Molecular Characterization of piroplasms in the Black (*Diceros bicornis michaeli*) and White rhinoceros (*Ceratotherium simum simum*) Meta-population in Kenya

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2014

DECLARATION

I declare that this thesis is my original work and has not been presented for a degree in any other university or for any other award

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DEDICATION

To my loving Dad Festus Kivata, Mum Angelica Kivata, Husband Zadock Wambua, Son Adrian Wambua, Brothers and Sisters Benson Mutua, Emmanuel Ndaka, Gabriel Muthiani, Christopher Musyoka, Winfred Nthoki, Catherine Mwongeli and Elizabeth Nundu, Nephews Gregory Mbithi, Alphine Kyalo and Wayne Nzioka for their continued love, encouragement and support during the time I was carrying out this project.

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ABBREVIATIONS AND ACRONYMS

BLAST	Basic Local Alignment Search Tool
BPP	Bayesian Posterior Probability
CDC	Centre for Disease Control
DNA	Deoxyribonucleic Acid
DIC	Disseminated Intravascular Coagulation
EDTA	Ethylenediaminetetraacetic acid
IPZ	Intensively Protected Zones
IUCN	International Union for Conservation of Nature
Kg	Kilogram
PCR	Polymerase chain Reaction
Rhino	Rhinoceros
RLB	Reverse Line Blot
RNA	Ribonucleic acid
rRNA	Ribosomal Ribonucleic acid
TBE	Tris-Borate-Ethylenediaminetetraacetic acid
TEMED	Tetramethly-Ethlenediamine
Spp	Species
LNNP	Lake Nakuru National Park
MNP	Meru National Park
NRS	Ngulia Rhino Sanctuary
NNP	Nairobi National Park

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ABSTRACT

The eastern black (*Diceros bicornis michaeli*) and the white (C*eratotherium simum*) rhinoceros are critically endangered species mainly due to poaching and loss of habitat. Conservation strategies such as creation of highly secured sanctuaries, intensive management and linking meta-populations by translocations, are yielding positive population growth rate in Kenya. However, diseases are still another impediment to the rhinoceros population growth. Lethal infection with *Babesia bicornis* have been linked to mortality and morbidity of rhinoceroses post-translocation in South Africa and Tanzania. Presence of such lethal *Babesia* or other piroplasms in the Kenyan rhinoceros meta-population has not been investigated. Such information is necessary in guiding health monitoring, translocation, diagnosis and veterinary care for the species. The aim of this study was to determine the species and genetic diversity of piroplasms in selected rhino sub-populations in Kenya. Blood samples were collected from 114 (82 black and 32 white) rhinoceroses during scheduled management activities by Kenya Wildlife Service between 2011 and 2012. The samples were collected from males and females of all age groups. Genomic DNA was extracted from whole blood using DNA extraction kit (DNeasy blood and Tissue Kit, QIAGEN) followed by a nested amplification of the 18S small subunit ribosomal RNA (18S ssrRNA) gene of *Babesia* and *Theileria*. The PCR products were analyzed by gel electrophoresis, and a subset of the positive secondary PCR products sequenced for both forward and reverse strands. Results showed that *Theileria. bicornis* was the only infecting species with a prevalence of 49.1%. No *Babesia* species were identified. White rhinoceroses (65.6%) were significantly infected compared to black rhinoceroses (42.7%, χ^2 =0.028). More males were infected compared to females in both rhinoceros species but the difference was not statistically significant (χ^2 = 0.353). There were variations in infection rates among the age categories with more sub adults infected compared to adults and juveniles but the difference was not significant (χ^2 = 0.465). Results showed variations in infections among the sub-populations with Meru National Park having the highest infection rate (66.7%) and Solio Game Ranch the least (12.5%), but the differences were not statistically significant (χ^2 = 0.140). Three new haplotypes of *T. bicornis* H1, H2, and H3 were identified in this study. H3 was the most predominant (66.7%) and was distributed in all the sampled sub-populations. H1was only found in Lake Nakuru National Park's black rhinoceroses whereas H2 was identified in Lake Nakuru and Meru National Parks, and Ngulia Rhino Sanctuary. This study concludes that *T. bicornis* infects both black and white rhinoceroses and it is distributed in most of the conservational areas in Kenya. This being the first report of new *T. bicornis* haplotypes, the findings have important ecological and conservational implications, especially for future population management and translocation programs.

