria* and *Babesia* involve mammalian and vector stages, they differ in the cell subsets that they invade in the vertebrate hosts, the specific tick vectors that transmit them, as well as the mode of transmission (Brayton et al., 2007).

Wildlife, including felids and bovids, are competent hosts for blood-borne parasites (Irvin et al., 1996). In addition, a lot of emerging and re-emerging diseases comprise of pathogens that are zoonotic, and wildlife species play an important role as reservoirs of causative agents of diseases (Parola and Raoult, 2001). Thus, understanding the occurrence, identity and prevalence of such organisms is critical in their control especially at the wildlife-livestock interface.

Molecular assays, primarily those based on application of polymerase chain reaction (PCR), allow the detection and identification of pathogens with enhanced specificity and sensitivity compared to the traditional methods of microscopy and serology (Criado-Fornelio, 2007). Various modifications to PCR have been developed to enhance its utility in clinical/veterinary diagnostics and in pathogenic identification. Reverse line blot (RLB) is a practical diagnostic and epidemiological tool for identifying haemoparasites (Gubbels et al., 1999; Tait and Oura, 2004) that enables screening of a large number of samples for multiple parasite species at low cost and in parallel (Kong et al., 2006). RLB hybridization assay is capable of detecting up to 43 molecular targets in 43 specimens using one multiplex PCR reaction followed by probe

hybridization. Oligonucleotide probes are 5' amine-labeled to allow fixation to a nylon membrane, which is re-usable up to 20 times. Primers are 5' biotin-labeled which allows detection of hybridized PCR products using streptavidin-peroxidase and a chemiluminescent substrate via photosensitive film. A combination of low setup and consumable costs (approximately US\$2 per sample), high throughput (multiple membranes can be processed simultaneously) and has a short turnaround time (approximately 10 hours) make this technique suitable for large-scale screening for pathogens even for laboratories with limited resources (Kong et al., 2006). Combined with DNA sequencing, RLB can enable the detection and identification of novel pathogens from host blood (Nijhof et al., 2003, 2005; Oosthuizen et al., 2008). The 18S ribosomal RNA (18S rRNA) is frequently used in the design of RLB DNA probes and for subsequent parasite identification through sequencing. Both 18S rRNA, and the internal transcribed spacer (ITS) region, have both conserved and variable regions that make them suitable for detection and genetic differentiation of blood parasites in the presence of mammalian DNA (Allsopp and Allsopp, 2006; Oosthuizen et al., 2008).

Besides transmitting harmful microorganisms, heavy tick infestation can cause considerable reduction in animal body weight gain, milk production, and fertility (Josson et al., 1998) through blood sucking. This combined with losses resulting from diseases amounts to huge economic losses especially in the tropic and subtropic regions where ticks are endemic (De Castro et al, 1997; de la Fuente et al., 2008). Tick control is predominantly by chemical acaracides worldwide. However, the emergence of resistant tick strains, mounting public concerns over food safety and environmental contamination, as well as the high cost of these pesticides, call for alternative vector control methods (Lovis et al., 2013). Integrated vector control management using anti-tick vaccines has already been shown to reduce the amount and frequency of acaracide use in field conditions (Willadsen and Kemp, 2003). However,

existing anti-tick vaccines are limited to few tick species and geographical. Thus, there is need to identify and evaluate tick antigens with broader application (Parizi et al., 2012).

The tick gut is an essential organ for blood digestion, and serves as the primary interface between the vector and the pathogen (Sojka et al., 2013). Structural and functional proteins in the gut that aid the complex process of digesting and absorbing a blood-meal are targets for developing anti-tick vaccines due to their ‘concealed’ nature (Willadsen, 1988; Hajdusek et al., 2010). Iron from lysis of host erythrocytes is essential for tick reproduction (Hajdusek et al., 2009); however, iron overload can be toxic to ticks leading to suppressed blood uptake whereas improper iron transport within the tick body can impair ovary development and egg production (Hajdusek et al., 2009; Galay et al., 2013). Thus, proteins that facilitate iron homeostasis are candidate antigens for anti-tick vaccine development (Hajdusek et al., 2009, 2010).

In chapter 1, published oligonucleotide probes were utilized to determine piroplasms infection in wild felids sampled in Kenya. Both RLB and sequence characterization found evidence of previously reported *Babesia* spp., and unknown *Theileria* sp. among wild feline species from Kenya. In chapter 2, genetic variation among *Theileria* spp. occurring in the giraffes was investigated. A combination of two newly designed oligonucleotide probes and analyses of the 18S rRNA genes indicated the occurrence of at least two *Theileria* populations in the giraffes from Kenya and those from Southern Africa, and extensive genetic heterogeneity within field isolates of these two groups. The work detailed in chapter 3 investigated the nature of *Theileria* infections occurring in waterbuck resident from an ECF-endemic locality in Kenya. Two detection assays for *Theileria parva*, nested PCR and RLB, found no evidence that these animals are naturally infected with *T. parva*. However, these animals were found to be prevalently infected with *Theileria* species that have not been reported before, including some that are highly similar to known pathogens of domestic animals. Overall, these studies

indicate that besides the occurrence of previously unknown haemoparasites, extensive intra-species heterogeneities exist in piroplasm species infecting wild hosts.

In chapter 4, ferritin 2 (FER2), a recently characterized soluble iron-transporter was identified from two hard ticks, *I. persulcatus* the vector for Lyme borrelia in Japan, and *Rhipicephalus appendiculatus*, a tick vector of great veterinary and economic importance to eastern, central and southern Africa. Recombinant FER2 from *I. persulcatus* was utilized in a preliminary vaccine trial that indicated this protein is highly efficacious against tick blood feeding, consistent with past studies, and justifies its inclusion in future anti-vaccines evaluation. Moreover, amino acid conservation in FER2 from unrelated tick genera makes it a suitable antigen for development of a universal anti-tick vaccine, an attractive prospect especially for African regions where multiple tick species occur.

Finally, the findings of the foregoing chapters and future prospects regarding the research questions investigated herein are summarized under the conclusion section.

PART I:
MOLECULAR INVESTIGATION OF TICK-BORNE PROTOZOAN PARASITES
AT THE LIVESTOCK-WILDLIFE INTERFACE IN KENYA

The original papers of this part appeared in *Acta Trop.* 124:71-78 (2012), *Parasitol. Int.* 62: 448-453 (2013), and *Vet. Parasitol.* in press (2013).

CHAPTER 1

**MOLECULAR DETECTION AND CHARACTERISATION OF POTENTIALLY
NEW *BABESIA* AND *THEILERIA* SPECIES/VARIANTS IN WILD FELIDS
FROM KENYA**

1.1 INTRODUCTION

The Eastern African savannah rangelands are home to several wild carnivores classified as vulnerable or threatened under the 2008 International Union for the Conservation of Nature (IUCN) Red List of Threatened Species (<http://www.iucnredlist.org>, accessed on April 2012), including the lions, leopards and cheetahs. These and other wildlife species in most parts of Africa are currently susceptible to anthropological and ecological adverse effects such as habitat loss and fragmentation (Ogutu et al., 2008), poaching (Burn et al., 2011) and infectious diseases (Nijhof et al., 2003, 2005; Oosthuizen et al., 2008). Ecotourism, is the second largest foreign exchange earner for Kenya, and is heavily dependent on the diverse wildlife fauna, including feline species. Therefore, there is urgent need to identify and monitor infectious diseases of wildlife, some of which are transmissible to livestock and humans (Thompson et al., 2009; Polley and Thompson, 2009).

Blood-borne parasites are prevalent in lions in the wild, but normally do not cause clinical disease manifestation. *Babesia leo* and *Babesia felis*, which are classified as small piroplasms (<1.5 µm) can occur as mixed infections in free-ranging lions and have been reported extensively in southern Africa (Lopez-Rebollar et al., 1999; Penzhorn, 2006; Penzhorn et al., 2001; Bosman et al., 2007), while *B. leo* has also been reported in Tanzania (Nijhof et al., 2005). Although these parasites are not known to cause clinical disease in otherwise healthy lions, recrudescence can occur in wild hosts triggered by external factors such as stress (Nijhof et al., 2005), immunosuppression as a result of co-infection with viral agents (Munson

et al., 2008) or re-introduction of immunologically naïve hand-bred animals into the wild (Adamson, 1962). *B. leo* has been reported in a South African leopard (Bosman et al., 2007) where it is not known to cause clinical disease following experimental infection (Penzhorn, 2006). In addition, a large piroplasm, *Babesia pantharae*, was previously described in a leopard in Kenya (Dennig and Brocklesby, 1972), but was never characterized at molecular level.

In cheetahs, piroplasms have been reported (Bosman et al., 2007). Prevalence of *B. felis* and *B. leo* in free-ranging cheetahs in southern Africa was reported at 7.5% (Bosman et al., 2007) whereas that of *Babesia lengau* was 28.5% (Bosman et al., 2010). In addition, a *Cytauxzoon*-like parasite was previously reported in a pair of captive juvenile cheetahs (Zinkl et al., 1981) while *Theileria*-like piroplasms were reported in cheetahs at Serengeti National Park and Ngorongoro Crater Conservation Area, Tanzania (Averbeck et al., 1990). From these and other studies, it is apparent that information on the occurrence, prevalence and genetic relationship of haemoparasites infecting wild felids in Kenya is scant and therefore, the current study was envisaged to partly address this deficiency.

Currently, molecular diagnostic assays, primarily those based on polymerase chain reaction (PCR) allow detection and diagnosis of pathogens with enhanced specificity and sensitivity compared to the traditional methods of microscopy and serology (Abdul-Ghani et al., 2012; Piron et al., 2007). Reverse line blot (RLB) is a practical epidemiological and diagnostic tool for identifying haemoparasites (Gubbels et al., 1999) that enables screening of a large number of samples for multiple parasite species both cheaply and simultaneously (Kong et al., 2006). Moreover, the 18S ribosomal RNA (18S rRNA) gene, consisting of both conserved and variable regions makes a suitable marker for detection and genetic characterisation of blood parasites in the presence of mammalian DNA (Allsopp and Allsopp, 2006; Salim et al., 2010). This study was carried out to detect and characterise haemoparasites infecting wild felids in

Kenya using parasite 18S rRNA genes and consequently, deduce their phylogenetic relationship with other related organisms. The findings herein indicate the occurrence of potentially new species or genotypes of piroplasms in all three feline species.

1.2 MATERIAL AND METHODS

1.2.1 Blood samples and preparation of genomic DNA

The animal orphanage at Kenya Wildlife Service (KWS) headquarters in Nairobi is a captive facility for rehabilitation of animals rescued from the wild. In 2009, a pair of lions kept at this facility was treated for suspected mineral deficiency and babesiosis, during which the animals were sampled for haematological investigations. In the same year, five cheetahs and two leopards were sampled during translocation to new habitats following frequent conflict with livestock farmers. The information regarding these animals is summarized in Table 1-1. Blood sampling procedures were conducted in accordance with the KWS institutional guidelines for animal welfare and the International Livestock Research Institute (ILRI) guidelines on using animals for research purposes. In all cases, blood was obtained from the brachial vein into ethylenediaminetetraacetic acid (EDTA)-vacutainers, was transported on ice to the laboratory, and was frozen at -20 °C. DNA was extracted from 500 µl of blood sample using the Wizard Genomic DNA purification Kit (Promega, Madison, WI, USA). DNA pellets were resuspended in 100 µl of DNA rehydration buffer and incubated for 1 hr at 65 °C, and stored at 4 °C until use.

1.2.2 PCR and RLB hybridization

The primers RLB F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') and RLB R2 (5'-Biotin-CTA AGA ATT TCA CCT CTA ACA GT-3') that are specific for *Theileria* and *Babesia* genera were used to amplify the hypervariable (V4) region of parasite 18S rRNA gene using a touch-down PCR recipe as previously described (Nijhof et al., 2003) with slight

modifications using the KOD-Plus high fidelity DNA polymerase kit (Toyobo, Osaka, Japan). Reagents for PCR reactions consisted of 2.5 µl of 10x KOD Plus buffer, 1µl of 25 mM MgSO₄, 1 µl of each primer (10 pmol), 2 µl of dNTPs (2.5 mM), 2 µl of DNA (100 ng/µl), and 1 µl (1.0 U/µl) of KOD Plus DNA polymerase. The volumes were made up to 25 µl with molecular biology grade water. In addition to the nine samples, DNA from cultured *Babesia gibsoni* (HUVIM isolate, kindly provided by Dr. Masahiro Yamasaki, Laboratory of Internal Medicine, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University) parasites and double-distilled water were used as positive and negative controls, respectively. The amplification steps consisted of preheating at 94 °C for 2 min, 10 cycles of 94 °C for 20 sec, 67 °C for 30 sec, and 68 °C for 30 sec with annealing temperature decreasing at every cycle by 1 °C until 57 °C. This was followed by 40 cycles of 94 °C for 20 sec, 57 °C for 30 sec and 68 °C for 30 sec using Perkin Elmer 9600 thermocycler (Applied Biosystems, Carlsbad, CA, USA). PCR amplification was confirmed by electrophoresing the PCR products through a 2% agarose gel stained with ethidium bromide, and visualised with a UV-trans-illuminator.

RLB was carried out as previous described (Gubbels et al., 1999) with modifications. A Biotodyne C membrane (Pall Life Sciences, Ann Arbor, MI, USA) was activated by incubating in 16% 1-ethyl-3-(3-dimethyl-amino-propyl) carbodiimide (EDAC) (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 10 min. The membrane was then washed with double-distilled water for about 2 min and placed on a screen-blotter (Sanplatec, Osaka, Japan). Oligonucleotide probes (Table 1-2), covalently linked with an N-(monomethoxy-trityl (MMT))-C6 amino linker were obtained from Hokkaido System Science, Japan, and reconstituted into 100pmol stocks. Ten µl of stock probes were diluted with 0.5 M NaHCO₃ to final volume of 120 µl, and bound on the activated membrane by incubating for 5 min at room temperature. The membrane was inactivated with 100 mM NaOH for exactly 8 min at

room temperature. The membrane was then washed with 100 ml of 2xSSPE/0.5% sodium dodecyl sulphate (SDS) for 5 min at 60 °C with gentle shaking. The membrane was either used immediately or stored at 4 °C in a sealed plastic containing 20 mM EDTA, pH 8. A volume of 15 µl of the PCR products, irrespective of concentration, was diluted to a final volume of 120 µl with 2xSSPE/0.1% SDS, and denatured by heating at 99.9 °C for 10 min on a Perkin Elmer 9600 thermocycler, and immediately snap-cooled on ice. A *Theileria parva* PCR sample was also included as positive control for the *Theileria*-specific probe. The denatured products were applied on the pre-prepared membrane containing DNA probes and incubated for 60 min at 42 °C. The membrane was washed twice using 2xSSPE/0.5% SDS buffer at 52 °C for 10 min. The membrane was then incubated with peroxidase-labelled NeutrAvidin (Thermo Fisher Scientific, Waltham, MA, USA) for 30-60 min at 42 °C, followed by washing twice with pre-warmed 2xSSPE/0.5% SDS for 10 min at 42 °C with gentle shaking. To detect the probe-amplicon hybridization, the membrane was incubated with 15 ml of ImmobilonTM Western Chemiluminescent horseradish peroxidase (HRP) Substrate, (Millipore, Billerica, MA, USA) for 2 min at room temperature, and the membrane exposed to Chemiluminescent imager (Bio-Rad laboratories, Hercules, CA, USA). To strip off the PCR products, the membrane was washed twice with pre-heated 1% SDS 90 °C for 30 min, and rinsed with 20 mM EDTA. The membrane was stored in 2 ml of 20 mM EDTA at 4 °C for future use.

1.2.3 Cloning and sequencing of 18S rRNA

The primers Nbab_1F (5'-AAG CCA TGC ATG TCT AAG TAT AAG CTT TT-3') and Nbab_1R (5'-CTT CTC CTT CCT TTA AGT GAT AAG GTT CAC-3') (Oosthuizen et al., 2008) were used to amplify the near full-length parasite 18S rRNA gene from the DNA samples using the KOD-Plus high fidelity DNA polymerase kit (Toyobo, Osaka, Japan). Reaction mixtures were prepared as described above, and reactions performed in quadruples

for each sample. PCR cycling conditions were as follows: pre-denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C denaturation for 15 sec, annealing at 55 °C for 30 sec, extension at 68 °C for 2 min, and hold at 4 °C. The four PCR replicates were pooled together to give 100 µl, and purified with GeneClean III Kit, (Q-BIOgene, Heidelberg, Germany) according to the manufacturer's instructions. The pooling of the PCR products is such that, the resulting amplicon contains less than 25% of any erroneous sequence arising from any of the reactions (Oosthuizen et al., 2009). Eight µl of purified PCR product was incubated with 1 µl of 2.5 mM dNTPs and 1 µl of 10x A-attachment mix (Toyobo, Osaka, Japan) for 10 min at 60 °C, followed by cloning into pGEMT-Easy vector (Promega, Madison, WI, USA) according to the manufacturer's manual instructions. Recombinant bacterial clones were identified by PCR screening with vector primers. Four recombinant colonies per isolate were grown overnight in Luria broth (LB) base medium and plasmid DNA extracted with the QIAprepTM Spin Miniprep kit (Qiagen, Hilden, Germany). DNA concentration was determined with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). 300-350 ng of plasmid DNA was subjected to sequencing PCR (5 pmol of primer) with the Quick Start kit (Beckman Coulter, Fullerton, CA, USA.) according to manufacturer's instruction, followed by ethanol-precipitation. The purified products were sequenced by CEQ 2000 Dye Terminator Cycle Sequencing with the primers Nbab_1F, Nbab_1R, BT18S_2F (5'-GGG TTC GAT TCC GGA GAG GG-3'), BT18S_3F (5'-GGG CAT TCG TAT TTA ACT GTC AGA GG-3'), BT18S_4F (5'-CGG CTT AAT TTG ACT CAA CAC GGG-3') and BT18S_4R (5'-CCC TCT CCG GAA TCG AAC CC-3') (Oosthuizen et al., 2009).

1.2.4 Sequence analysis

DNA sequences for the near-full-length 18S rRNA gene were assembled and edited manually using Geneious Pro version 5.5.6 available at <http://www.geneious.com> (Drummond et al., 2011). The new sequences were deposited in the GenBank under accession numbers

JQ861961, JQ861962, JQ861963, JQ861964, JQ861965, JQ861966, JQ861967, JQ861968, JQ861969 JQ861970, JQ861971, JQ861972, JQ861973, JQ861974, JQ861975, and JQ861976.