CHAPTER ONE

INTRODUCTION

1.1 Background

Rhinoceros were abundant in Kenya in the early 20th Century (Brett, 1993) and they were viewed as agricultural pests that impeded human settlement in the eastern part of Kenya. The eastern black rhinoceros (*Diceros bicornis michaeli*) numbered around 20,000 individuals in the 1970's and still had a wide range distribution throughout Kenya. However, the population declined catastrophically due to authorized hunting, poaching and human settlements during the following 20 years, to less than 400 individuals by 1990 (Okita *et al*., 2007). This decline resulted in small, isolated, demographically unviable populations scattered across fragmented regions in Kenya, with many facing local extinction. An ambitious translocation program for isolated rhinoceros populations that focused on moving rhinoceroses into high security breeding nucleus sanctuaries enhanced their security and breeding prospects (Merz, 1994). Gradually new sanctuaries were established by translocating offspring from the nucleus sanctuaries and by 2008, the black rhinoceros population in Kenya had grown to over 650 animals (Emslie *et al*., 2009) in 16 subpopulations.

The current number of free ranging black and white rhinos in Kenya is around 670 and 370 individuals respectively. Although the population size of the Eastern black rhinos in Kenya is small, it represents about 90% of the subspecies global

population (Emslie, 2011). The Southern white rhinoceros (*Ceratotherium simum simum*) population was introduced in Kenya from South Africa (Okita *et al.,* 2007; Emslie *et al*., 2009) and their small number represents a steadily growing population. Demand for rhino horns of both species drive the high poaching incidence. The black rhinoceroses' horn is highly valued making the species a prime target for poaching. In Kenya, both species are intensively managed as a meta-population with sub-populations occurring in different eco-zones (Brett, 1990). Translocation of individual rhinos from various sub-populations is therefore frequently undertaken to promote gene flow and also manage population size as well as balance sex ratio (Muya & Oguge, 2001).

The process of capture and translocation is a highly stressful event, which elicits variable negative effects on the physiology and immunology of the animal (Woodford & Rossiter, 1993). A group of blood parasites known as piroplasms, in the order *Piroplasmidae*, which includes *Babesia* and *Theileria*, have been linked to morbidity and mortality of rhinos in Tanzania, South Africa and Kenya (Nijhof *et al*., 2003; Penzhorn, 2006; Obanda *et al*., 2011), which suggests that besides poaching, disease is an emerging impediment to rhinoceros recovery strategies (Ramsey *et al*., 1993; Penzhorn *et al*., 1994).

The connection of translocation to onset of disease such as piroplasmosis, which is transmitted by the nearly ubiquitous ticks, is of interest because translocation is

a vital tool frequently employed to manage the meta-population *in situ* (Emslie *et al*., 2009). The species of *Babesia* and *Theileria* associated with mortalities in black rhino in South Africa and Tanzania are *T. bicornis* and *B. bicornis* that have been named as new species (Nijhof *et al*., 2003). To date there is no evidence whether these lethal species infect Kenyan rhinoceroses. Moreover, it is not known which other species of piroplasms latently infect rhinoceroses in the different Kenyan sub-populations.

The objective of this study was therefore to evaluate the occurrence, prevalence, diversity, and spatial distribution of piroplasms in rhinoceros sub-populations in Kenya.

1.2 Problem Statement

Black and white rhinos are critically endangered because of their small numbers yet poaching and diseases still remains a threat that may drive the species to extinction. Kenya has established a robust rhinoceros conservation programme with the goal of reducing poaching, promoting population growth and achieving genetically viable and breeding population. The creation of secure sanctuaries is providing appreciable protection to the rhinoceroses. However, achieving maximum population growth is still a challenge and unlikely because factors that affect growth rate of a small population are both multiple and variable. Parasite infections and parasitic diseases are now known to play a negative role in the

health and population size of wild animals. Parasitic infections may reduce host fitness, reproduction, immune competence, and sometimes alter behavior. Therefore, in the case of rhinos, blood-borne protozoa's in the genus, *Babesia* and *Theileria* commonly known as piroplasms have been implicated in the post-translocation deaths of rhinos. Translocation is thought to elicit stress induced-immuno-suppression that allows proliferation of latent infection leading to clinical state. Since translocation is key to the rhino population management, its frequent use is thus seen as trigger for deadly result contrary to the desired intent. However, translocation is always known to be inherently associated with risks of spread and introduction of pathogens. Its continued use globally is therefore guided by IUCN protocols that require pre-translocation surveys or epidemiological studies of pathogen risks in the area. Currently, the status of piroplasms that are circulating in Kenyan population is unknown. The proposed study therefore seeks to provide useful information towards health management of the rhinos in Kenya.

1.3 Justification

In Kenya, there are two species of rhinoceroses, the southern white rhinoceros (*Ceratotherium simum simum*) and the eastern black rhinoceros (*Diceros bicornis michaeli*). The eastern black rhinos are critically endangered and are on the verge of extinction. Besides poaching, infectious diseases are potential threats to populations as they can cause acute or chronic infections that may reduce fitness

or cause death (Nijhof *et al*., 2003). Wild animals occupying areas endemic to certain parasites usually develop endemic stability and rarely develop clinical disease (Penzhorn *et al*., 1994; Penzhorn, 2006). However, when stressed by natural conditions or human induced factors, such as capture and translocation, which is an important management tool, wild animals develop clinical disease. Moreover, translocation is known to spread pathogens and vectors to other areas, thereby risking infection to immunologically naive populations (Woodford & Rossiter, 1993; Leighton, 2002). Tick-borne piroplasms in the genus *Babesia* and *Theileria* cause disease and death in rhinoceroses, and they have been associated with post-translocation deaths. Nevertheless, the occurrence, distribution and prevalence of these parasites in rhinoceroses are not well described in Kenya. Therefore it was imperative to have knowledge of their epidemiology and spatial distribution to help in prevention and control during and after rhino translocation. The findings of the study will provide information that will be directly beneficial to the conservation of the endangered rhino species in Kenya.

1.4 Hypothesis

Null Hypothesis: There is no host species and rhinoceros sub-population difference in the infection rates of piroplasms in Kenya.

1.5 Objectives

1.5.1 General objective

To determine the genetic diversity, prevalence and spatial distribution of piroplasms infecting Kenyan rhinoceroses

1.5.2 Specific objectives

i. To genetically identify species of *Babesia* and *Theileria* among sub-populations of black and white rhinos in Kenya.
ii. To determine prevalence of *Babesia* and *Theileria* infection in black and white rhinoceros sub-populations in Kenya.
iii. To determine the spatial distribution of *Babesia* and *Theileria* among the sampled rhino sub-populations

CHAPTER TWO

LITERETURE REVIEW

2.1 Piroplasms

Piroplasms are parasitic apicomplexan protozoa that invade erythrocytes and sometimes other cells of vertebrates (Levine, 1971; Brown & Torres, 2008). All piroplasms are small, round or pear- shaped, and are parasitic on amphibians, birds, and mammals (Levine, 1971). The Order *Piroplasmidae* is divided into four families with *Babesiidae* and *Theileriidae* comprising species of major veterinary and medical importance. Piroplasms are transmitted by haematophagus ixodid ticks.

2.1.1 *Babesia*

Babesia is a tick-borne intra-erythrocyte and generally host specific protozoan parasite that causes hemolytic disease referred to as Babesiosis (Brown & Torres, 2008). To date, there are about 99 species of *Babesia* which have been recognized based on their morphology, serologic tests, and molecular characteristics (Levine, 1971; Moody & Chiodini, 2000; Hunfeld *et al*., 2008). These organisms have a wide geographical range, and the range of ticks and animals upon which the vectors feed is equally wide (Maria & Jeffrey, 1996). They are parasitic on variety of vertebrates, and are transmitted through the saliva of tick when it bites to feed (Uilenberg, 2006).

Although it is possible for a single *Babesia* species to infect more than one vertebrate host, most *Babesia* species are host specific (Brown & Torres, 2008). In general *B. bovis, B. bigemina, B. divergens,* and *B. major* infect cattle and buffalos, *B. equi* and *B. caballi* infect horses, *B. canis* infect dogs, *B. felis* infect cats, *B. microti* infect rodents while *B. trautmanni* and *B. Perroncitoi* infect *pigs* (Maria & Jeffrey, 1996; Uilenberg, 2006). Ixodid ticks in the genus *Boophilus*, *Rhipicephalus*, *Ixodes*, *Haemaphysalis*, *Dermacentor* and *Hyalomma* are the major vectors of the *Babesia* parasites (De Vos *et al*., 2004; Bock *et al*., 2004; Marc *et al*., 2010; Clive, 2012). These protozoan parasites survives the moulting stages of ticks, hence all life stages of ticks can transmit them to susceptible hosts.