Basic local alignment search tool (BLAST) (Altschul et al., 1990) searches were conducted with the new sequences for related parasite. Further, the new sequences were aligned with related parasites retrieved from the Genbank using CLUSTAL W (Thompson et al., 1994) in Geneious Pro 5.5.6. The alignment was truncated manually to the size of the shortest sequence and similarity matrices performed using two-parameter method of Kimura (Kimura, 1981) alongside the Jukes-Cantor correction model for multiple base changes (Jukes and Cantor, 1969). Phylogenetic trees were constructed using neighbor-joining (Saitou and Nei, 1987) and maximum parsimony (Guindon and Gascuel, 2003) methods implemented in Geneious Pro 5.5.6, with bootstrap at 1,000 replicates with *Plasmodium falciparum* used as outgroup to root the tree in both cases.

1.3 RESULTS

The current study was conducted with blood samples obtained through opportunistic sampling of symptomatic and asymptomatic feline species (Table 1-1).

The lions were housed in an open enclosure adjacent to other wild species. Blood smears from the lion pair indicated the presence of haemoparasites of *Babesia* type according to the report by the attending veterinarian. In addition, these animals were anemic and were also diagnosed with presumptive copper deficiency characterised by neurological symptoms (wobbling). The animals recovered fully following diet supplementation and treatment with anti-*Babesia* drug, imidocarb dipropionate. The leopards and cheetahs were captured and relocated from the wild following persistent attacks on livestock in nearby farms. The animals in the latter group were all in good health condition though anxiety and alopecia associated

with capture was visible (Dr. E. Kariuki, KWS, personal comm.). Blood smears were not available from either the cheetahs or the leopard and no ticks were recovered from either of the study animals.

A touch-down PCR targeting *Theileria* and *Babesia* 18S rRNA V4 hypervariable region indicated the presence of parasite DNA in seven out of nine blood samples, including the two lions, three cheetahs and the two leopards (Figure 1-1A). The PCR products were hybridized with a panel of species- and genus-specific probes by RLB. This revealed the presence of unspecified *Babesia* spp. or genetic variants of a species in the lions and the leopards, while the cheetahs samples hybridized with a *Theileria* catch-all probe (Figure 1-1B). The two leopard isolates, isolate 78 and 80 hybridized with *Babesia* catch-all probes in the RLB, while faint signals were observed with the *B. leo* probe.

PCR amplification of the near-full-length 18S rRNA gene produced visible bands in five animals only; the lion pair, the two leopards and one cheetah, presumably due to low parasitemia. The amplicons from these five isolates were cloned and sequenced as described in materials and methods section. Four different clones of the 18S rRNA gene for one of the lions were sequenced; three were identical while two deletions were found on the fourth one, perhaps due to *Taq* polymerase errors. Sequences from the other lion, isolate 90, were of poor quality and were excluded from analyses. BLASTn searches with the lion-derived sequences found no identical sequences, with the closest match being *Babesia canis* (98%). Eight plasmid clones were sequenced between the two leopard isolates and the resulting 1,686-bp long sequences were identical (Figure 1-2). BLASTn searches with these leopard-derived sequences found no matching parasite, with the closest match being *B. felis* (96.9%). Four clones derived from the cheetah sample had length and sequence variations (indels), reflecting intra-isolate polymorphisms (Figure 1-2). The cheetah clones showed 99% nucleotide match with *Theileria* sp. (giraffe) found in the GenBank database.

The feline isolates in the present study were aligned with sequences retrieved from GenBank. The final consisted of 43 sequences with a total of 1,884 bp aligned. Both joint-neighboring and maximum parsimony phylogenetic analyses gave trees with analogous topographies (Figure 1-3). The parasites from the lions clustered in a clade carrying *B. canis* spp. complex with high bootstraps support (93.2%) whereas the leopard-derived sequences cluster in a clade bearing *Babesia* species occurring in African wild felines. The cheetah isolates falls in the *Theileria sensu stricto* group, consistent with BLASTn search results.

1.4 DISCUSSION

Occurrence and prevalence of protozoan parasites in wild felids is widely reported in southern Africa (Penzhorn et al., 2001; Bosman et al., 2007, 2010). However, the status of feline piroplasmosis in Kenya is poorly understood at the present. This study was conceived to narrow this knowledge gap on haemoparasites occurring in wild felids in East Africa. *Theileria* and *Babesia*, collectively referred to as piroplasms, normally infect wild felids without causing untoward effects in otherwise healthy hosts (Bosman et al., 2007).

In lions, *Babesia* is prevalent in free-ranging prides (Lopez-Rebollar et al., 1999; Penzhorn et al., 2001). However, these parasites normally occur as clinically unapparent infections in immuno-competent hosts (Penzhorn et al., 2004; Schoeman et al., 2001). Though rarely, mortalities have been reported in free-ranging and captive-bred lions (Munson et al., 2008; Adamson, 1962). Presently, a parasite corresponding to *Babesia* was detected and identified in a pair of anemic captive lions. These animals were housed in a facility adjacent to other wildlife species, including buffalo calves, a wild cat (*Felis silvestris*) and ungulates, perhaps exposing them to infected ticks though it is likely they got infected in the wild. A RLB assay was negative for either *B. leo* or *B. felis* in these animals, although these parasites are commonly reported in lions in southern Africa (Penzhorn et al., 2001). The two lions were

successfully treated with imidocarb dipropionate, marked by the disappearance of clinical symptoms and negative blood smears several weeks following treatment (Dr. E. Kariuki, KWS, personal comm). Fatalities associated with infection from haemoparasites in lions have only been reported in unusual circumstances. The famous “Elsa” lioness is believed to have succumbed to babesiosis associated with impaired immunocompetence, following stressful confrontations with other lions after she was introduced into the wild (Barnett and Brocklesby, 1968). More recently, large-scale mortalities associated with babesiosis in wild lions were reported in Tanzania (Munson et al., 2008). During that outbreak, the high fatalities among the lions were linked to co-infection with canine distemper virus, a rapidly lethal infection that causes severe immunosuppression. Although the two lions in the present study were born in the wild, they have been bred in captivity, and therefore, it is conceivable that they are susceptible to piroplasm infections on coming in contact with infected ticks from animals in adjacent containments. Moreover, nutritional deficiencies can occur in wild animals held in captivity, including felids which may lead to impaired growth, reduced fertility, suppression of the immune system and ultimately, the death of the animals (Ofstedal and Allen, 1996). This could lead to recrudescence of otherwise non-pathogenic parasites as suggested by observations made from the present study. A novel *B. canis* genotype that infects domestic cats has also been reported in the Middle-East (Baneth et al., 2004). This parasite was distinct from other genotypes infecting felines and canines suggesting greater diversity within the *B. canis* complex. Canine babesiosis is particularly virulent in southern Africa (Penzhorn, 2011) reflecting geographic and vector specificity of the causative genotype of *B. canis*. Eastern and southern Africa are geographically and climatically different regions, and consequently, parasites infecting felids in these areas could be genetically distinct causing different disease manifestations, though this need to be investigated.

Signals corresponding to *B. leo* were detected on the RLB membrane. However, upon sequencing a total of eight plasmid clones bearing 18S rRNA from the two leopard isolates, none of them matched exactly with *B. leo* sequences in the GenBank, suggesting the sequencing was suboptimal. This in turn could be as a result of poor quality oligonucleotide probe synthesis (Kong et al., 2006). A large *Babesia* has previously been isolated from a leopard in Kenya (Dennig and Brocklesby, 1972). This parasite is morphologically and serologically distinct from *B. felis* but can infect splenectomized domestic cats. While it is tempting to ascribe the sequences obtained from the leopards in the present study to the above large piroplasm, in hindsight, blood smears should have been prepared to support the sequence data.

Although three *Babesia* spp. have been reported in cheetahs previously (Bosman et al., 2007, 2010), none of our cheetah isolates hybridized with *Babesia*-specific probes. Furthermore, attempts to amplify the near full-length 18S rRNA gene were successful with isolate number 91 only, possibly due to low parasitemia in the remaining two animals that gave visible bands by touch-down PCR. Intra-isolate heterogeneity was found among the four clones sequenced from this animal sample, perhaps reflecting the presence of mixed genotypes, arising from parasite recombination in tick vectors (Bishop et al., 2004) or existence of different rDNA transcription units (Kibe et al., 1994).

It was inferred from phylogenetic analyses that the detected parasite is closely related to *Theileria* spp. that infect sheep and giraffes. *Theileria*-like parasites have been reported in free-ranging cheetahs in Serengeti National Park and Ngorongoro Crater Conservation Area, Tanzania (Averbeck et al., 1990). There are no reports, at the moment, of clinical piroplasmosis in cheetahs, although cheetahs are unusually vulnerable to infectious diseases (Eaton et al., 1993; Munson et al., 1999; O'Brien et al., 1985) due to an innate immune defect (O'Brien et al., 1985). A parallel could be drawn between this *Theileria* sp. and a *Theileria*

sp. recently identified in dogs (Matjila et al., 2008). The latter was phylogenetically identical to a pathogenic *Theileria* sp. identified in a sable antelope, *Hippotragus niger* (Oosthuizen et al., 2008). Although the 18S rRNA gene is widely used in defining new genotypes of piroplasms, it may not resolve between closely-related parasite species or genetic variants (Lack et al., 2012).

In summary, the identity of piroplasms occurring in wild felid species in Kenya was investigated. Phylogeny inference from 18S rRNA gene sequences indicates at least one unique parasite is present in each of all three felids. The pathophysiology and the tick vectors for these parasites are unknown at the present. Although the study utilised a statistically inadmissible number of samples, the newly-generated sequence materials will enable designing of species-specific probes to facilitate large-scale epidemiological investigations to determine the prevalence and the tick vector (s) of these protozoan parasites.

1.5 SUMMARY

Piroplasms frequently infect domestic and wild carnivores. At present, there is limited information on the occurrence and molecular identity of these tick-borne parasites in wild felids in Kenya. In 2009, a pair of captive lions (*Panthera leo*) was diagnosed with suspected babesiosis and mineral deficiency at an animal orphanage on the outskirts of Nairobi, Kenya. Blood smears indicated presences of haemoparasites in the erythrocytes, however, no further investigations were conducted to identify the infecting agent. The animals recovered completely following diet supplementation and treatment with anti-parasite drug. In this report, we extracted and detected parasite DNA from the two lions and seven other asymptomatic feline samples; two leopards (*Panthera pardus*) and five cheetahs (*Acinonyx jubatus*). Reverse line blot with probes specific for *Babesia* spp. of felines indicated the presence of new *Babesia* species or genotypes in the lions, a *Babesia* in the leopards, and unknown *Theileria* sp. in the cheetahs. Phylogenetic analyses using partial sequences of 18S ribosomal RNA (18S rRNA) gene showed that the parasite infecting the lions belong to the *Babesia canis* complex, and the parasite variant detected in the leopards clusters in a clade bearing other *Babesia* spp. reported in wild felids from Africa. The cheetah isolates falls in the *Theileria sensu stricto* group. These findings indicate the occurrence of potentially new species or genotypes of piroplasms in all three feline species.

Table 1-1. List of samples used in this study and geographical place of origin

Host	Description	Geographical origin	Clinical condition
Leopard # 78	Adult, female,	Nairobi National Park	Asymptomatic
Leopard # 80	Adult, female,	Nairobi National Park	Asymptomatic
Cheetah #1	Juvenile, male	Nairobi National Park	Asymptomatic
Cheetah #83	Juvenile, male	Salama-Malili Ranch	Asymptomatic
Cheetah # 91	Juvenile, male	Salama-Malili Ranch	Asymptomatic
Cheetah # 92	Adult, female	Salama-Malili Ranch	Asymptomatic
Cheetah # 93	Adult, female	Salama-Malili Ranch	Asymptomatic
Lion # 84	Adult, male	Nairobi Orphanage	Anemia, lethargy, wobble movement, dry eyes, and piroplasms on thin blood smears.
Lion # 90	Adult, male	Nairobi Orphanage	Anemia, lethargy, wobble movement, dry eyes, and piroplasms on thin blood smears.

Table 1-2. List of oligonucleotide probes used in RLB assay

Probe specificity	18S sequence (5'-3')
<i>Theilera/Babesia</i> catch-all	TAA TGG TTA ATA GGA RCR GTT G
<i>Theileria</i> genus	ATT AGA GTG CTC AAA GCA GGC
<i>Babesia</i> genus 1	ATT AGA GTG TTT CAA GCA GAC
<i>Babesia</i> genus 2	ACT AGA GTG TTT CAA ACA GGC
<i>B. lengau</i>	CTC CTG ATA GCA TTC
<i>B. canis</i>	TGC GTT GAC CGT TTG AC
<i>B. felis</i>	TTA TGC GTT TTC CGA CTG GC
<i>B. leo</i>	ATC TTG TTG CCT TGC AGC T
<i>B. gibsoni</i> (Japan)	TAC TTG CCT TGT CTG GTT T
<i>B. gibsoni</i> (USA)	CAT CCC TCT GGT TAA TTT G

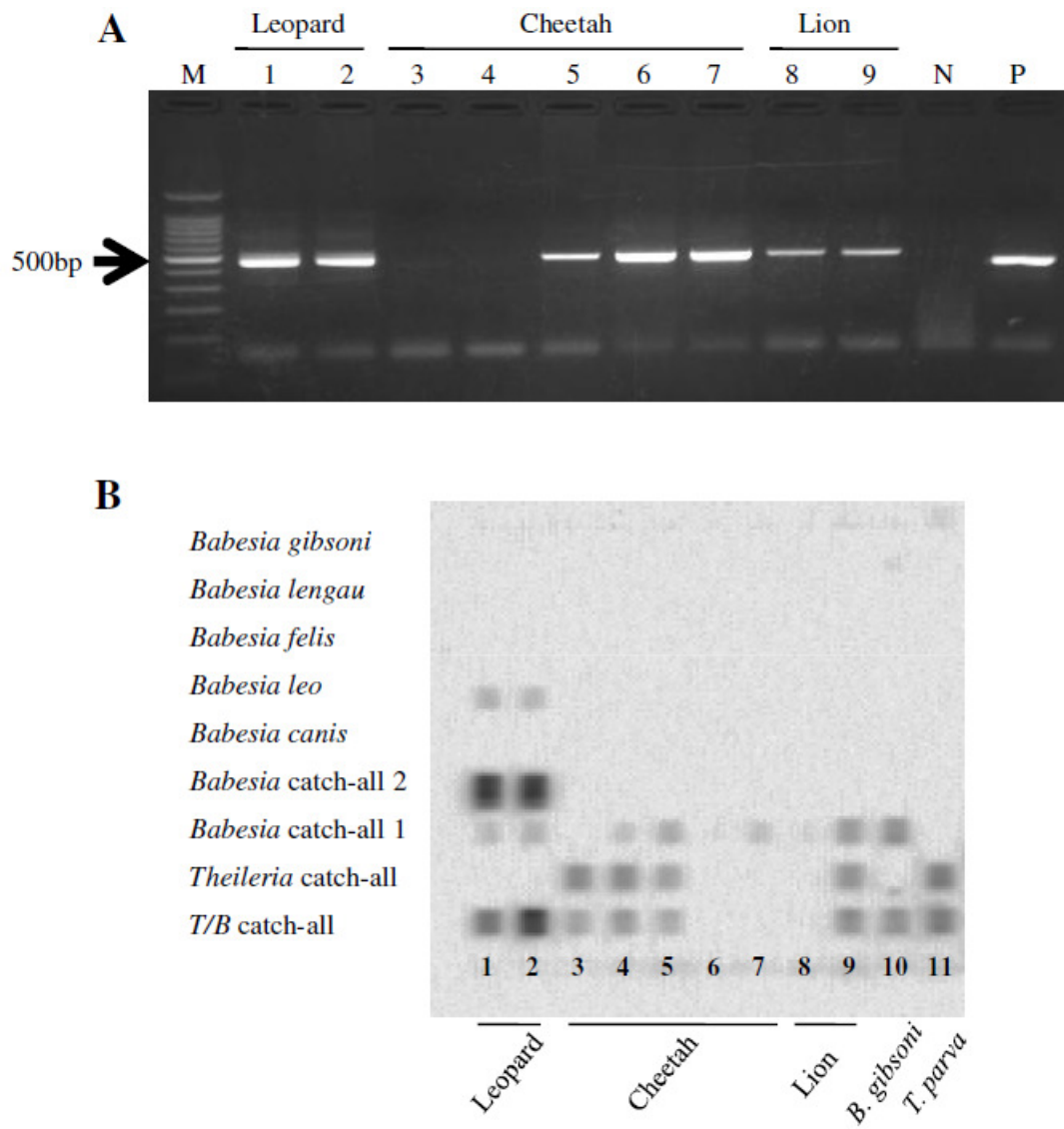


Figure 1-1. Infection of feline species with piroplasms

A: Detection of piroplasm infections in wild felids by touch-down PCR. M: 100 bp ladder size marker. 1-2: Leopard isolates; 3-7: Cheetah isolates; 8-9: Lion isolates. N: non-template negative control. P: *B. gibsoni* cell-culture-derived gDNA positive control. B: A representative RLB: 1-2: Leopard isolates; 3-7: Cheetah isolates; 8-9: Lion isolates; 10: *B. gibsoni* cell-culture-derived gDNA; 11: *Theileria parva*-positive bovine derived gDNA; T/B: *Theileria/Babesia*.