2.1.1.1 The life cycle of *Babesia*

The life cycle of *Babesia* is complex and involves three stages of reproduction that takes place in arthropod the intermediate host and definitive vertebrate host. The development begins when the tick ingests infected blood meal from a definitive host (Figure 2.1). The blood meal contains gametes and once inside the ticks gut, they undergo gamogony which is the fusion of the gametes. This is followed by sporogony which involves an asexual reproduction in salivary glands of the tick, a life stage that involves formation of a zygote followed by meiosis and multiple fissions that lead to formation of a sporozoite. The final stage, merogony which is an asexual reproduction occurs in the definitive vertebrate host. Merogony involves the development of a trophozoite inside the cell where it

increases in size while repeatedly replicating its nucleus and other organelles. The development is followed by cytokinesis, which subdivides the multinucleated schizont into numerous daughter cells called merozoites that are released into blood when the host cell ruptures (Hunfeld *et al*., 2008). Some of the trophozoites develop into gametocytes which are taken up by the ticks when they are feeding.

The general mode of transmission of *Babesia* spp is similar regardless of species, although minor differences exist (Maria & Jeffrey, 1996). The parasites are transmitted by ticks; however as with most blood pathogens, surgical procedures such as dehorning, castration and needle vaccination procedures are sometimes implicated in accidental transfer of infected blood from one animal to another, thereby transmitting infection (Homer *et al*., 2000; Brown & Torres, 2008). Ticks acquire *Babesia* infection during feeding through bites (Bock *et al*., 2004) or transovarially whereby after the zygotes have entered the hemolymph they may invade cells such as fat cells and nephrocytes and undergo second cycle of division (Telford *et al*., 1993; Hunfeld *et al*., 2008; Brown & Torres, 2008). The resulting ookinetes can then invade the eggs in the ovaries and be transmitted to the next generation (Bock *et al*., 2004).

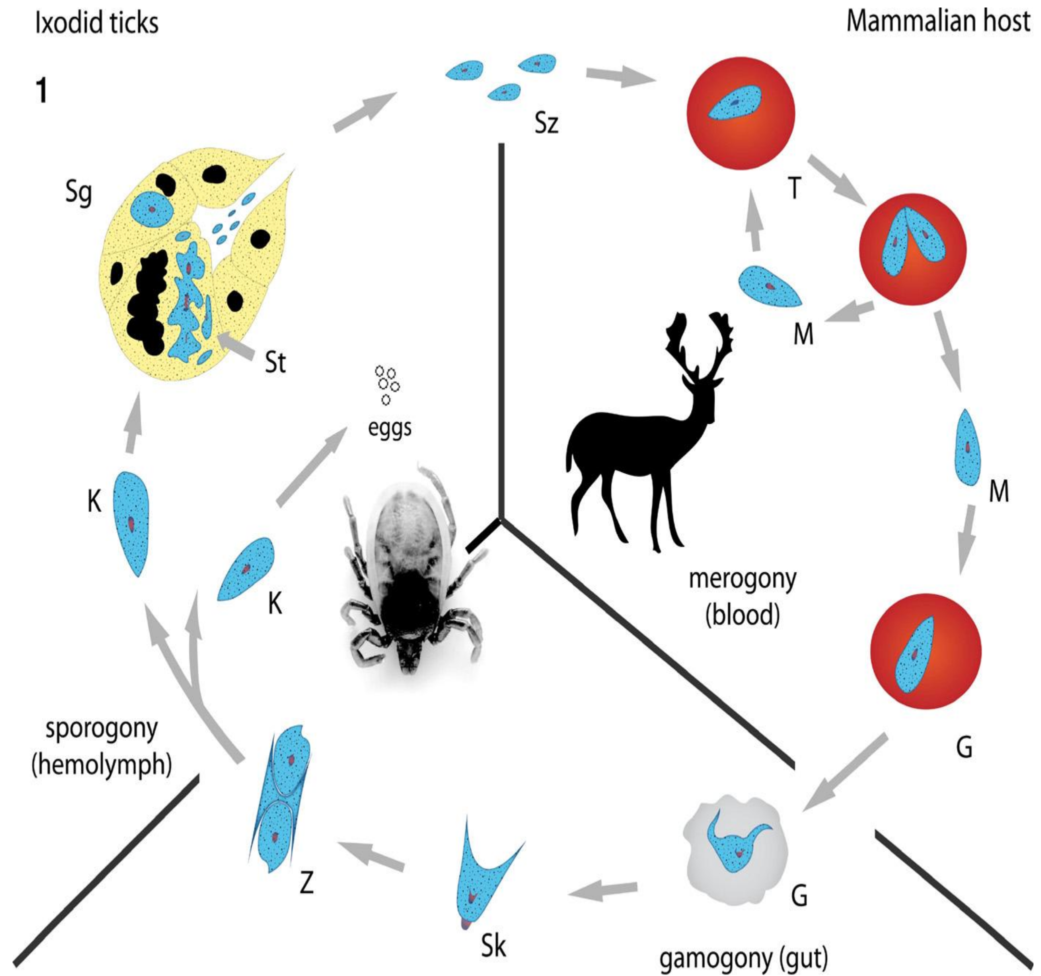

Figure 2.1: Life cycle of *Babesia* spp. Infection is acquired when sporozoites (Sz) are transferred during tick feeding. Sporozoites then invade erythrocytes and develop into trophozoites (T). Trophozoites divide by binary fission and produce merozoites (M) which continue infection and reinitiate the replicative cycle in the host. Some trophozoites develop into gametocytes (G) which can initiate infection in the tick vector. In the tick gut gametocytes develop into ‘‘Strahlenko¨rper” (Sk) which fuse to form a zygote (Z) which develops into a kinete (K). Kinetes gain access to the hemolymph of the tick, replicate, and invade various organs. Sporogony is initiated when kinetes invade the salivary glands (Sg). Here, the parasite forms a multinucleated sporoblast (St). Newly developed sporozoites (Sz) will then be injected into the host with tick saliva upon the next blood meal (Hunfeld *et al*., 2008).

Transovarial transmission constitutes a large population of ticks infected with *Babesia* (Homer *et al*., 2000; Hunfeld *et al*., 2008). Not all larvae carry the infection and the proportion of larvae infected, referred to as the 'infection rate' varies considerably. The infection rate in larval ticks depends mainly on the concentration of parasites in the blood of the animal on which the 'mother' tick fed. The infection rate in larvae from adult female ticks that fed on 'carrier' host is likely to be extremely low and may be zero (Brown & Torres, 2008). However, if female ticks engorge on an animal which has babesiosis, the larval progeny will have a high infection rate. Other factors which influence the infection rate in larval ticks are temperature and time after hatching (Bock *et al*., 2004). The parasite multiplies in the host cells damaging them until the animal dies or its immune system overcomes the infection (Hunfeld *et al*., 2008). The incubation period of *Babesia* species is 2-3 weeks for natural disease but can be as short as 4-14 days in experimental inoculation depending on the size of the inoculums (Brown & Torres, 2008)

2.1.1.3 *Babesia* infection in wildlife

Clinical infection by *Babesia* species causes a disease known as Babesiosis. In wild animals *Babesia* species infection has been documented in several animal species in different geographical locations (Levine, 1971; Penzhorn, 2006; Uilenberg, 2006). *Babesia* species have been reported in both black and white Rhino (Mugera & Wandera, 1967; Brocklesby, 1967; McCullogh &Achard, 1969;

Bigalke *et al*., 1970; Ramsey & Zainuddin, 1993; Meltizer, 1994; Knapp, 1997; Govender *et al.*, 2011) specifically *Babesia bicornis* in South Africa and Tanzania. They have been associated with rhino mortalities (Nijhof *et al*., 2003; Zimmerman, 2009). Babesiosis has been described in wild felids including the African lion (*Panthera leo*), Leopards (*Panthera pardus*), and cheetahs (*Acinonyx jubatus*) and it is caused by *B. felis*, *B. cati*, *B. leo*, *B. canis presentii*, *B. canis canis*, *B. canis vogeli*, *B. pantherae*, and *B. herpalluri* (Cornelia *et al*., 2003; Lopez *et al*., 2005; Penzhorn *et al*., 2006; Uilenberg, 2006; Bosman, 2010; Marc *et al*., 2010; Githaka *et al*., 2012; Samson *et al*., 2012). In Tanzania co-infection of *Babesia (B. felis, B. leo and B. gibsoni)* and Canine Distemper Virus (CDV) has been correlated with lion mortalities (Munson *et al*., 2008).