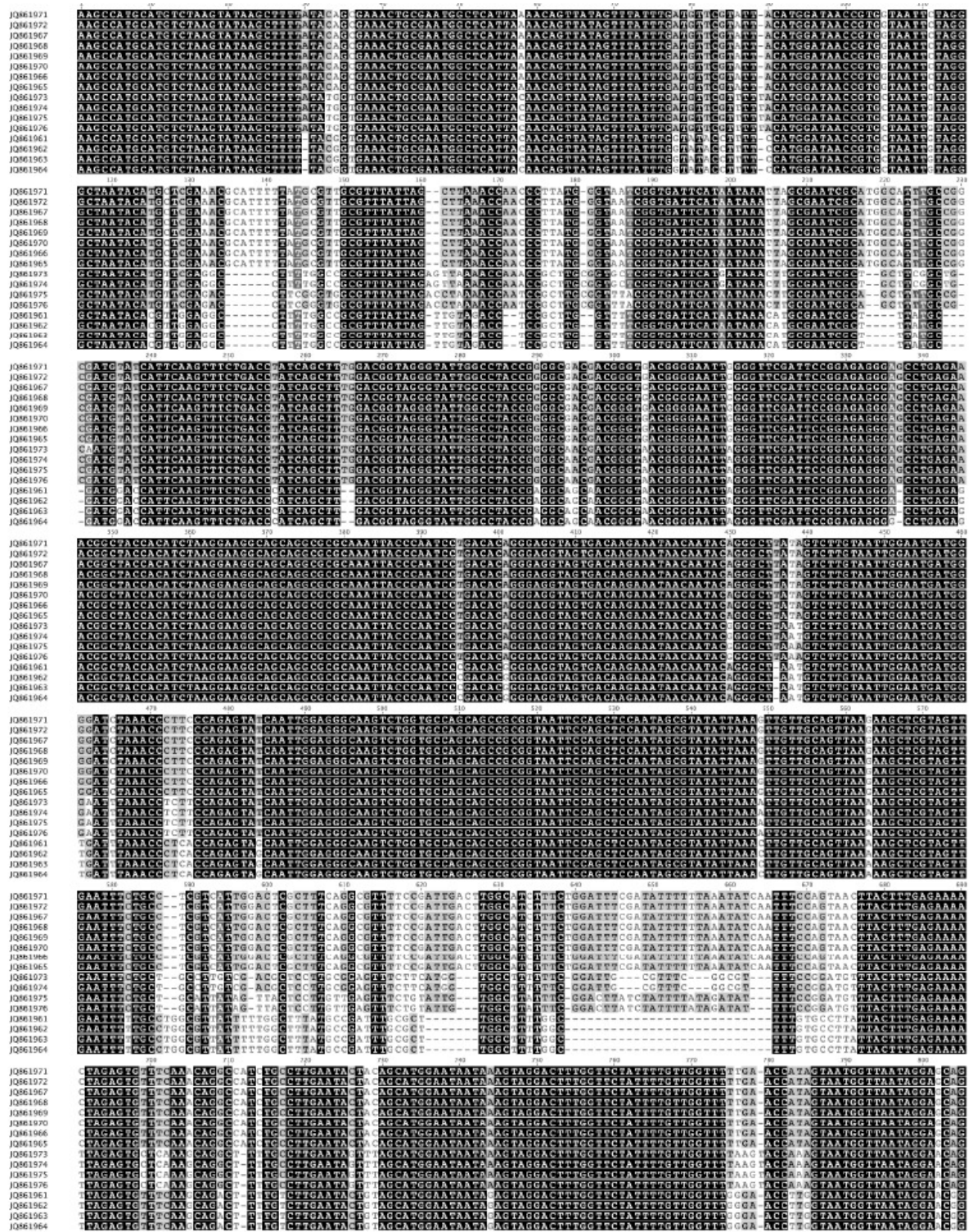


Figure 1-2. Nucleotide sequence alignment of all new 18S rRNA sequences derived from the feline isolates. Accession JQ861961-JQ861964: the lion-derived sequences; JQ861965-JQ861972: leopard-derived isolates; JQ861973-JQ861976: the cheetah-derived sequences. Nucleotide substitutions are shown by light shadings whereas gaps indicate insertions/deletions. Non-variant regions of parasite 18S rRNA genes represented by the dark shades.

(Figure 1-2 continued)

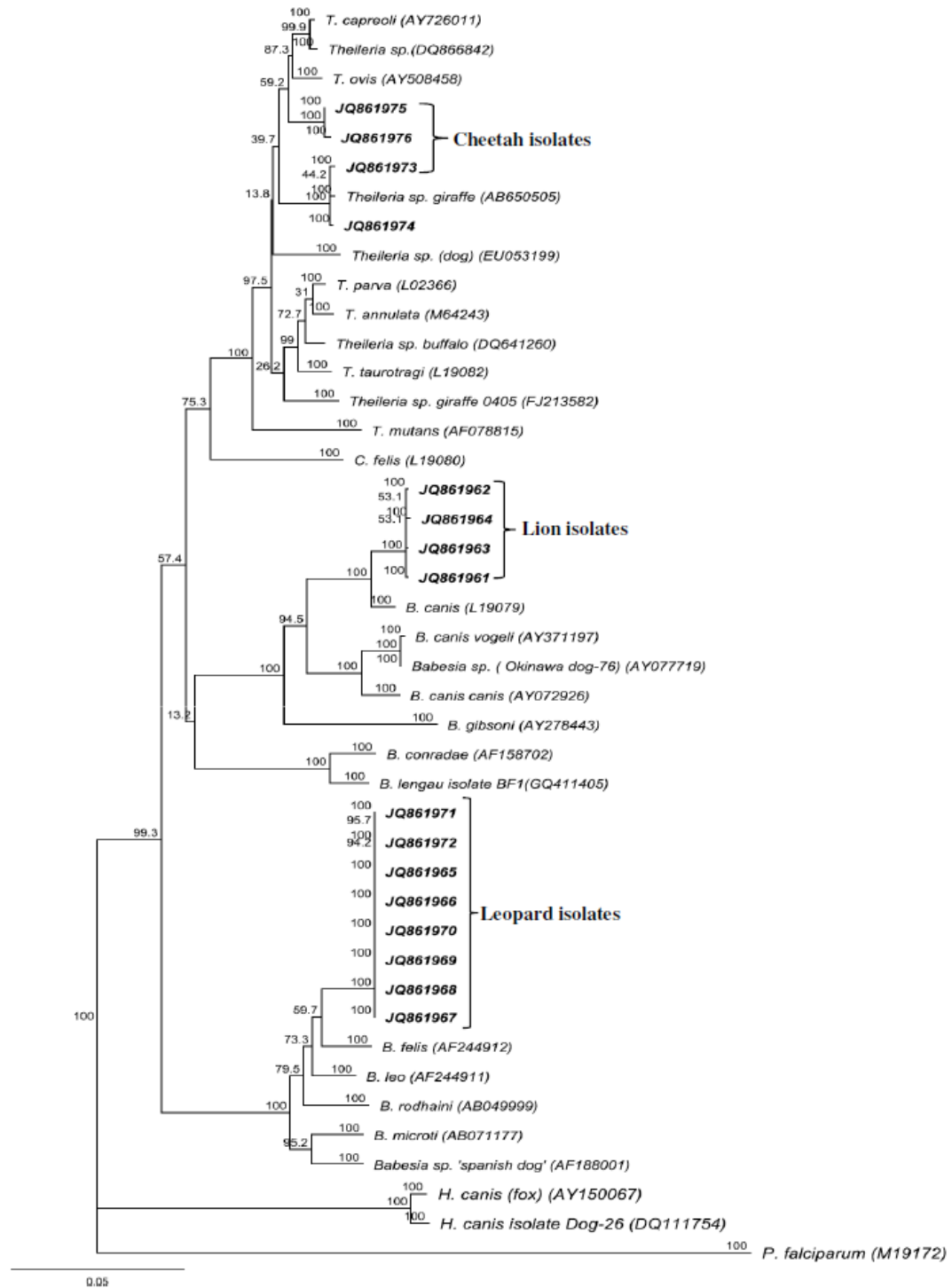


Figure 1-3. A rooted phylogenetic tree showing the relationship between the parasites identified in this study and other related piroplasmid species/variants sequenced previously. *P. falciparum* was included to root the tree. Tree topography was inferred by neighbor-joining method. The numbers represent the percentage of 1,000 replications (bootstrap support) for which the same branching patterns were obtained. Isolates from the present study are shown in bold.

CHAPTER 2

GENOTYPIC VARIATIONS IN FIELD ISOLATES OF *THEILERIA* SPECIES INFECTING GIRAFFES (*GIRAFFA CAMELOPARDALIS TIPPELSKIRCHI* AND *G. C. RETICULATA*) IN KENYA

2.1 INTRODUCTION

Giraffes, like feline species, once inhabited most of the African savanna (Dagg and Bristol, 1982); however, their numbers have decreased drastically in key habitats such as the Maasai Mara National Reserve and the Serengeti National Park in East Africa (Ogutu et al., 2008). Among the sub-species of giraffes, the Rothschild's giraffe (*Giraffa camelopardalis rothschildi*) and the West African giraffe (*G. c. peralta*) are classified as endangered under the IUCN Red List of Threatened Species (Fennessy and Brown, 2008; Fennessy and Brenneman, 2012). This decline is attributable to decreasing natural habitat, poaching and, possibly, climate change (Ogutu et al., 2008). In addition, infectious diseases, such as those transmitted by ticks, are now recognized as serious threats to wildlife, including giraffes (Krecek et al, 1990; Nijhof et al., 2003, 2005; Oosthuizen et al., 2008, 2009; Alasaad et al., 2012).

Piroplasms in Kenyan giraffes were first reported by Brocklesby and Vidler (1965), who identified *Theileria*-like piroplasms in both the Maasai (*G. c. tippelskirchi*) and reticulated (*G. c. reticulata*) giraffes, and one *Babesia* sp. in reticulated giraffes. Recently, Oosthuizen et al. (2009) reported potentially pathogenic species of *Babesia* and *Theileria* suspected of causing fatalities in young giraffes in South Africa. In their report, each animal was found to be infected with a unique parasite, as deduced from 18S rRNA gene sequences, and suggested that more piroplasms may occur in giraffes (Oosthuizen et al., 2009). The tick vectors, as well as, the epidemiology and pathology associated with these organisms in East African giraffes

are not yet known.

Extensive genetic variation has been reported among field populations of different *Theileria* spp. (Kibe et al., 1990; Bhoora et al, 2009, 2010; Salim et al., 2010; Kamau et al., 2011; Weir et. al, 2011). Bhoora et al. (2010) and Salim et al. (2010) reported the occurrence of numerous genotypes of *Theileria equi*, the causative agent of equine piroplasmosis in horses, across geographic regions within a single country. Several unique genotypes and variants associated with disease outbreaks have also been al Australia (Kamau et al., 2011). This genetic heterogeneity within *Theileria* spp. is believed to, among other factors; arise from sexual recombination during gametogony in the vector-ticks (Katzner et al., 2011).

In the current study, RLB oligonucleotide probes specific for *Theileria* species of giraffes were designed and used to screen giraffe blood samples from Kenya for haemoparasites. The 18S rRNA genes were sequenced and used to determine genetic variation among the detected parasites and their relationship with related organisms.

2.2 MATERIALS AND METHODS

2.2.1 Blood samples and extraction of DNA

Thirteen giraffe blood samples in EDTA-coated tubes were provided by the Veterinary Department, KWS, Nairobi, Kenya (Table 2-1). All the animals were clinically healthy with no obvious signs of piroplasmosis; one animal designated as #115, was being treated for a broken forelimb at the time of sampling. Blood was collected through jugular venipuncture and stored at -20 °C except for sample #115, which was processed immediately after collection. Blood sampling was conducted in accordance with the KWS institutional guidelines for animal welfare and the International Livestock Research Institute (ILRI) guidelines on use and care of animals for research purposes. Genomic DNA was extracted from 500 µl of frozen blood or 300 µl of fresh blood with the Wizard Genomic DNA

purification Kit (Promega, Madison, WI, USA) according to recommended protocol. Extracted DNA pellets were dissolved in 100 µl of DNA rehydration buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA [pH 8.0]) and stored at 4°C.

2.2.2 PCR and RLB hybridization

Primers RLB F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') and biotin-labeled RLB R2 (5'-Biotin-CTA AGA ATT TCA CCT CTA ACA GT-3') (Georges et al., 2001) were used to PCR amplify the V4 hypervariable region (V4) of the 18S rRNA gene from the giraffe DNA samples using a touchdown PCR protocol based on a previously described method (Nijhof et al., 2005). The PCR assay was performed using 100-150 ng of genomic DNA in a total volume of 25 µl and Platinum™ Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA). Genomic DNA from *T. parva*-infected cattle, Cape buffalo (*Syncerus caffer*) and eland (*Taurotragus oryx*) were used as positive controls at the PCR step for the subsequent RLB hybridization. The buffalo and eland positive controls were field samples from which *Theileria* sp. (buffalo) and *Theileria taurotragus*, respectively, had been previously detected. Control genomic DNA for other *Theileria* spp. was not available. Sterile, deionized water was used as a negative control. PCR cycling consisted of a denaturation step at 94 °C for 2 min, followed by 10 cycles of touch-down PCR at 94 °C for 30 sec for denaturation, annealing at 67 °C for 30 sec (with temperature decreasing by 1 °C per cycle), and extension at 68 °C for 30 sec. This was followed by 40 cycles of denaturation at 94 °C for 30 sec, annealing at 57 °C for 30 sec, and extension at 68 °C for 30 sec. Five µl of PCR products were analyzed by electrophoresis on a 2% agarose gel. DNA in the gel was stained with ethidium bromide, and visualized under UV light.

To identify *Theileria* spp. occurring in giraffes, 18S rRNA gene sequences of *Theileria* spp. previously described in giraffes (Oosthuizen et al., 2008) were obtained from GenBank and used to design two species-specific probes, designated as *Theileria* sp. (giraffe) 1 and

Theileria sp. (giraffe) 2. In addition to these two, probes specific for all *Theileria* and *Babesia* spp. (*Theileria/Babesia* catch-all), all *Theileria* spp. (*Theileria* catch-all), all *Babesia* spp. (*Babesia* 1 and 2) as well as several others that are specific for different *Theileria* spp. were included on the membrane for screening blood parasites (Table 2-2). RLB was conducted based on methods described by Gubbels et al. (1999). The probes, containing an N-(monomethoxytrityl)-C₆ amino linker (Hokkaido System Science, Japan) were bound to a Biodyne C membrane (Pall Life Sciences, Ann Arbor, MI, USA) on a screen-blotter (Sanplatec, Osaka, Japan) at a final concentration of 10pmol. PCR products (20 µl) were diluted to final volume of 125 µl with 2xSSPE/0.1% SDS, and were then denatured at 99.9 °C for 10 min, and immediately snap-frozen on ice for several minutes. Denatured products (120 µl), including the control samples, were hybridized to the membrane-bound probes at 42 °C for 60 min. The membrane was washed twice using 2xSSPE/0.5% SDS buffer at 52 °C for 10 min. The membrane was then incubated with peroxidase-labeled NeutrAvidin (Thermo Fisher Scientific, Rockford, IL, USA) for 30-60 min at 42 °C, followed by two washes with pre-warmed 2xSSPE/0.5% SDS for 10 min at 42 °C with gentle shaking. To detect the probe-amplicon hybridization, the membrane was incubated with 15 ml Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) for 2 min at room temperature, and then exposed to a Chemiluminescent imager (Bio-Rad laboratories, Hercules, CA, USA). For reuse of the membrane, bound PCR products were removed by washing twice with pre-heated 1% SDS at 90 °C for 30 min and rinsing with 20 mM EDTA for 15 min. The membrane was stored in 2 ml of 20 mM EDTA at 4 °C.

2.2.3 Cloning and sequencing of 18S rRNA gene

Primers Nbab_1F (5'-AAG CCA TGC ATG TCT AAG TAT AAG CTT TT-3') and Nbab_1R (5'-CTT CTC CTT CCT TTA AGT GAT AAG GTT CAC-3') (Oosthuizen et al., 2008) were used to amplify near full-length of the 18S rRNA gene from giraffe DNA samples

by PCR. PCR was performed with KOD Plus high fidelity DNA polymerase (Toyobo, Osaka, Japan) following the manufacturer's instructions with 2 µl (100-150 ng) of genomic DNA in a final volume of 25 µl. For each isolate, PCR was performed in quadruplicates and subjected to thermocycling: initial denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 15 sec of denaturation, annealing at 55 °C for 30 sec, and extension at 68 °C for 2 min followed by a final hold at 4 °C. PCR products were pooled for a total volume of 100 µl, and purified with the GeneClean III Kit, (Q-BIOgene, Heidelberg, Germany) according to the manufacturer's instructions. The resulting blunt-ended amplicons were modified with 10x A-attachment mix (Toyobo, Osaka, Japan) as recommended by the manufacturer. The products were then ligated into pGEMT-Easy vector (Promega, Madison, WI, USA) and transformed into chemically-competent DH5a *Escherichia coli* cells. Two to four recombinant bacterial colonies per transformation were identified by colony-PCR screening and inoculated into LB medium supplemented with ampicillin, and incubated overnight at 37 °C. Recombinant plasmids were extracted from 3 ml of culture using the QIAprepTM Spin Miniprep kit (Qiagen, Hilden, Germany). Approximately 350 ng of plasmid DNA for the selected bacterial clones was sequenced with 2.5 pmol of the primers T7 and SP6 (Promega, Madison, WI, USA), BT18S_2F, BT18S_3F (Oosthuizen et al., 2008), and RLB R2 (Georges et al., 2001) using the Quick Start kit (Beckman Coulter, Fullerton, CA, USA). Purified products were analysed with the CEQ 2000 Dye Terminator Cycle Sequencer (Beckman Coulter, Fullerton, CA, USA), and the resultant sequences for the nearly full-length 18S rRNA gene assembled and edited manually using Geneious Pro version 5.5 (<http://www.geneious.com>) (Drummond et al., 2011). Sequence ends of low quality were trimmed and excluded from analyses. GenBank accession numbers for the new sequences and corresponding animal sources are listed in Table 2-1.

2.2.4 Sequence analysis

BLAST (Altschul et al., 1990) was used to search for sequences similar to the 18S rRNA gene sequences determined in the present study. A nucleotide sequence alignment of the new sequences and a number of related organisms obtained from the GenBank was constructed using CLUSTAL W (Thompson et al., 1994) in Geneious Pro 5.5 and the alignment trimmed to the size of the shortest sequence (1,471bp). Similarity matrices were performed using the two-parameter method of the Jukes-Cantor correction model for multiple base changes (Jukes and Cantor, 1969). Phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei, 1987) implemented by Geneious Pro 5.5 in combination with the bootstrap method at 1,000 replicates/tree.

2.3 RESULTS

2.3.1 Detection of haemoparasites in giraffe specimens by microscopy and PCR

Blood samples for the present study were obtained from clinically healthy animals during translocation or in the course of treatment (Table 2-1). Blood smears revealed the presence of haemoparasites in the erythrocytes of specimen #115 whereas the rest of the blood specimens were too haemolysed for microscopy. Genomic DNA was successfully extracted from all samples. A touchdown PCR with primers targeting the V4 region in the 18S rRNA gene gave visible bands with 12/13 (92%) of the giraffe-derived DNA, and for the three positive controls. No contamination was detected in the water negative control (Figure 2-1).

2.3.2 Detection of haemoparasites in giraffe specimens by PCR-RLB hybridization

A total of sixteen generic and species-specific probes were included in the RLB assay. Among the positive controls, strong signals corresponding to *T. parva*, *Theileria* sp. (buffalo) and *T. taurotragi* were visible on the membrane while additional bands were also observed in the case of the buffalo and eland genomic DNA. In the study samples, strong and distinct

RLB signals were obtained with the *Theileria/Babesia* catch-all and *Theileria* catch-all probes. Between the two newly developed probes, *Theileria* sp. (giraffe) 1 hybridized with PCR amplicons from a total of 11 giraffe samples (#30, 43, 44, 63, 64, 66, 70, 81, 86, 94 and 115) whereas the second probe, *Theileria* sp. (giraffe) 2 hybridized with 7 samples (44, 81, 86, 94, 104, 108 and 115). Moreover, out of the 13 samples, 5 (44, 81, 86, 94 and 115) yielded signals with both new probes. No signal was detected with the water negative control and as well as the other species-specific probes on the membrane (Figure 2-1).

2.3.3 Sequence analysis of 18S genes from *Theileria* infecting the giraffe

Almost-full length 18S rRNA genes were successfully amplified from a total of nine samples. The resultant PCR amplicons were cloned and multiple plasmid clones for each animal specimen were sequenced (Table 2-1). In total, 28 sequences, representing parasite isolates from Kenyan giraffes, were assembled. BLAST searches with these 18S rRNA gene sequences indicated that these were of *Theileria* origin; however, none of the 28 clones from this study yielded 100% identity with sequences in GenBank. In addition, among the five samples with dual infections, clones corresponding to the *Theileria* sp. (giraffe) 2 probe were overrepresented among the randomly sequenced clones, perhaps reflecting higher parasitemia, which is supported by stronger signals on the RLB. Nucleotide sequence alignment of the 18S rRNA gene sequences obtained in the present study, and those reported in South Africa revealed extensive heterogeneity within and between isolates, largely within the V4 region (Figure 2-2). Genotypes identical to the ones reported in South African giraffes were found among isolates sequenced in the current study, consistent with the RLB data. Moreover, clones without sequences matching to the two probes, were also found upon aligning 18S rRNA gene sequences obtained from the Kenyan giraffes and giraffe isolates reported previously.

Phylogenetic analyses were conducted with the aligned 18S rRNA gene sequences (Figure 2-

3). Further, a phylogenetic tree comprised of 18S rRNA gene sequences from *Theileria* and *Babesia* spp. placed isolates from giraffes within the *Theileria senso stricto* clade (data not shown). From these analyses, *Theileria* spp. from the Kenyan giraffes were found to cluster into two major clades, which also carry *Theileria* isolates that were previously reported in giraffes in South African. Importantly, the South African genotypes (accession number FJ213582, FJ213584 and FJ213583) clustered with sequences derived from the Kenyan giraffes in a manner consistent with the RLB observation.

2.4 DISCUSSION

In this study, the presence and identity of haemoparasites in blood samples from giraffes in Kenya were investigated. RLB with oligonucleotide probes designed to detect *Theileria* spp. of giraffes showed that *Theileria* DNA was present in all 13 samples. Subsequently, the 18S rRNA gene was successfully amplified and cloned from 9 of the samples. BLAST searches and phylogenetic analyses with the resultant sequences confirmed the presence of potentially new *Theileria* spp. in the giraffe samples.

Infectious diseases, including those transmitted by ticks and other haematophagous arthropods, are increasingly recognized as threats to wildlife species in East Africa (Brocklesby and Vidler, 1965; Munson et al., 2008). Although disease agents have co-evolved alongside wildlife, the latter were once both more numerous and more genetically diverse, and therefore, presumably, less susceptible to disease outbreaks. Without a known cause for the reported decrease in the number of wild giraffes in East Africa, there is a need to investigate the possible role of infectious diseases in their decline.

The finding of haemoparasites in a blood smear from one of the giraffe was consistent with a study by Brocklesby and Vidler (1965) that reported finding *Theileria* and *Babesia* in Kenyan giraffes, and a recent study that found pathogenic *Theileria* and *Babesia* in South African

giraffes (Oosthuizen et al., 2009). The giraffe blood samples analysed in the present study yielded positive signals for the *Theileria* generic probe and with one or both of the newly designed probes that are specific for *Theileria* spp. (giraffe) in the RLB assay, suggesting high prevalence of these parasites among giraffes. Oosthuizen et al. (2009) reported novel *Theileria* from both healthy and clinically ill giraffes following translocation. RLB, in this study, indicated that *Theileria* genotypes identical to one causing fatalities among South African giraffes are present in Kenya. This suggests a need for surveillance against fatalities associated with these parasites. This is urgently important given the ongoing restocking program through translocation of giraffes in Kenya (Dr. E. Kariuki, KWS, unpublished data). Although *Babesia* spp. have previously been reported among the giraffes (Oosthuizen et al., 2009; Brocklesby and Vidler, 1965) none of the specimens hybridized with the two *Babesia*-specific probes in the present study. This could suggest the absence of the tick vectors for these organisms at the localities where the study animals were resident.

Although the vectors for these piroplasms are currently unknown, parasitisation of giraffes by multiple species of ticks is reportedly very common (Horak et al., 2007) potentially leading to high infection rates with blood-borne parasites. The two species-specific probes designed in this study might enable the detection of these parasites from ticks collected from the field; however, this was not evaluated since the ticks were not available.

The specificity and geographic coverage observed with the new probes further suggest that they could be used reliably to detect *Theileria* spp. occurring in giraffes. However, there is need for more empirical data from large epidemiological surveys since RLB with some blood-parasites such as *T. equi* and *Babesia caballi* has been found to discriminate between geographically diverse isolates (Bhoora et al. 2009). In addition, presently, only three positive controls (*T. parva*, *T. sp* (buffalo) and *T. taurotragi*) were included in the RLB. Consequently, cross-hybridization between the new probes and other *Theileria* spp. cannot be ruled out

entirely. However, there was no such cross-hybridization with *Theileria* spp. that occur benignly in Cape buffaloes and elands in the field, suggesting that the new probes are specific to the *Theileria* spp. of giraffes.

Singular and mixed infections were evident from RLB, and were confirmed by sequencing of the 18S rRNA genes from parasite isolates in the current work. Moreover, more samples hybridized with the *Theileria* sp. (giraffe) 1 probe than *Theileria* sp. (giraffe) 2, whereas genotypes identical to both probes were also found in some isolates. The significance of this observation can only be postulated at the present; however, it is worth noting that the *Theileria* sp. (giraffe) 1 probe was derived from a South African isolate that was associated with acute disease onset and death in a young giraffe (Oosthuizen et al. 2009).

Mans et al. (2011) characterized 18S rRNA gene variants, by random sequencing, in some *Theileria* spp. that occur in cattle and Cape buffalo. Approximately 1,000 clones, from a cohort of 62 buffaloes and 49 cattle were analyzed in that study. In both species, no new genotypes were obtained from sequencing beyond 200 clones indicating genotypic saturation within the sample sets. In contrast, a large number of variants were observed from a small number of clones sequenced in the present study perhaps reflecting the fact that these animals originated from different geographic regions in the country, and further underlining the extensive genotypic variation that exist in *Theileria* from giraffes.

A significant number of sequence variants was found within and among isolates when the 18S rRNA gene sequences obtained from the Kenyan giraffes were aligned with isolates reported in South Africa. It is possible that these are mere variants rather than completely new species or sub-species since the observed heterogeneity did not impair the RLB assay. Mans et al. (2011) has also shown that heterogeneity in the 18S rRNA gene does not seem to impair a real-time PCR detection assay specific for *T. parva*. Such genetic variation in haemoparasites is attributable to either parasite sexual recombination during gametogony (Katzer et al.,

2011), the presence of more than one transcription unit in the 18S rRNA gene (Kibe et al., 1994) or point mutations (Kamau et al., 2011).

The 18S rRNA gene sequences acquired in the present study were aligned with closely related parasites and subjected to phylogenetic analysis. The isolates from the present study cluster into two major clades that include the South African genotypes from which the new probes were designed. When analyzed alongside *Babesia* spp., *Theileria* isolates from giraffes fall well within the *Theileria sensu stricto* clade. Collectively, the RLB data and the phylogeny suggest that the South African parasites represent a sub-set of the genotypes present in Kenya, and it is likely that more isolates will be found if a larger number of giraffes from Southern Africa are sampled.

Although the 18S rRNA gene is helpful in identifying and differentiating between species of piroplasms, its resolution power diminishes at isolate level and for closely related parasites (Salim et al., 2010). The single copy S5 gene (Gardner et al., 2005; Pain et al., 2005) may become an alternative marker suitable for differentiating between closely related species as shown recently by Mans et al. (2011). However, its use in phylogenetic characterization of blood parasites is currently limited by the small number of S5 sequences available in nucleotide databases.

In summary, this study reports the occurrence of genetically diverse *Theileria* spp. in otherwise healthy giraffes in Kenya. Two RLB probes were designed and used to detect *Theileria* spp. in giraffes. Phylogeny of the giraffe *Theileria* suggests the existence of two distinct populations, and extensive heterogeneity within these two groups. Further studies are necessary to clarify the observed genetic diversity among the *Theileria* occurring in the giraffes, and to investigate the possible role of *Theileria* spp. in disease incidence among giraffes in East Africa.

2.5 SUMMARY

Recently, mortalities among giraffes, attributed to infection with unique species of piroplasms were reported in South Africa. Although haemoparasites are known to occur in giraffes of Kenya, the prevalence, genetic diversity and pathogenicity of these parasites has not been investigated.

In this study, blood samples from 13 giraffes in Kenya were investigated microscopically and genomic DNA extracted. PCR amplicons of the hyper-variable region 4 (V4) of *Theileria* spp. small subunit ribosomal RNA (18S rRNA) gene were hybridized to a panel of genus- and species-specific oligonucleotide probes by reverse line blot (RLB). Two newly designed oligonucleotide probes specific for previously identified *Theileria* spp. of giraffes found single infections in eight of the specimens and mixed infections in the remaining five samples.

Partial 18S rRNA genes were successfully amplified from 9 samples and the PCR amplicons were cloned. A total of 28 plasmid clones representing the Kenyan isolates were analyzed in the present study and compared with those of closely-related organisms retrieved from GenBank. In agreement with RLB results, the nucleotide sequence alignment indicated the presence of mixed infections in the giraffes. In addition, sequence alignment with the obtained 18S rRNA gene sequences revealed extensive microheterogeneities within and between isolates, characterized by indels in the V4 regions and point mutations outside this region. Phylogeny with 18S rRNA gene sequences from the detected parasites and those of related organisms places *Theileria* of giraffes into two major groups, within which are numerous clades that include the isolates reported in South Africa. Collectively, these data suggest the existence of at least two distinct *Theileria* species among giraffes, and extensive genetic diversity within the two parasite groups.

Table 2-1. Summary of animal hosts from which giraffe blood samples were obtained, and Genbank accession numbers of corresponding *Theileria* isolates identified in this study

Sample identification	Age (years)	Geographical origin	RLB screening	18S rRNA clone # (GenBank accession no.) [bp]
Giraffe # 30	1	Garrisa, N.E.P [†]	Single infection	2(JQ928928)[1665], 5(JQ928927)[1361]
Giraffe # 43	2	Garrisa, N.E.P	Single infection	ND
Giraffe # 44	5	Garrisa, N.E.P	Mixed infection	1(JQ928926 [‡])[1433], 2(JQ928925)[1665], 4(JQ928924)[1672]
Giraffe # 63	1.5	Garrisa, N.E.P	Single infection	5(JQ928922 [*])[1649], 6(JQ928921)[1665], 4(JQ928923)[1665]
Giraffe # 64	2	Nairobi National Park	Single infection	ND
Giraffe # 66	2	Nairobi National Park	Single infection	2(JQ928920)[1665], 3(JQ928919)[1653], 4(JQ928918 [*])[1665], 6(JQ928917)[1665]
Giraffe # 70	1.5	Nairobi National Park	Single infection	1(JQ928916)[1665], 6(JQ928914 [*])[1644], 2(JQ928915)[1665]
Giraffe # 81	Adult	Giraffe Centre, Nairobi	Mixed infection	1(JQ928913)[1665], 3(JQ928912)[1656], 6(JQ928911)[1665]
Giraffe # 86	Adult	Giraffe Centre, Nairobi	Mixed infection	1(JQ928910)[1570], 3(JQ928909)[1665], 5(JQ928908)[1665], 6(JQ928907)[1614]
Giraffe # 94	Juvenile	Giraffe Centre, Nairobi	Mixed infection	ND
Giraffe # 104	Adult	Garrisa, N.E.P	Single infection	ND
Giraffe # 108	Adult	Garrisa, N.E.P	Single infection	4(JQ928933 [*])[1658], 1(JQ928934 [*])[1664], 6(JQ928932)[1609]
Giraffe # 115	Adult	Giraffe Centre, Nairobi	Mixed infection	6(JQ928929) [1665], 2(JQ928931 [‡])[1665], 3(JQ928930)[1184]

Samples that were positive in both touch-down PCR and RLB but failed to yield visible bands with 18S rRNA PCR were not cloned (ND).

N.E.P[†]: North-Eastern Province. ND: No data. * Isolates matching RLB probe *Theileria* sp. (giraffe) 1. ‡ Isolates matching RLB probe *Theileria* sp. (giraffe) 2.

Table 2-2. List of oligonucleotide probes used in the RLB assay

Probe specificity	18S sequence (5'-3')	Reference
<i>Theileria/Babesia</i> catch all	TAATGGTTAATAGGARCRTTG	Gubbels et al., 1999
<i>Theileria</i> catch-all	ATTAGAGTGCTCAAAGCAGGC	Nagore et al., 2004
<i>Babesia</i> catch-all 1	ATTAGAGTGTTTCAAGCAGAC	Bhoora et al., 2009
<i>Babesia</i> catch-all 2	ACTAGAGTG TTT CAA ACAGGC	Brothers et al., 2011
<i>Theileria parva</i>	GGACGGAGTTCGCTTTG	Gubbels et al., 1999
<i>Theileria taurotragi</i>	TCTTGGCACGTGGCTTTT	Gubbels et al., 1999
<i>Theileria</i> sp.(buffalo)	CAGACGGAGTTTACTTTGT	Oura et al., 2004
<i>Theileria annulata</i>	CCTCTGGGGTCTGTGCA	Georges et al., 2001
<i>Theileria buffeli</i>	GGCTTATTTCCGWTGATTTT	Gubbels et al., 1999
<i>Theileria mutans</i>	CTTGCGTCTCCGAATGTT	Gubbels et al., 1999
<i>Theileria velifera</i>	CCTATTCTCCTTTACGAGT	Gubbels et al., 1999
<i>Theileria ovis</i>	TTGCTTTTGCTCCTTTACGAG	Nagore et al., 2004
<i>Theileria</i> sp. (kudu)	CTCCATTGTTTCTTTCCTTTG	Nijhof et al., 2005
<i>Theileria</i> sp. (sable)	GCTGCATTGCCTTTTCTCC	Oosthuizen et al., 2008
<i>Theileria</i> sp. (giraffe)1 (FJ213582 & FJ213584)	TTATTTCTCCTTGACGAGTT	This work
<i>Theileria</i> sp. (giraffe) 2 (FJ213583)	CTCTTTGATGGGCTTTTG	This work

Genbank accession numbers of *Theileria* spp. (giraffe) used to derive the newly designed probes are shown in parentheses.

The degenerate position R denotes either A or G, and W denotes either A or T.

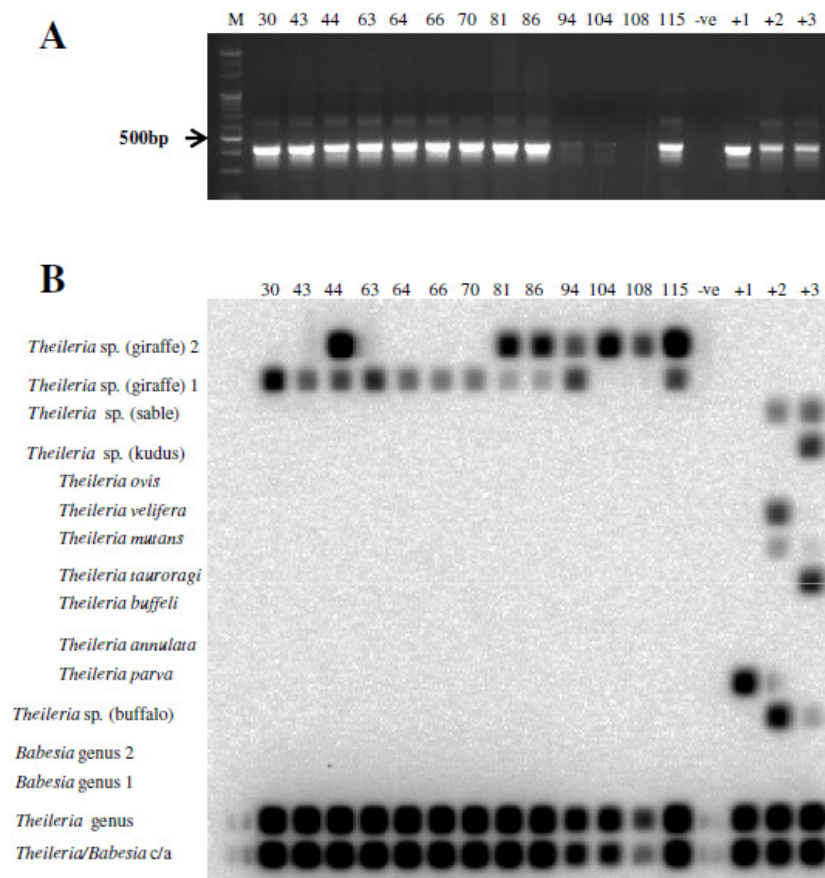


Figure 2-1. Detection of haemoparasites in giraffes by RLB assay

A: Touch-down PCR applied to giraffe-derived gDNA samples. +1, +2 and +3 are positive control samples from cattle, Cape buffalo and eland, respectively. *M* is a 100-bp DNA ladder. Lanes are labeled according to animal number. B: RLB hybridization of PCR products derived from giraffe DNA. Control DNA samples from animals with mixed infection are shown in Lane +2 and +3.

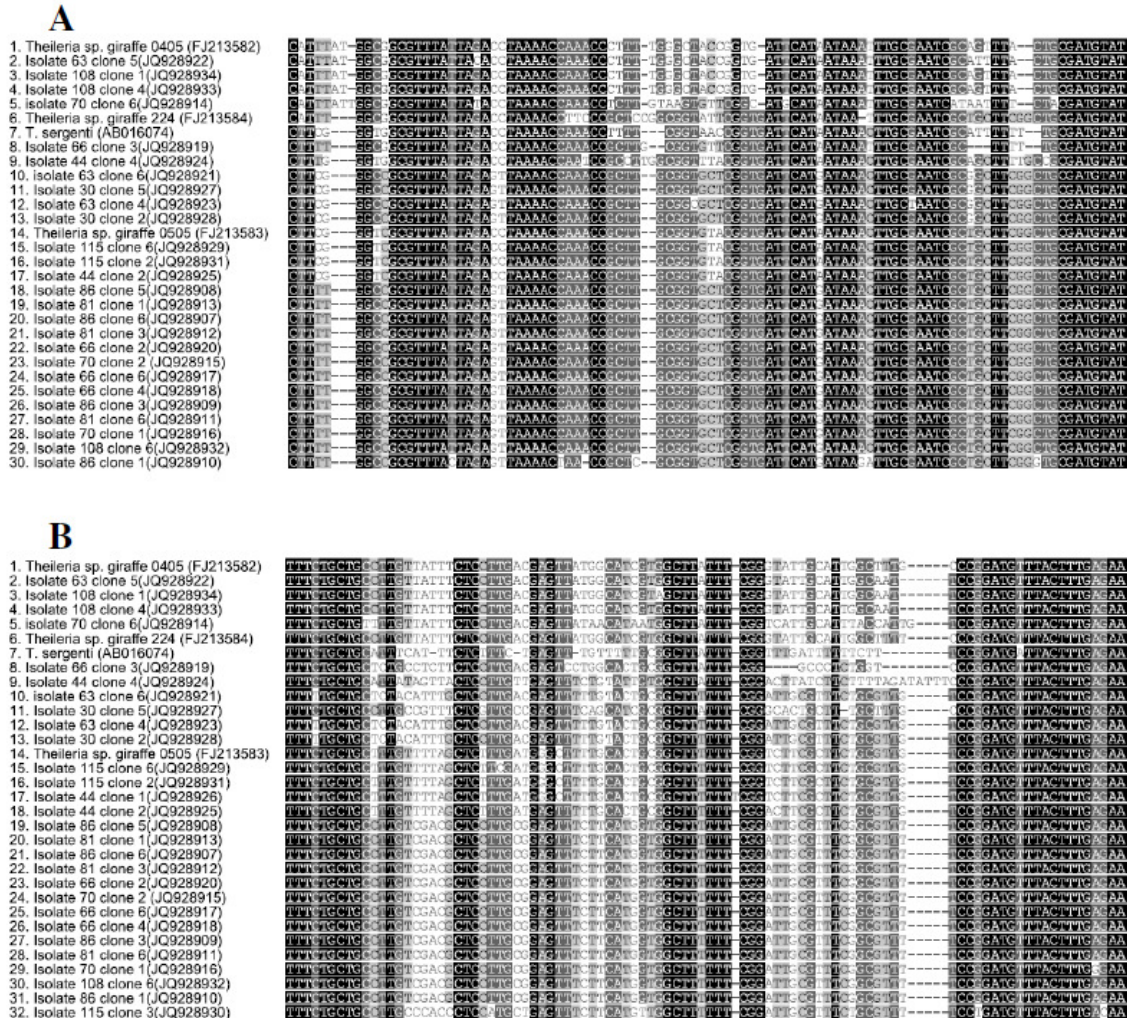


Figure 2-2. Multiple sequence alignment extraction of the most variable regions of 18S rRNA genes obtained from *Theileria* sp. (giraffe) in the present study, and those previously identified in South Africa (GenBank accession numbers FJ213583, FJ213582 and FJ213584)
 Full-length 18S rRNA gene from *Theileria sergenti* (GenBank accession number AB016074) was used as reference for sequence length. A: Nucleotide positions 180-279. B: Nucleotide positions 620-719. The obtained length for sequences with accession numbers JQ928926 and JQ928930 fell short of the region shown in A. Nucleotide substitutions are shown by light shadings whereas gaps indicate insertions/deletions. Conserved regions represented by the dark shadings.

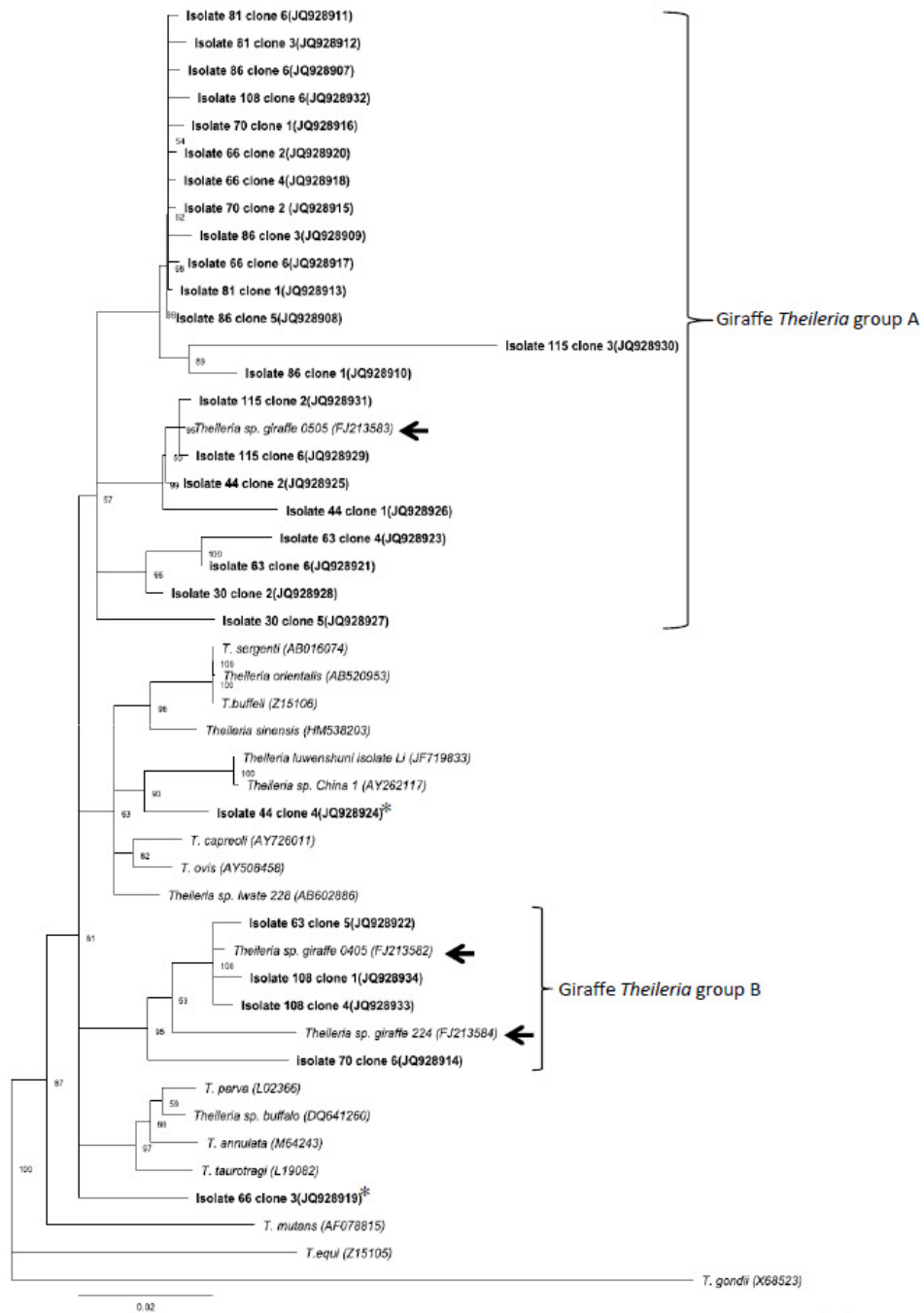


Figure 2-3. Rooted neighbor-joining phylogenetic tree showing the relationship between genotypes of *Theileria* spp. (giraffe) identified in the present study and closely related *Theileria* species based on 18S rRNA gene sequences
The numbers represent the percentage of 1000 replications (bootstrap support) for which the same branching patterns were obtained. GenBank accession numbers for parasite sequences are in parentheses. Genotypes identified in this study are in bold, while the arrow indicates *Theileria* spp. (giraffe) that were previously reported in South Africa. The scale bar indicates substitutions per site. * these sequences are likely due to sequencing errors rather than unique isolates.

CHAPTER 3

IDENTIFICATION AND PHYLOGENETIC CHARACTERISATION OF NEW *THEILERIA* SPECIES FROM WATERBUCK (*KOBUS DEFASSA*) IN A *THEILERIA* *PARVA*-ENDEMIC AREA IN KENYA

3.1 INTRODUCTION

Whereas a majority of *Theileria* species such as those reported in wild felids or giraffes are clinically benign (Githaka et al., 2012, 2013), others like *T. parva* and *Theileria annulata* cause high livestock mortality in endemic areas especially among exotic cattle breeds (reviewed by Bishop et al., 2004). In eastern, central and southern Africa, the primary cause of theileriosis in cattle is the sporozoan parasite *T. parva* that occurs naturally in the Cape buffalo (*Syncerus caffer*) (Gardner et al., 2005; Oura et al., 2011a, 2011b). In addition, the waterbuck (*Kobus defassa*), an ungulate species endemic to the eastern African savannah, is also regarded as a potential host for *T. parva* (Stagg et al., 1994) although this requires confirmation.

Waterbuck can be infested by large numbers of *Rhipicephalus appendiculatus* ticks, sometimes resulting in host mortality (Melton et al., 1982). Cell cultures originating from peripheral blood monocytes from waterbuck can be infected and immortalized by *Theileria* from buffalo but not by cattle-derived *T. parva* (Stagg et al., 1983), although the schizont-infected/immortalized waterbuck cells failed to establish infection in autologous animals (Stagg et al., 1994). Experimentally infected naïve waterbuck develop a mild infection following inoculation with a *T. parva* stabilate at a dose ten times higher than that which would be lethal to cattle (Stagg et al., 1994). The animals become persistently infected as demonstrated by the ability of feeding ticks to acquire the parasite up to a year post-infection. However, there is no evidence that *T. parva* is transmissible to the waterbuck under field conditions (Bishop et al., 1992). Furthermore, although *Theileria* spp. are known to occur in

the waterbuck (Fawcett et al., 1987; Stagg, 1992), there is currently no genetic information regarding these parasites.

The aim of the present study was to investigate the nature of *Theileria* infections in waterbuck and cattle blood samples that originated from an East Coast fever (ECF)-endemic locality in Kenya with frequent waterbuck/cattle interaction. A second objective was to characterise at molecular level, the *Theileria* spp. found in the waterbuck.

3.2 MATERIALS AND METHODS

3.2.1 *Blood samples and DNA extraction*

Marula, the site of the present study, is a private ranching farm located in Nakuru, Rift Valley in Kenya. Currently, large populations of bovids share pasture in this farm, including the waterbuck, cattle and the Cape buffaloes. Waterbuck blood samples ($n=26$) were collected at the farm by the KWS at four different time points (Table 3-1). Cattle ($n=86$) were sampled from a herd grazing in the vicinity with buffaloes at Marula during an epidemiological study on tick-borne haemoparasites in the region. In all cases, blood was obtained through jugular venipuncture into EDTA-vacutainers and transported on ice to the laboratory, and frozen at -20 °C until the present analyses. DNA was extracted from 500 µl blood sample using the Wizard Genomic DNA purification Kit (Promega, Madison, WI, USA). DNA pellets were resuspended in 100 µl DNA-rehydration buffer (10 mM Tris, 1mM EDTA) and incubated for 1hr at 65 °C, and stored at 4 °C until use.

3.2.2 *Nested PCR for detection of T. parva*

A nested PCR assay was set up to detect *T. parva* from waterbuck and cattle samples. PCR cycling conditions and primers used in the primary PCR were as described previously (Skilton et al., 2002). For cattle samples, primary PCR products were diluted 10-fold while the waterbuck PCR products were incorporated into the second PCR reaction undiluted. Two

µl of PCR primary products were used as template in secondary nested PCR using Ex-Taq DNA polymerase kit (Takara, Kyoto, Japan). The primers and cycling conditions for the nested PCR were as described previously (Odongo et al., 2009) except that the number of cycles was revised upwardly to 35 to enhance sensitivity. Cycling was performed on a Perkin Elmer 9700 thermocycler (Applied Biosystems, Carlsbad, CA, USA) and PCR products resolved by 2% agarose gel electrophoresis. Products were visualised under UV after ethidium bromide staining.

3.2.3 PCR and RLB hybridization

Primers RLB F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') and biotin-labeled RLB R2 (5'-Biotin-CTA AGA ATT TCA CCT CTA ACA GT-3') (Georges et al., 2001) were used to amplify the V4 hypervariable region (V4) of the 18S rRNA gene from the DNA samples using a touchdown PCR protocol based on a previously described method (Nijhof et al., 2005). Briefly, PlatinumTM Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA) was used to amplify 100-150 ng of genomic DNA in a total volume of 25 µl. DNA for *T. annulata*, *T. parva*, *T. taurotragi*, *Theileria mutans*, *Theileria buffeli*, *T. equi*, *Theileria* sp. (giraffe), *Theileria* sp. (buffalo), *Theileria velifera*, and *Theileria* sp. (sable) were used as positive controls at the PCR step for the subsequent RLB hybridization. Sterile, deionized water was used as a negative control. Filter tips and a dedicated micropipette set were used at all steps to minimise cross-contaminations. PCR cycling consisted of a denaturation step at 94 °C for 2 min, followed by 10 cycles of touch-down PCR at 94 °C for 30 sec for denaturation, annealing at 67 °C for 30 sec (with temperature decreasing by 1 °C per cycle), and extension at 68 °C for 30 sec. This was followed by 40 cycles of denaturation at 94 °C for 30 sec, annealing at 57 °C for 30 sec, and extension at 68 °C for 30 sec. Five µl of PCR products were analyzed by electrophoresis on a 2% agarose gel. DNA in the gel was stained with ethidium bromide, and visualized under UV light.

To identify the *Theileria* spp. present in the waterbuck and cattle samples, a total of 13 genus- and species-specific probes (Table 3-2) targeting *Theileria* spp. commonly found in Eastern Africa, were selected for RLB. RLB was conducted based on methods described by Gubbels et al. (1999). The probes, containing an N-(monomethoxytrityl)-C₆ amino linker (Hokkaido System Science, Sapporo, Japan) were bound to a Biodyne C membrane (Pall Life Sciences, Ann Arbor, MI, USA) on a protein screen-blotter (Sanplatec, Osaka, Japan) at the final concentration of 10 pmol. PCR products (15 µl) irrespective of concentration, were diluted to final volume of 125 µl with 2xSPPE/0.1% SDS, and were then denatured at 99.9 °C for 12 min, and immediately snap-frozen on ice for several minutes. Denatured products (120 µl), including the control samples, were hybridized to the membrane-bound probes at 42 °C for 60 min. The membrane was washed twice using 2xSSPE/0.5% SDS buffer at 52 °C for 10 min. The membrane was then incubated with peroxidase-labeled NeutrAvidin (Thermo Fisher Scientific, Rockford, IL, USA) for 30-60 min at 42 °C, followed by two washes with pre-warmed 2xSSPE/0.5% SDS for 10 min at 42 °C with gentle shaking. To detect the probe-amplicon hybridization, the membrane was incubated with 8 ml of ImmobilonTM Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) for 1-2 min at room temperature, and then exposed to a Chemiluminescent imager (Bio-Rad, Hercules, CA, USA). For reuse of the membrane, bound PCR products were removed by washing twice with pre-heated 1% SDS at 90 °C for 30 min and rinsing with 20 mM EDTA for 15 min. The membrane was stored in 2 ml of 20 mM EDTA at 4 °C.

3.2.4 Cloning and sequencing of 18S rRNA gene

Primers Nbab_1F and Nbab_1R (Oosthuizen et al., 2008), were used to amplify almost full-length of the 18S rRNA gene from the waterbuck DNA samples by PCR. PCR was performed with KOD Plus high fidelity DNA polymerase (Toyobo, Osaka, Japan) following the manufacturer's instructions with 2 µl (100-150 ng) of genomic DNA in a final volume of 25

µl. For each isolate, PCR was performed in quadruplicates and subjected to thermocycling: initial denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 15 sec of denaturation, annealing at 55 °C for 30 sec, and extension at 68 °C for 2 min followed by a final hold at 4 °C. PCR products were pooled for a total volume of 100 µl, and purified with the GeneClean III Kit (Q-BIOgene, Heidelberg, Germany) according to the manufacturer's instructions. The resulting blunt-ended amplicons were modified with 10x A-attachment mix (Toyobo, Osaka, Japan) as recommended by the manufacturer. The products were then ligated into pGEMT-Easy vector (Promega, Madison, WI, USA) and transformed into chemically-competent DH5α *E. coli* cells. Two to four recombinant bacterial colonies per transformation were identified by colony-PCR screening and inoculated into LB medium supplemented with ampicillin, and incubated overnight at 37 °C. Recombinant plasmids were extracted from 3 ml of culture using the QIAprep™ Spin Miniprep kit (Qiagen, Hilden, Germany). Approximately 350 ng of plasmid DNA for the selected bacterial clones was sequenced with 2.5 pmol of the primers T7 and SP6 (Promega, Madison, WI, USA), BT18S_2F, BT18S_3F (Oosthuizen et al., 2008) and RLB R2 (Nijhof et al., 2003) using the Quick Start kit (Beckman Coulter, Fullerton, CA, USA). Purified products were analysed with the CEQ 2000 Dye Terminator Cycle Sequencer, and the resultant sequences for the nearly full-length 18S rRNA gene assembled and edited manually using Geneious Pro version 6.1 (<http://www.geneious.com>) (Drummond et al., 2011). Sequence ends of low quality were trimmed and excluded from analyses. These sequences have been submitted to the Genbank pending accession numbers assignment.

3.2.5 Cloning and sequence analysis of partial ribosomal internal transcribed spacer (ITS) region

ITS regions were amplified from a total of six DNA samples representing the three waterbuck *Theileria* groups designated from the 18S rRNA gene. A forward strand Small subunit rRNA

(SSU rRNA) gene primer 1055F (5'-GGT GGT GCA TGG CCG-3'), and a reverse strand large subunit rRNA gene primer, LSUR300 (5'-T(A/T) GCG CTT CAA TCC C-3') (Holman et al., 2003) were used to perform the primary PCR. Thermal cycling included initial denaturation at 96 °C for 3 min followed by 30 cycles at 94 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 2 min, and extension at 72 °C for 10 min and hold at 4 °C. Nested PCR was performed as previously described (Holman et al., 2003) with forward primer ITSF (5'-GAG AAG TCG TAA CAA GGT TTC CG-3') and reverse primer LSUR50 (5'-GCT TCA CTC GCC GTT ACT AGG-3'). The thermal cycling protocol was as detailed above, except for the initial denaturation at 96 °C for 2 min and annealing at 55 °C. The nested PCR products were evaluated and cloned as described above. Recombinant plasmids were sequenced with T7 and SP6 vector primers (Promega, Madison, WI, USA) as well as nested primers ITSF and LSU50 (Holman et al., 2003).

3.2.6 *Phylogenetic analysis*

BLAST (Altschul et al., 1990) was used to search for sequences similar to the 18S rRNA gene sequences obtained by cloning and sequencing, as described above. A nucleotide sequence alignment of the new sequences and a number of related organisms obtained from the Genbank was constructed using CLUSTAL W (Thompson et al., 1994) in Geneious 6.1. Similarity matrices were performed using the two-parameter method of Kimura (Kimura, 1981) alongside the Jukes-Cantor correction model for multiple base changes (Jukes and Cantor, 1969). Phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei, 1987) implemented by Geneious 6.1 in combination with the bootstrap method at 1,000 replicates/tree.

3.3 RESULTS

3.3.1 *Detection of T. parva by Nested PCR*

A nested PCR targeting the single-copy gene p104 of *T. parva* was initially used to detect infection with the parasite in the study samples. In the cattle samples, the assay detected *T. parva* infections in nearly all samples (data not shown); however there were no discernible amplicon bands with the waterbuck samples. The detection limit of the current nested PCR was previously reported to be approximately 0.4 parasites/μl of host blood (Odongo et al., 2009). Therefore, to enhance the detection of *T. parva* DNA in the waterbuck samples, primary PCR products were not diluted prior to setting the secondary PCR; this too did not yield any visible bands on separating amplification products on agarose gel.

3.3.2 Detection of Theileria parasites in cattle and waterbuck samples by RLB

The RLB assay, incorporating probes among those that are specific for *Theileria* spp. that are known to occur at the study site, was subsequently applied to both sample sets. In all cases, the RLB assays were conducted with a protein miniblottedter that was adapted to perform the present study. Unlike the blotter routinely used to perform RLB studies, the one used in the present study was rectangular, limiting the loading capacity to a maximum of 19 samples per assay. A further variation was implemented during the RLB; the oligonucleotide probes were synthesized with an N-(monomethoxytrityl)-C₆ (MMT) amino linker instead of the TFA amino linker commonly reported in published RLB protocols. These oligo probes were found to function satisfactorily (Figure 3-1A-G).

Similar to the nested PCR, the RLB revealed high infection rate of *T. parva*, and even higher rate of infection with *Theileria* sp. (buffalo) among the cattle samples (Figure 3-1C-G). In addition, other species of *Theileria* that infect cattle were found in some of the cattle samples. These included benign *Theileria* species such as *T. mutans*, *T. buffeli* and *T. taurotragi* (Figure 3-1C, D, E, and G). In contrast, none of the species-specific probes hybridized with DNA derived from the waterbuck specimens (Figure 3-1A-B). Two faint signals, one corresponding to *Theileria* sp. (giraffe) 1 and another to *T. taurotragi*, were visible upon RLB

with the waterbuck DNA (Figure 3-1A). Among the species-specific probes on the membrane, the last one was for *Theileria* sp. (kudus). However, a positive control sample for this species was not available. Similarly, positive controls for *Theileria* sp. (buffalo), *T. velifera* and *Theileria* sp. (sable) were not available individually. A buffalo DNA specimen previously confirmed to have multiple infections with the three parasite species was used as positive control instead for all three. Interestingly, the *Theileria/Babesia* and *Theileria* genus-specific catch-all probes hybridized strongly with 25/26 (96%) of the waterbuck samples, suggesting the presence of haemoparasites other than those specified by the probes on the membrane (Figure 3-1A). One waterbuck sample gave a signal with the *Theileria/Babesia* universal probe alone, perhaps indicating the presence of a *Babesia* species in this animal sample.

3.3.3 Identification of novel Theileria in the waterbuck by sequencing of parasite ribosomal genes

Nearly full-length 18S rRNA genes were successfully amplified and cloned from 19 of the waterbuck samples following RLB. Plasmid clones for 18S rRNA were sequenced with vector-specific, and gene-specific internal primers published previously. BLASTn searches using the newly generated parasite sequences identified found no identical sequences in the GenBank. However, sequences from the present study had high homology with three *Theileria* spp., *T. equi*, *Theileria ovis* and *Theileria luwenshuni* (Table 3-3). Multiple sequence alignment of the new sequences with those of related organisms from Genbank revealed heterogeneity especially within the V4 hypervariable region consistent with being new and distinct parasites (Figure 3-2A). 18S rRNA gene sequences from the waterbuck *Theileria* were aligned with sequences from related protozoan parasites retrieved from GenBank and phylogenetic analyses undertaken (Figure 3-2B). Neighbour-joining analyses of

18S rRNA placed the waterbuck isolates into three distinct clades, all falling within the *Theileria sensu stricto* group.

Since the discriminatory utility of the 18S rRNA gene has been shown to diminish for closely-related parasites (Chansiri et al., 1999), a highly variable region of the ITS sequence from six clones representative of the waterbuck isolates was sequenced. Similar to the analyses with the 18S rRNA gene sequences, ITS sequences depicted three parasite populations among the waterbuck, distinct phylogenetic clusters and both sequence and length (data not shown). Interestingly, with the exception of waterbuck sample #1 and 2 that were provided on a later date and from which the amplification of the ribosomal genes was not done, there was strong correlation between sample collection period and the identity of the detected *Theileria* parasite (Table 3-1 and 3-3). This pattern was further confirmed by the clustering of the 18S rRNA sequences obtained from the waterbuck specimens upon phylogenetic analysis (Figure 3-2B). Among the three 18S rRNA clades identified from the waterbuck samples, one clade, designated as group A, clustered with *T. equi*, a pathogenic *Theileria* species and the causative agent of piroplasmosis in domestic and wild equine species. A second cluster, designated as group B, lies closer to *T. luwenshuni*, a highly pathogenic parasite that infects sheep and goats, and was discovered previously in China (Yin et al., 2008). BLASTn searches with the sequences forming the third clade indicated their closest match is *T. ovis*, a benign parasite of sheep.

3.4 DISCUSSION

Wildlife is an important reservoir of tick-transmissible blood-parasites including those of the genus *Theileria* (Han et al., 2009; McKeever, 2009; Oura et al., 2004, 2011; Pienaar et al., 2011). *T. parva* causes a rapidly fatal bovine theileriosis known as ECF, with severe economic losses in eastern, central and southern Africa (Bishop et al., 2004). Although the

role of the Cape buffalo in the maintenance and transmission of this pathogen is well documented (Conrad et al., 1987; Sibeko et al., 2008; Pienaar et al., 2011), evidence exists to suggest that other *bovidae*, particular the waterbuck, may play a role in the epidemiology of ECF (Stagg et al., 1994). Furthermore, *Theileria* spp. that are considered otherwise benign could also contribute to theileriosis of cattle when present as mixed infections (Oura et al., 2004). The present study was conceived to address the questions whether waterbuck are reservoirs for *Theileria* species that may be pathogenic to cattle and at the same time, and to investigate which *Theileria* spp. are found in waterbuck. A *T. parva*-specific nested PCR and RLB assays found no evidence of infection with *T. parva* in the waterbuck, while a majority of the cattle sampled from the same area were infected with *T. parva* and *Theileria* sp. (buffalo). Importantly, the RLB indicated the occurrence of previously uncharacterised *Theileria* spp. in the waterbuck that were subsequently confirmed by sequencing of 18S rRNA and ITS regions. Sequence analysis suggested the occurrence of at least three distinct populations of *Theileria* in waterbuck from a single locality in Kenya.

It is currently believed that waterbuck may play a role in the epidemiology of ECF (Bishop et al., 1992; Stagg et al., 1994) although data to support this hypothesis remains limited. To date natural infection of waterbuck with *T. parva* has not been confirmed. In this study we failed to detect *T. parva* DNA in blood samples from 26 waterbuck resident in an ECF-endemic locality in Kenya at which *T. parva* is present (Pelle et al., 2011), using both nested PCR and RLB assays, whereas potentially novel *Theileria* spp. were detected in the majority of the waterbuck samples by RLB. Two faint signals corresponding to *Theileria* sp. (giraffe) and *T. taurotragi* were observed with waterbuck sample # 20 and 28, respectively. Although these were evaluated as possible backgrounds on the RLB membrane, the signal with sample # 28 could be a positive for *T. taurotragi* which is known to infect peripheral blood leucocytes from a wide range of Bovidae, including the waterbuck (Stagg et al., 1983).

Marula farm, the site for the present study, is a commercial cattle ranch with a substantial population of bovids including buffalo and the waterbuck. This site is therefore suitable for investigating transmission of pathogens at the wildlife-livestock interface (Smith and Parker, 2010). The farm lies in an ecological zone suitable for *R. appendiculatus*, necessitating farmers to apply acaricides weekly during the dry seasons and bi-weekly in the wet seasons (Dr. E. Kariuki, KWS, personal comm). The presence of vector and multiple hosts make this site attractive for investigating pathogen prevalence among the bovids that share pastures at the ranch.

Consistent with previous studies (Pelle et al., 2011), cattle samples from the present study site had high infection rates with both *T. parva* and *Theileria* sp. (buffalo), singly or as mixed infections. However, waterbuck sampled from the same site presented no evidence of infection with either of the two parasites. Whereas cattle-derived *T. parva* do not infect waterbuck cells, buffalo-derived genotypes readily infect and transform them (Stagg et al., 1983, 1994), although the infected/immortalized cells failed to establish infection in autologous animals (Stagg et al., 1994). Moreover, the observation that waterbuck cell cultures are infected and transformed less efficiently by *Theileria* sp. (buffalo) than buffalo or cattle cells (Stagg et al., 1983) is also consistent with the failure to find *T. parva* in waterbuck from the field.

Extensive genetic diversity occurs in field populations of *T. parva* in cattle (Katzner et al., 2010), with the buffalo parasite population being more heterogeneous (McKeever, 2009; Oura et al., 2011a). Variation in the vectorial capacity of the tick may also influence *T. parva* population structure on passage through *R. appendiculatus* (Ochanda and Young, 2003; Katzner et al., 2010). Past studies in Kenya revealed that a genotype that is identical at the p67 locus with *T. parva* from cattle was infective to the waterbuck (Stagg et al., 1994). Although the waterbuck samples in the present study were relatively few, the failure to detect *T. parva*

or *Theileria* sp. (buffalo) in these animals does not support a role for this host in ECF epidemiology at this site. This conclusion is backed by the fact that we detected DNA for other haemoparasites among the waterbuck, suggesting that the sensitivity of the assays was not a problem. Melton et al. (1982) reported that waterbuck, especially the young, are frequently infested with *R. appendiculatus* ticks, which theoretically can inject a large parasite inoculum. However, in the field, some populations of *R. appendiculatus* are refractory to infection with *T. parva* (Odongo et al., 2009) and therefore, heavy infestation may not necessarily lead to parasite acquisition by the vector.

Novel *Theileria* spp., including pathogenic ones, are frequently reported in wild ungulates (Höfle et al., 2004, 2005; Schnittger et al., 2004). During the present investigations, an infection rate of 96% with *Theileria* spp. of unknown identity was observed in the waterbuck using a *Theileria* genus-specific RLB probe. This was consistent with a previous study that reported a high prevalence of theilerial piroplasms in waterbuck (Stagg, 1992). A single waterbuck sample yielded a signal with the *Theileria/Babesia* universal probe only, where another specimen may have *T. taurotragi*. Since genus-specific probes for *Babesia*, *Anaplasma* or *Erhlichia* spp. were not present in the RLB, it is likely that infection with other haemoparasites besides *Theileria* can not be excluded.

The identification and taxonomic characterisation of new haemoparasites is frequently inferred from the 18S rRNA gene (Allsop and Allsop, 2006; Chansiri et al., 1999; Lack et al., 2012). On the basis of the 18S rRNA gene sequences, the waterbuck *Theileria* genotypes clustered into three distinctive clades on phylogeny. Interestingly, with the exception of waterbuck sample #1 and 2 that were provided on a later date and from which the amplification of the ribosomal genes was not done, a strikingly correlation between sample collection dates and the identity of the detected *Theileria* parasite was observed. This pattern was further confirmed by the clustering of the 18S rRNA and ITS sequences obtained from

the waterbuck specimens upon phylogenetic analysis. This finding is consistent with the waterbuck blood samples having originated from three independent animal herds within Marula farm, which is also supported by the specific dates on sample collection.

Among the three clades carrying the 18S rRNA sequences for the *Theileria* parasites detected in the waterbuck, one clade, designated as group A, clustered with *T. equi*, a pathogenic *Theileria* species and causative agent of piroplasmosis in horses and donkeys. This association is significant since recent data suggests that *T. equi* occupies an intermediate phylogenetic position with respect to *Theileria* and *Babesia* (Kappmeyer et al., 2012), and moreover, *T. equi* exhibits extensive heterogeneity in field populations (Bhoora et al., 2010; Salim et al., 2010). However, a *T. equi* probe included in the RLB did not hybridize with any of the waterbuck DNA samples, including those from group A, suggesting the presence of a different *Theileria* species or a genetic variant of *T. equi*. *T. equi* is highly heterogeneous in the field (Bhoora et al., 2009, 2010; Salim et al., 2010), and RLB has been shown to discriminate between isolates (Bhoora et al., 2009).

Fatalities related to infection with *Theileria* spp. have been reported in wildlife (Nijof et al., 2003, 2005; Höfle et al., 2004; Oosthuizen et al., 2009). A recent KWS report found mortalities of up to 40% during translocations of waterbuck (Dr. Isaac Lekolool, unpublished data). Presently, *Theileria* spp. that are highly similar at sequence level with known pathogens of livestock and wildlife were identified. Besides the *T. equi*-like genotype, a *Theileria* species with high sequence homology with *T. luwenshuni* was found. *T. luwenshuni* is a highly pathogenic parasite of sheep and goats that was reported in China (Yin et al., 2008). Detailed clinical and pathological data for the fatalities reported by KWS are not available and therefore, further studies are needed to establish whether the detected parasites are pathogenic. This is of importance since high mortality rates have been reported in calves of another waterbuck species, *Kobus ellipsipyrmnus*, in the Umfolozi Game Reserve in South

Africa (Melton, 1987). These fatalities were associated with aggravated tick infestations, suggesting a possible role of tick-borne pathogens, consistent with the occurrence of such pathogens in several other antelope species (Nijhof et al., 2005). A *Theileria* species whose closest BLASTn matches were *T. ovis* was also detected in the present study. Future studies could establish whether it represent a benign species or a pathogenic form.

In summary, this study investigated the possible role of waterbuck as a reservoir of *Theileria* spp. that are infective to livestock. Taken together, these findings suggest that waterbuck may not play a role in ECF epidemiology but harbours multiple other *Theileria* that may be pathogenic.

3.5 SUMMARY

Waterbuck (*Kobus defassa*), a wild bovid species endemic to the Eastern African savannah, is suspected of harboring tick-transmitted parasites infective to livestock. In addition to being infested by large numbers of *Rhipicephalus appendiculatus*, laboratory studies have previously suggested that waterbuck may be a reservoir of *Theileria parva*.

In the present study, blood samples from 26 waterbuck and 86 cattle originating from Marula Farm, a site in Rift Valley, Kenya, and an area endemic for East Coast fever (ECF), were investigated for the presence of cattle-infective *Theileria* parasites. Two assays, a nested PCR targeting the p104 gene of *T. parva*, and reverse line blot (RLB) incorporating 13 oligonucleotide probes specific for *Theileria* spp. of livestock and wildlife in Kenya were performed on DNA extracted from both sets of samples. Both assays found no evidence of *T. parva* or *Theileria* sp. (buffalo) infection in the waterbuck samples. In contrast, the cattle samples were overwhelmingly positive for both parasites.

On the RLB, a generic catch-all probe for *Theileria* spp. gave strong signals in 25/26 (96%) of the waterbuck samples while none of the 11 species-specific probes hybridized with the waterbuck-derived touch-down PCR products. 18S ribosomal RNA (18S rRNA) and internal transcribed spacer (ITS) genes amplified from some of the RLB-positive waterbuck samples indicate the occurrence of three uncharacterised *Theileria* spp. from waterbuck populations from a single locality in Kenya. Among the three 18S rRNA clades identified from the waterbuck samples, one clade, designated as group A, clustered with *Theileria equi*, a pathogenic *Theileria* species infective to domestic and wild equids. These parasites did not hybridize with the probe for *T. equi*, suggesting occurrence of a novel genetic variant. A second cluster, designated as group B, lies closer to *Theileria luwenshuni*, a highly pathogenic parasite that infects sheep and goats that has been reported in China. BLASTn searches with the sequences forming the third clade indicated they were closer to *T. ovis*, a benign parasite

infective to sheep. Taken together, the findings of this study suggest that waterbuck may not play a role in ECF epidemiology but harbours multiple other *Theileria* spp. whose veterinary importance is unknown at the present.

Table 3-1. Summary of animal hosts from which blood samples were obtained

	Waterbuck sample	Date of collection	Geographical origin	RLB screening	18s rRNA cloning
1.	Waterbuck # 1	12.2010	Marula	++	N/A
2.	Waterbuck # 2	12.2010	Marula	++	N/A
3.	Waterbuck # 10	23.5.2009	Marula	++	Yes
4.	Waterbuck # 11	23.5.2009	Marula	++	ND
5.	Waterbuck # 12	23.5.2009	Marula	++	Yes
6.	Waterbuck # 15	15.5.2009	Marula	++	Yes
7.	Waterbuck # 17	23.5.2009	Marula	++	Yes
8.	Waterbuck # 19	15.5.2009	Marula	++	Yes
9.	Waterbuck # 20	12.6.2009	Marula	+++	Yes
10.	Waterbuck # 21	13.6.2009	Marula	++	ND
11.	Waterbuck # 22	15.6.2009	Marula	++	ND
12.	Waterbuck # 23	13.6.2009	Marula	++	Yes
13.	Waterbuck # 24	12.6.2009	Marula	++	Yes
14.	Waterbuck # 25	10.6.2009	Marula	++	Yes
15.	Waterbuck # 26	13.6.2009	Marula	++	Yes
16.	Waterbuck # 27	13.6.2009	Marula	++	ND
17.	Waterbuck # 28	10.6.2009	Marula	+++	ND
18.	Waterbuck # 34	10.6.2009	Marula	+	ND
19.	Waterbuck # 39	14.10.2008	Marula	++	Yes
20.	Waterbuck # 42	9.10.2008	Marula	++	ND
21.	Waterbuck # 47	14.10.2008	Marula	++	Yes
22.	Waterbuck # 50	8.10.2008	Marula	++	Yes
23.	Waterbuck # 53	10.10.2008	Marula	++	Yes
24.	Waterbuck # 57	14.10.2008	Marula	++	Yes
25.	Waterbuck # 61	14.10.2008	Marula	+	Yes
26.	Waterbuck #71	10.10.2008	Marula	++	Yes
	Cattle sample #1-86	-	Marula	++	N/A

Waterbuck samples that were positive in both touch-down PCR and RLB but failed to yield visible bands with 18S rRNA PCR were not cloned (ND). 18S rRNA was not amplified from the cattle samples and two waterbuck specimens (N/A).

+= RLB positive with *Theileria/Babesia* catch-all only.

++= RLB positive with *Theileria/Babesia* catch-all and *Theileria* catch-all.

+++= RLB positive with *Theileria/Babesia*, *Theileria*-genus, and a species-specific probe.

Table 3-2. Identity and DNA sequence of oligonucleotide probes used in the RLB assay

Probe specificity	18S sequence (5'-3')	Reference
<i>Theileria/Babesia</i> catch all	TAATGGTTAATAGGARCRTTG	Gubbels et al., 1999
<i>Theileria</i> catch all	ATTAGAGTGCTCAAAGCAGGC	Nagore et al., 2004
<i>Theileria annulata</i>	CCTCTGGGGTCTGTGCA	Georges et al., 2001
<i>Theileria parva</i>	GGACGGAGTTCGCTTTG	Gubbels et al., 1999
<i>Theileria taurotragi</i>	TCTTGGCACGTGGCTTTT	Gubbels et al., 1999
<i>Theileria mutans</i>	CTTGCGTCTCCGAATGTT	Gubbels et al., 1999
<i>Theileria buffeli</i>	GGCTTATTTTCGGWTTGATTTT	Gubbels et al., 1999
<i>Theileria equi</i>	TTCGTTGACTGCGYTTGG	Butler et al., 2008
<i>Theileria</i> sp. (giraffe 1)	TTATTTCTCCTTGACGAGTT	Githaka et al., 2013
<i>Theileria</i> sp.(buffalo)	CAGACGGAGTTTACTTTGT	Oura et al., 2004
<i>Theileria velifera</i>	CCTATTCTCCTTTACGAGT	Gubbels et al., 1999
<i>Theileria</i> sp. (sable)	GCTGCATTGCCTTTTCTCC	Oosthuizen et al., 2008
<i>Theileria</i> sp. (kudus)	CTCCATTGTTTCTTTCCTTTG	Nijhof et al., 2005

The degenerate position R denotes either A or G. The degenerate position W denotes either A or T, and Y denotes either C or T.

Table 3-3. Blastn search results with 18S rRNA sequences of three *Theileria* spp. obtained from the waterbuck

Parasite clade	Waterbuck 18S rRNA clone	Sequence length (bp)	Highest Blastn match	Accession no. of match	% similarity
Group A	W26C1	1,666	<i>T.equi</i>	JQ390047	99
	W24C2	1,671	<i>T.equi</i>	AB515307	99
	W20C1	1,673	<i>T.equi</i>	HM229408	99
	W25C1	1,664	<i>T.equi</i>	HM229408	98
	W26C2	1,665	<i>T.equi</i>	HM229408	99
	W23C1	1,664	<i>T.equi</i>	HM229408	99
	W20C4	1,666	<i>T.equi</i>	HM229408	99
Group B	W19C7	1,656	<i>T. luwenshuni</i>	JX469518	99
	W15C4	1,658	<i>T. luwenshuni</i>	JX469518	99
	W10C3	1,665	<i>T. luwenshuni</i>	JX469518	99
	W15C5	1,656	<i>T. luwenshuni</i>	JX469518	99
	W17C4	1,657	<i>T. luwenshuni</i>	JX469518	99
	W15C8	1,657	<i>T. luwenshuni</i>	JX469518	99
	W17C8	1,657	<i>T. luwenshuni</i>	JX469518	99
	W19C3	1,658	<i>T. luwenshuni</i>	JX469518	99
	W10C2	1,661	<i>T. luwenshuni</i>	JX469518	98
	W12C6	1,656	<i>T. luwenshuni</i>	JX469518	99
	W12C8	1,656	<i>T. luwenshuni</i>	JX469518	99
Group C	W61C2	1,661	<i>T.ovis</i>	AY260171	98
	W71C4	1,664	<i>T.ovis</i>	AY260171	98
	W61C4	1,664	<i>T.ovis</i>	AY260171	98
	W50C1	1,662	<i>T.ovis</i>	AY260171	98
	W57C2	1,667	<i>T.ovis</i>	AY260171	98
	W71C7	1,664	<i>T.ovis</i>	AY260171	98
	W39C2	1,110	<i>T.ovis</i>	FJ603460	97
	W53C6	1,662	<i>T.ovis</i>	AY260171	98
	W47C3	1,664	<i>T.ovis</i>	AY260171	98
	W53C1	1,662	<i>T.ovis</i>	AY260171	98

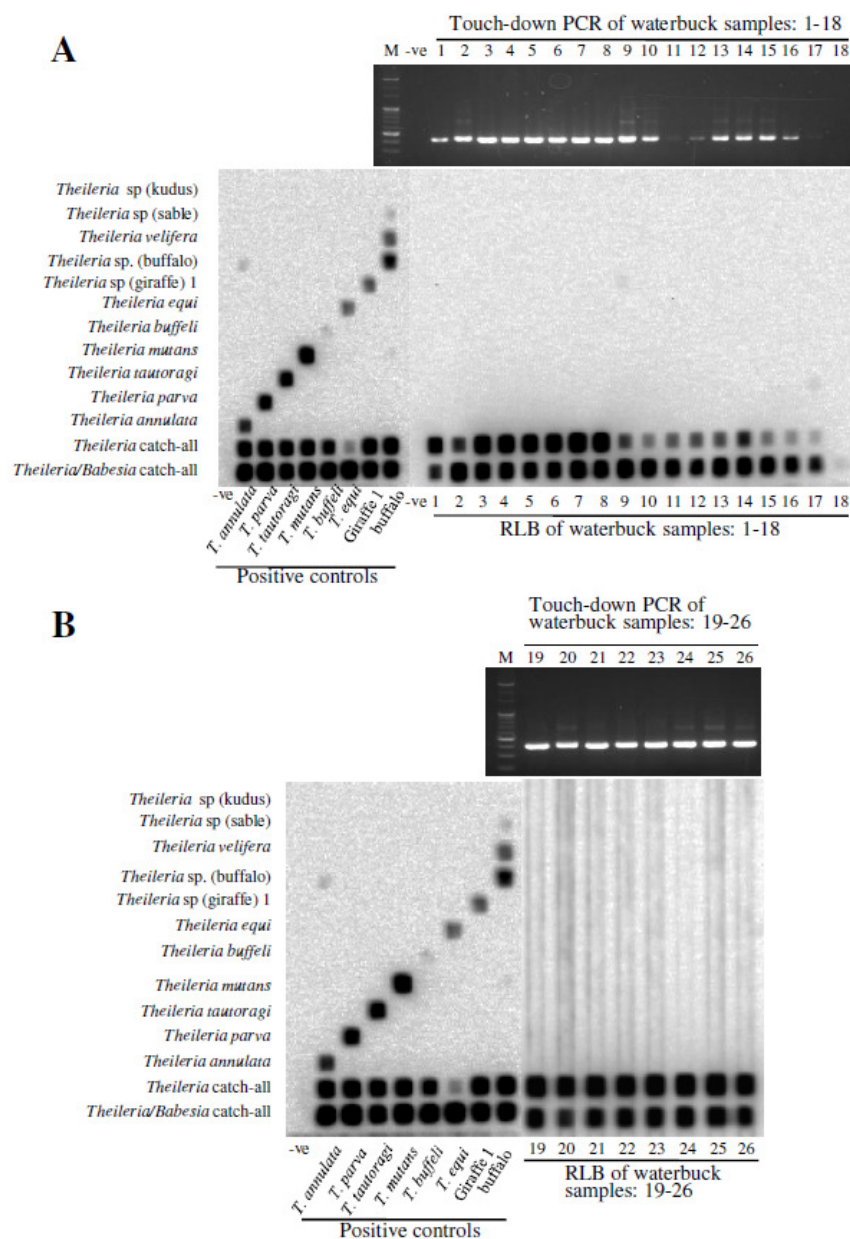
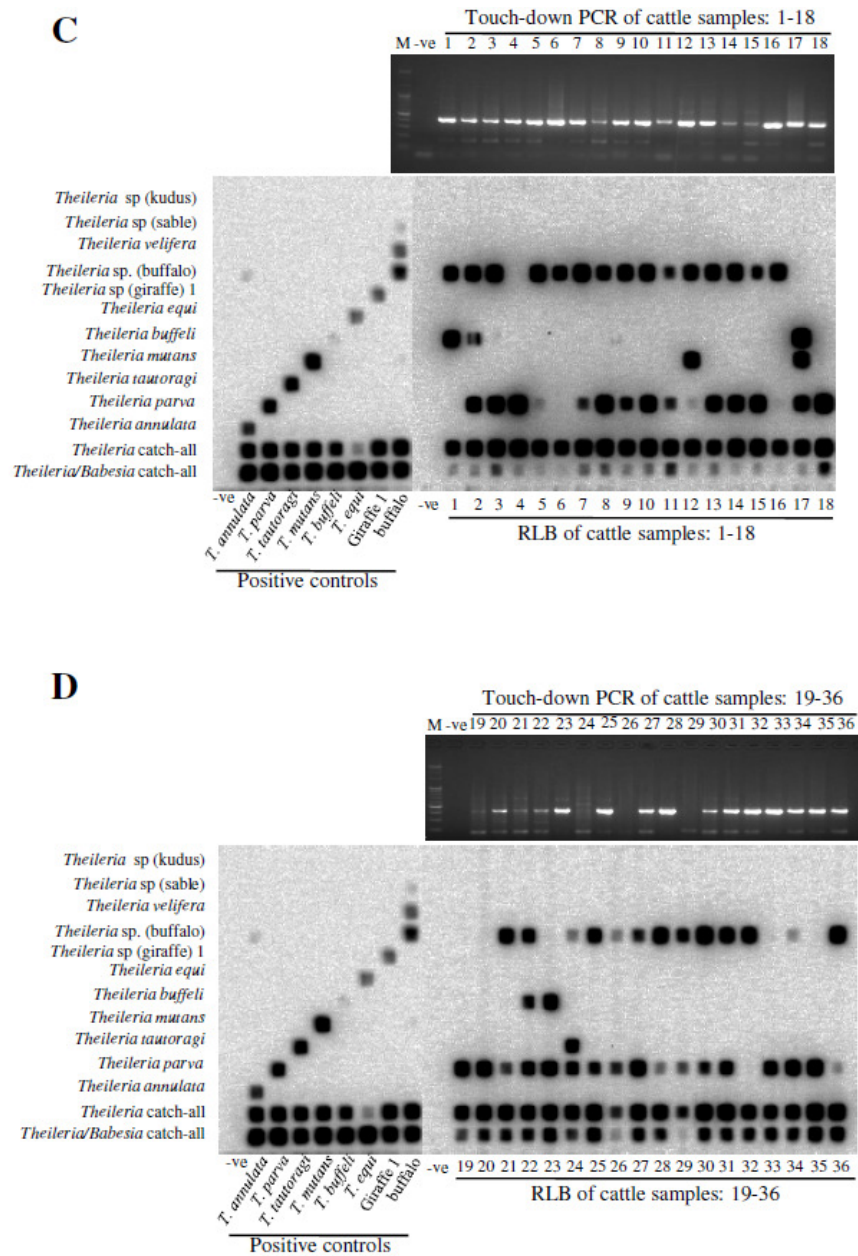
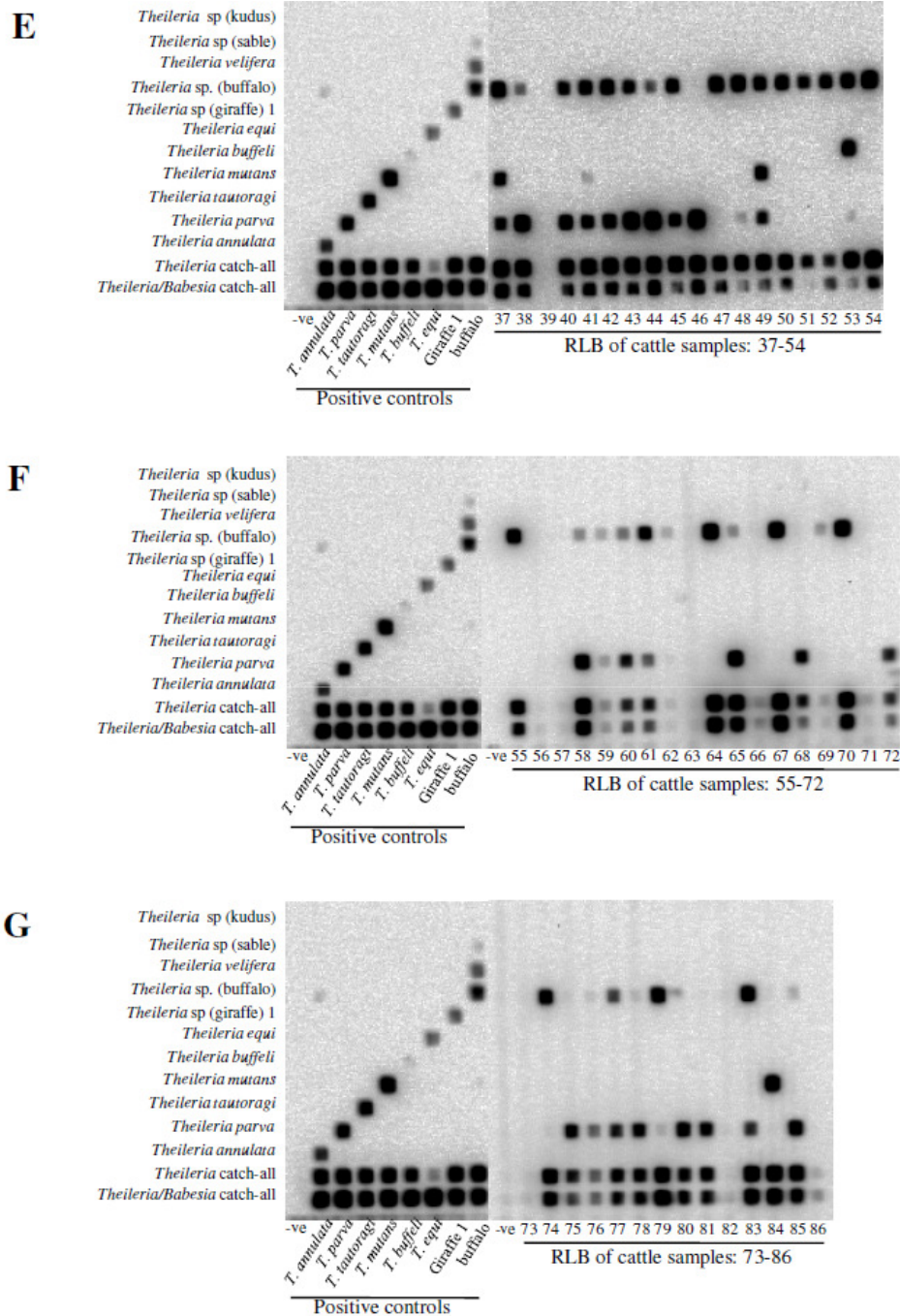


Figure 3-1. PCR-RLB of blood samples from waterbuck and cattle samples

A-B: Amplification of the V4 hypervariable region of the 18S rRNA gene from waterbuck-derived DNA samples and the corresponding RLB. The buffalo positive control (red) was previously shown to have *Theileria* sp (buffalo), *T. verifera* and *T. sable* all in one. Two faint spots at sample 9 and 17 are discussed in text. M is a 100-bp DNA ladder. C-D: Amplification of the V4 hypervariable region of the 18S rRNA gene from cattle DNA samples (1-36). PCR and RLB steps were conducted as with the waterbuck specimens. Positive reactions are shown by dark spots on the membrane. Probes were applied on the horizontal lanes while PCR products were applied vertically. More than one signal with the species-specific probes indicate mixed infections. E-G: RLB with cattle-derived DNA samples 37-86. PCR amplification was confirmed by 2% agarose electrophoresis prior to the RLB hybridisation (not shown). The negative control was omitted in panel E after the sample was exhausted. Data interpretations is as with previous panels.



(Figure 3-1. continued)



(Figure 3-1. continued)

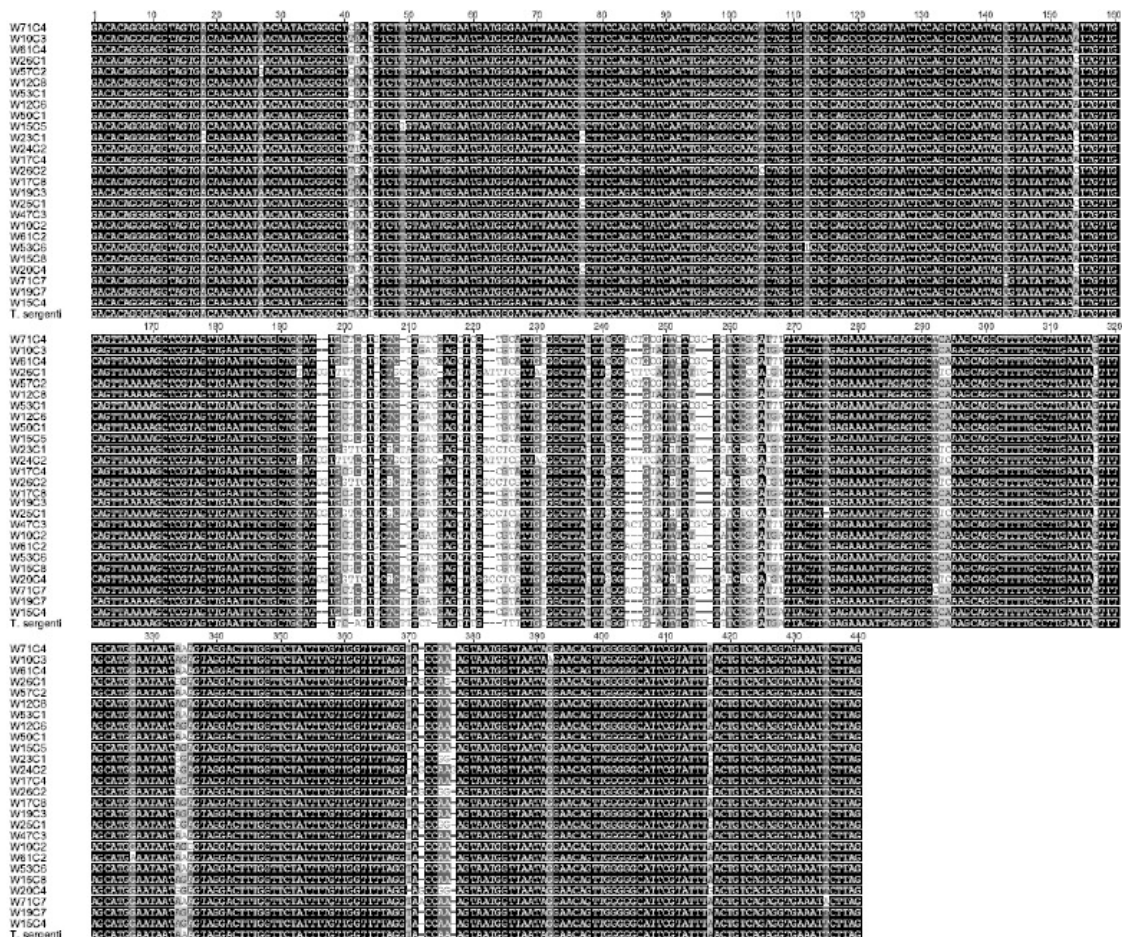


Figure 3-2 Multiple sequence alignment extraction of the V4 hypervariable region (nucleotide positions 446-886) of 18S rRNA genes of *Theileria* sp. (waterbuck) from the present study
 Full-length 18S rRNA gene from *Theileria sergenti* (Genbank accession number AB016074) was used as reference for sequence length. Light shadings denote nucleotide substitutions while dark shades represent non-variant region of the 18S gene. The shown region was extracted from a nucleotide sequence alignment comprising of all the 18S rRNA sequences obtained from the waterbucks.

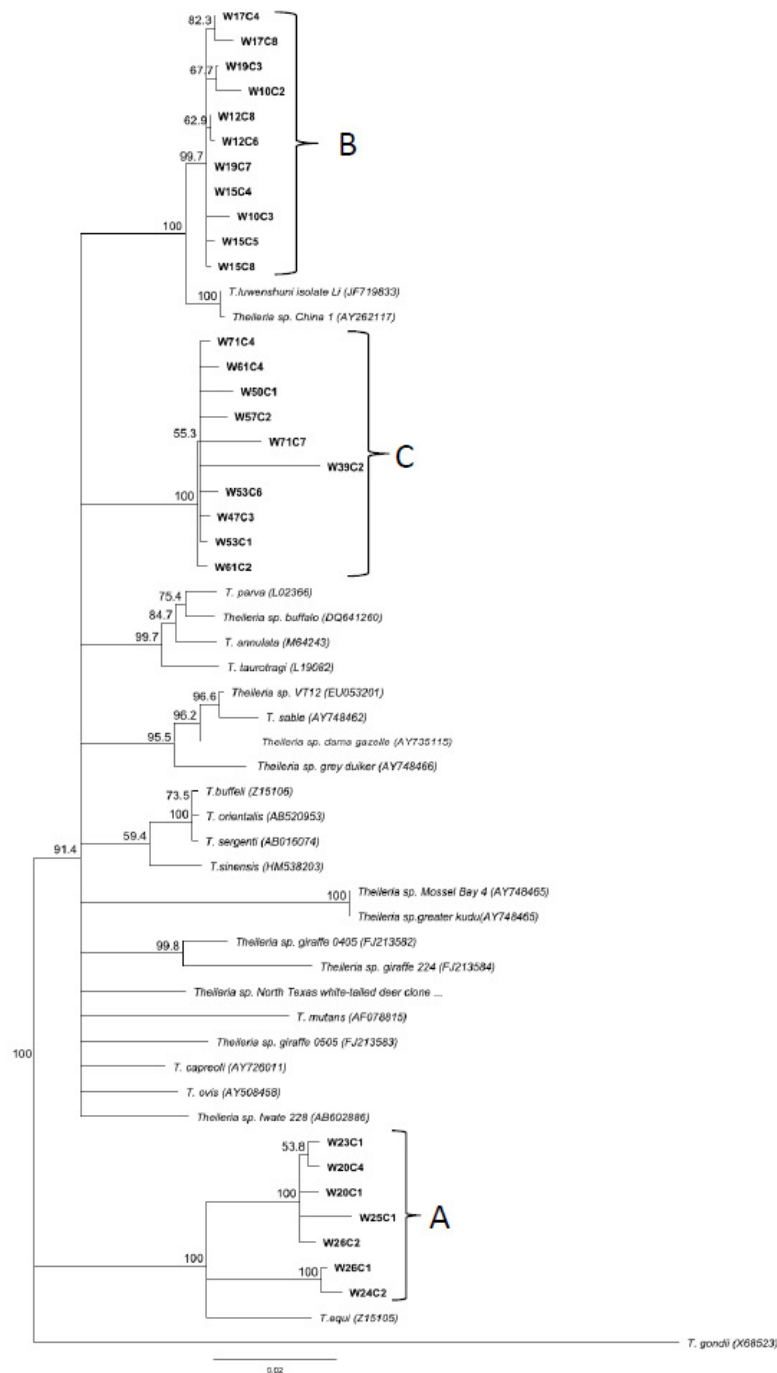


Figure 3-3. Rooted neighbor-joining phylogenetic tree showing the relationship between genotypes of *Theileria* spp. (waterbuck) identified in the present study and closely related *Theileria* species based on 18S rRNA gene sequences. The numbers represent the percentage of 1,000 replications (bootstrap support) for which the same branching patterns were obtained. GenBank accession numbers for parasite sequences are in parentheses. Genotypes identified in this study are in bold. The scale bar indicates substitutions per site.

PART II:
MOLECULAR AND IMMUNOLOGICAL CHARACTERISATION OF CANDIDATE
ANTI-TICK VACCINE ANTIGENS

The original paper of this part will be submitted for publication, and thus can not be shown at the time the thesis has been submitted to Hokkaido University.

CHAPTER 4
MOLECULAR CLONING AND EVALUATION OF RECOMBINANT FERRITIN 2
AGAINST HETEROLOGOUS CHALLENGE WITH *IXODES PERSULCATUS*
AND *IXODES OVATUS* TICKS

4.1 Abstract

The lack of highly immunogenic and conserved antigens among tick species is one of the major reasons why cross-protective tick vaccines have not been developed. In fact, Bm86, a tick antigen derived from *Rhipicephalus microplus*, used for a commercial anti-tick vaccine, had shown a limited efficacy against tick species which are distantly related to *R. microplus*. Recently, it has been found that ferritin 2 (FER2), which is a soluble iron transporter critical for successful blood feeding and fecundity in all tick species, could be applied for a cross-protective universal anti-tick vaccine. Thus, in this chapter, two additional FER2 homologues were identified from two Ixodid ticks, *Ixodes persulcatus* and *Rhipicephalus appendiculatus* ticks, which are important disease vectors in Japan, and many parts of Africa, respectively, and the vaccine potential of this antigen was evaluated against a heterologous tick challenge with *Ixodes* tick species.

RACE primers were designed based on the conserved regions of tick FER2, and used to identify FER2 homologues from *I. persulcatus* and *R. appendiculatus*. FER2 from *I. persulcatus* was highly homologous (99.5%) at amino acid level with that of *Ixodes ricinus*, which was shown to have vaccine efficacy of 98%. Similarly, FER2 identified in *R. appendiculatus* showed 89% amino acid identity with that of *R. microplus* which is reported to have vaccine efficacy of 76%. The computer-based analysis for the prediction of antigenic epitopes showed that these two proteins are highly immunogenic and suitable for immunization, and vaccination with recombinant FER2 from *I. persulcatus* expressed in

Escherichia coli (I.perFER2) was shown to induce high antibody response in hartley guinea pigs. Recombinant I.perFER2 had deleterious effects on *I. persulcatus* and *Ixodes ovatus* ticks when they were fed on vaccinated animals. These effects included decreased blood feeding and low oviposition rates. These results show that this antigen could be the best cross-species antigen yet for the development of universal tick vaccine. Future studies are necessary to evaluate FER2 as a cross-reactive antigen against other tick genera, and to examine protective efficacy of this antigen in cattle.

CONCLUSION

Ticks, and by extension tick-transmissible pathogens, are distributed globally. As obligate haematophagous feeders, ticks transmit a wide range of microorganisms, including many that are pathogenic to humans, livestock and wildlife species, leading to high mortalities and economic losses. Thus, epidemiological and genetic studies to characterise tick-borne pathogens present in a particular host species as well as developing alternatives to the use of chemical pesticides to control tick infestation are priority areas in many parts of the world.

Polymerase chain reaction-reverse line blot (PCR-RLB) is a simple and versatile method that enables simultaneous detection and differentiation of multiple pathogens from a host blood. Multiple probes on the membrane are useful in detecting mixed infections. Combined with sequencing of defined regions of pathogen genome, RLB can help detect novel parasites, as was shown in the course of this work. In the present studies, this assay was utilised to determine the occurrence and identity of haemoparasites from domestic and wildlife host species.

Wildlife is of particular economic importance to Kenya through ecotourism. However, infectious diseases, in addition to poaching and human-wildlife conflicts, have become a threat to many wild animals. Piroplasms, tick-borne haemoparasites comprising of *Babesia* and *Theileria* are widely reported among felids in southern Africa; however, the status of feline piroplasmosis in Kenya is poorly understood at the present, and hence the relevance of the study presented in chapter 1. Piroplasms DNA was detected from blood specimens of three wild feline species; lions, cheetah and leopards. Genetic variants of *B. canis* were found in blood samples from a pair of animals with suspected mineral deficiency. Although these animals were successfully treated with anti-parasite drug and diet supplementation, the finding of *B. canis*-like parasite is significant since fatal cases of babesiosis linked to co-

infection with canine distemper virus has been reported recently. From the leopard specimens, *B. leo* was detected by RLB; however, the limited number of 18S rRNA sequences obtained from plasmid clones did not match published sequences of *B. leo*, perhaps because the sequencing was of low quality. It is possible that sequencing of a larger numbers of clones could yield matching sequences. On the other hand, the sequences obtained from the leopard samples were clearly of *Babesia* as inferred from BLAST and phylogeny analyses. A *Babesia* of the big type has been reported in a leopard in Kenya previously, however, due to the absence of blood smears from these animals, it could not be concluded that these obtained sequences are of the said parasite.

In chapter 2, a combination of newly designed RLB probes and sequencing of parasite 18S rRNA genes revealed that at least two populations of *Theileria* occur in the giraffes from Kenya. Moreover, multiple genetic variants exist with the two populations, including genotypes that are nearly identical to pathogenic ones that were reported in giraffes from South Africa recently. There is need for further studies to confirm the utility of the new probes using samples of wider geographic coverage.

In chapter 3, the question of whether the waterbuck, a common ungulate species in many parts of Africa, carry parasites infective to cattle, including *T. parva* was investigated. In 26 waterbuck blood samples, there was no evidence of *T. parva* infection by both nested PCR and RLB specific to this parasite. Cattle blood specimens from the same sampling locality were however infected with numerous *Theileria* spp., including *T. parva* and *Theileria* sp. (buffalo) the causative agents of ECF and Corridor disease, respectively. The waterbuck samples however yielded unique sequences upon sequencing of 18S rRNA genes, which corroborated the detection of unknown *Theileria* species at the RLB step. The new sequences cluster into three distinct clades upon phylogeny, indicating they are perhaps three different species. One of these clades, designated as group A, closely cluster with *T. equi*, a known

pathogen of equines, which is highly heterogeneous in the field. It would be interesting to perform full genome sequencing of these *T. equi*-like isolates and compare them with that of *T. equi*.

Although wildlife species are generally resistant to piroplasmas, domestic hosts are highly susceptible to infections with *Theileria* and *Babesia*. Furthermore, ticks cause severe damage directly to animals through blood feeding resulting into decreased body weight, reduced meat/milk production, anemia and skin damage. Thus, vector control methods have dual purpose of reducing tick infestation and disease transmission. Integrated tick control with anti-tick vaccines has been shown to reduce acaricide use by two-third in some instances. These commercialized vaccines, based on the gut antigen Bm86, are however inadequate against tick species including those found in Japan and Africa.

The recent discovery of FER2, a highly conserved iron transport protein that is critical for tick blood feeding and reproduction, and that is highly immunogenic, present a unique opportunity to develop a universal anti-tick vaccine. Thus, there is need to evaluate the vaccine efficacy of FER2 homologues from other tick species of medical and veterinary importance, and importantly, determine cross-reactivity with other species. In this study, FER2 of *I. persulcatus*, a known vector of Lyme borrelia in Japan was identified through RACE cloning and the recombinant antigen used as a vaccine. I.persFER2 elicited a robust humoral response that likely correlate with protection against tick infestation. Indeed, blood feeding by *I. persulcatus* and *I. ovatus*, a related species was impaired in animals vaccinated with this antigen. Furthermore, oviposition in *I. persulcatus* ticks was clearly reduced, both in number of eggs and their appearance, consistent with past studies on the importance of FER2 in tick reproduction. Though preliminary in nature, these results clearly support the inclusion of FER2 in future vaccine trials against *I. persulcatus*. FER2 homologue from the African tick vector *R. appendiculatus* was also identified in the context of the present study. It is highly

similar to that of *R. microplus* both in the amino acid level and also the predicted antigenicity. Future studies will evaluate its utility as a vaccine antigen against this tick vector in Africa.

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APPENDIXES

Appendix 1: SDS-PAGE recipe.

Resolving gel for 2 gels (1gel)

	10%	11%	12%	13%	14%	15%
DDW	4,550 (2,275)	4,050 (2,025)	3,550 (1,775)	3,050 (1,525)	2,550 (1,275)	2,050 (1,025)
30% A.A	5,000 (2,500)	5,500 (2,750)	6,000 (3,000)	6,500 (3,250)	7,000 (3,500)	7,500 (3,750)
1.5M Tris pH 8.8	3,800 (1,900)	-	-	-	-	-
50% Glycerol	1,500 (750)	-	-	-	-	-
10% APS	150 (75)	-	-	-	-	-
TEMED	6 (3)	-	-	-	-	-

Stacking gel

	0.9ml	1.8ml	3.6ml	5.4ml
DDW	525	1,050	2,100	3,150
30% A.A	150	300	600	900
0.5M Tris pH 6.8	225	450	900	1,350
10% APS	3	6	12	18
TEMED	1.5	3	6	9

Appendix 2: Western blot recipe for DAB method.

50mM CoCl ₂	2.5ml
1M Imidazole	5ml
Double-distilled water	42.3ml
25mg/ml DAB	200µl
H ₂ O ₂	16.75µl

Molecular investigation of tick-borne protozoan parasites at the livestock-wildlife interface in Kenya and evaluation of a candidate anti-tick vaccine antigen

(ケニア共和国の家畜および野生動物緩衝地帯におけるダニ媒介性原虫の分子生物学的解析と抗ダニワクチンの開発)

マダニおよびマダニ媒介性疾患は、家畜の生産性低下や野生動物の死亡原因および人獣共通感染症などの公衆衛生上の観点から世界中で問題になっている。現在、マダニに対する制御法は抗ダニ剤によるものが主であるが、環境汚染や耐性ダニの出現などの問題も多く、新たな対策法の開発が求められている。

ケニア共和国（ケニア）では野生動物は貴重な観光資源である。しかし近年、密猟等に加え感染症を原因とする野生動物数の減少が危惧されている。南アフリカ共和国ではダニ媒介性原虫による感染が問題視されているが、ケニアにおいてはこれまで詳細な調査がなされていない。また、野生動物から家畜へのマダニ媒介性病原体の伝播も危惧されており、詳細な研究調査が求められている。そこで本研究では、第1章第1節でネコ科野生動物、第2節でキリンにおける *Babesia* 原虫および *Theileria* 原虫の疫学調査ならびに分子生物学的解析を行った。また第3節では、家畜と野生動物間のマダニ媒介性病原体の伝播の可能性を探るため、家畜および野生動物緩衝地帯において *Theileria parva* (*T. parva*) の分子疫学調査を行った。さらに第2章では、抗ダニワクチンの開発を目的として、*Ixodes persulcatus* (*I. persulcatus*) から候補抗原を同定し、その抗原の抗ダニ効果について検討した。

ケニア国内で採取した野生動物由来 DNA 検体を用いて、PCR 法および reverse line blot (RLB) 法により、*Babesia* 原虫および *Theileria* 原虫の分子疫学調査を行った。さらに陽性検体については、遺伝子比較解析を行った。その結果、ヒョウからは *Babesia leo* (*B. leo*) および新規の *Babesia* 原虫種の遺伝子が検出された。またライオンからは *B.canis* の感染が認められ、チーターからは新規の *Theileria* 原虫種の遺伝子が検出された。一方、マサイキリン

(*Giraffa camelopardalis tippelskirchi*) およびアミメキリン (*Giraffa camelopardalis reticulata*) を RLB 法によって診断した結果、13頭中12頭から *Theileria* 原虫遺伝子が検出された。18S ribosomal RNA 遺伝子の解析の結果、これらの原虫は2種類の新規 *Theileria* 原虫であることが明らかになった。

T. parva は、致死率が極めて高いウシの東海岸熱の起因病原体である。実験感染等により、ウォータバック (*Kobus defassa*) が *T. parva* のレゼルボア的一种である可能性が示唆されているが、野外における詳細な調査研究はない。そこで、ウォータバックと家畜であるウシが共存する Marula 地区において、*Theileria* 原虫感染の分子疫学調査を行った。その結果、検査したウシ86頭で高率な *T. parva* 感染が認められた。一方、ウォータバック26頭からは3種に大別される新規の *Theileria* 原虫が検出されたが、*T. parva* は検出されなかった。

抗ダニワクチンの開発を目的に、鉄貯蔵タンパクの一種である Ferritin 2 (FER2) に着目し *I. persulcatus* および *R. appendiculatus* から Ferritin 2遺伝子全長を同定した。得られた情報をもとに組換え *I. persulcatus*-Ferritin 2 (I.pFER2) を作製して、モルモットを用いて I.pFER2 の抗ダニワクチンの効果の検討を行った。その結果、I.pFER2免疫により *I. persulcatus* の有意な吸血量の減少と産卵量の減少が認められた。さらに I.pFER2は、異種のダニである *I. ovatus* に対しても抗ダニ効果が認められ、広いスペクトルを示す抗ダニワクチン抗原として期待された。

本研究で得られた知見から、ケニアに分布する野生動物には種々の *Babesia* 原虫や *Theileria* 原虫が感染していることが明らかとなった。今後、病原性等の詳細について検討する必要がある。一方で、高率にウシの *T. parva* 感染が認められた地区のウォータバックからは *T. parva* は検出されなかった。ウォータバックが *T. parva* のレゼルボアとなりうるかは、未だ不明であり、今後も検証が必要であると考えられた。また、鉄貯蔵タンパクである I.pFER2を用いたワクチン試験より、本因子がマダニの吸血や産卵を阻害する有用な抗ダニワクチン候補抗原であることが示された。今後、家畜を用いた試験や他のマダニに対する効

果について検討を重ね広いスペクトルを示す抗ダニワクチンとして応用可能か検討する必要があると考えられた。