Five *Babesia* spp have been reported in primates including *B. perodictici*, *B. galacolata*, and *B, microti* (Claive 2012; Jeneby *et al*., 2011) whereas *B. equi* has been reported in Kenyan and Tanzanian zebras. In several cases it has been associated with deaths of grevy's zebras (Claive, 2012). *Babesia* species has also been reported in the wild bovines from different geographical areas; *B .bovis* and *B. bigemina* has been reported in water buffalo (*Bubalus bubalis)* coexisting with crossbred cattle in Egypt, Argentina, and Thailand whereby the buffaloes had developed natural tolerance to the parasite and acted as reservoirs in Egypt (Yasser, 2012; Mohamad *et al*., 2011; Ferreri *et al*., 2008). The African buffalo

(*Syncerus caffer*), antelope (*Hippotragus niger, Kobus vordanii,*), and eland (*Taurotragus oryx*) have also been reported as hosts of *Babesia* (Karbe *et al*., 1979; Garcia *et al*., 2007; Brown & Torres, 2008; Oosthuizen *et al*., 2008). In South Africa, *Babesia* co-infection with, *Trypanosoma*, and *Theileria* species was reported in the African buffalo (Yusufmia *et al*., 2010). Thirteen *Babesia* species have also been shown to infect avians, with only one species, *B. shotti* being pathogenic (Pierce, 2000).

Babesiosis in wild animals is diagnosed by observing the clinical signs, microscopy and advanced molecular testing. To prevent and control the disease in animals, tick control, vaccination, chemoprophylaxis and chemotherapy strategies are applied (Brown & Torres, 2008). Several factors influence the occurrence of babesiosis, including over infestation by vector ticks resulting in a high inoculum of *Babesia*; long periods without ticks with resultant loss of immunity and vulnerability to infection; stress factors and nutritional deficiencies which can induce a drop in immunity and vulnerability to the disease (Brown & Torres, 2008). Immune response in babesiosis apparently involves both humoral and cell mediated components of the immune system (Moody *et al*., 2000) and not all antibodies developed after infection are protective. Infected animals develop a life-long immunity against re-infection with the same species (De Vos *et al*., 2004; Uilenberg, 2006; Brown & Torres, 2008)

2.1.2 *Theileria*

Theileria species are obligate intracellular protozoan parasites belonging to the genus *Theileria*, Family Theileriidae, Order Piroplasmida, Subclass Piroplasmia, Phylum Apicomplexa (Levine, 1971). *Theileria* infect both wild and domestic animals.There are two species, *Theileria annulata* and *Theileria parva* which are important animal parasites causing tropical Theileriosis and East Coast Fever respectively (Bishop *et al*., 2004; Brown & Torres, 2008). *Theileria* species are transmitted by ticks including *Rhiphicephalus*, *Hyalomma*, and *Amblyomma* tick species (Norval *et al*., 1992; Brown & Torres, 2008). *Theileria* parasites differ from *Babesia* in two ways. First, the former has both pre-erythrocytic stages in mononuclear cells and erythrocytic stage and secondly, they are smaller in size (Bishop *et al*., 2004; Uilenberg, 2006). These parasites are considered to be less pathogenic in most cases and for the few pathogenic cases; virulence varies according to the stock and dose of the parasite as well as the type of mammalian host (Norval *et al*., 1992).

2.1.2.1 Life cycle of *Theileria*

Theileria life cycle consists of both sexual and asexual stages. As illustrated in Figure 2.2 below, the life cycle of *Theileria* in the tick begins with the ingestion of piroplasm (gametocytes) infected erythrocytes with the blood meal. Gamete fusion takes place in the ticks gut and the zygote formed invades the gut epithelial cells and differentiates into a motile kinete which invades the salivary glands and

differentiates into sporozoites after the tick attaches to feed. Infective sporozoites are released from the host from day 3 to day 7 of tick feeding and are transferred to the host (Bishop *et al*., 2004). In the host the sporozoites invades the lymphocytes and monokines (Uilenberg, 2006), whereby they are asexually transformed into merozoites through trophozoites (merogony). The merozoites multiply in the cells and finally the lymphocytes burst releasing the merozoites which invades the erythrocytes and the cycle continues.

Theileria parasites are transmitted by ticks which acquires their infection during feeding through bites (Bock *et al*., 2004) or may be trans-stadially transmitted whereby the larval or nymphal instars of the tick acquires infections from a blood meal which is then transmitted to a new host after moulting by nymphs or adults respectively (Maria *et al*., 1996; Bishop *et al.,* 2004; Uilenberg, 2006). Ticks can remain infected on the pasture for up to two years depending on climatic conditions. For transmission to occur, the infected tick has to attach for several days to the host to enable sporozoite maturation and emission to the saliva of the feeding tick. However under high temperatures ticks on the ground may develop infective sporozoites which can be transmitted to the host within a few hours after tick attachment (Brown & Torres, 2008)

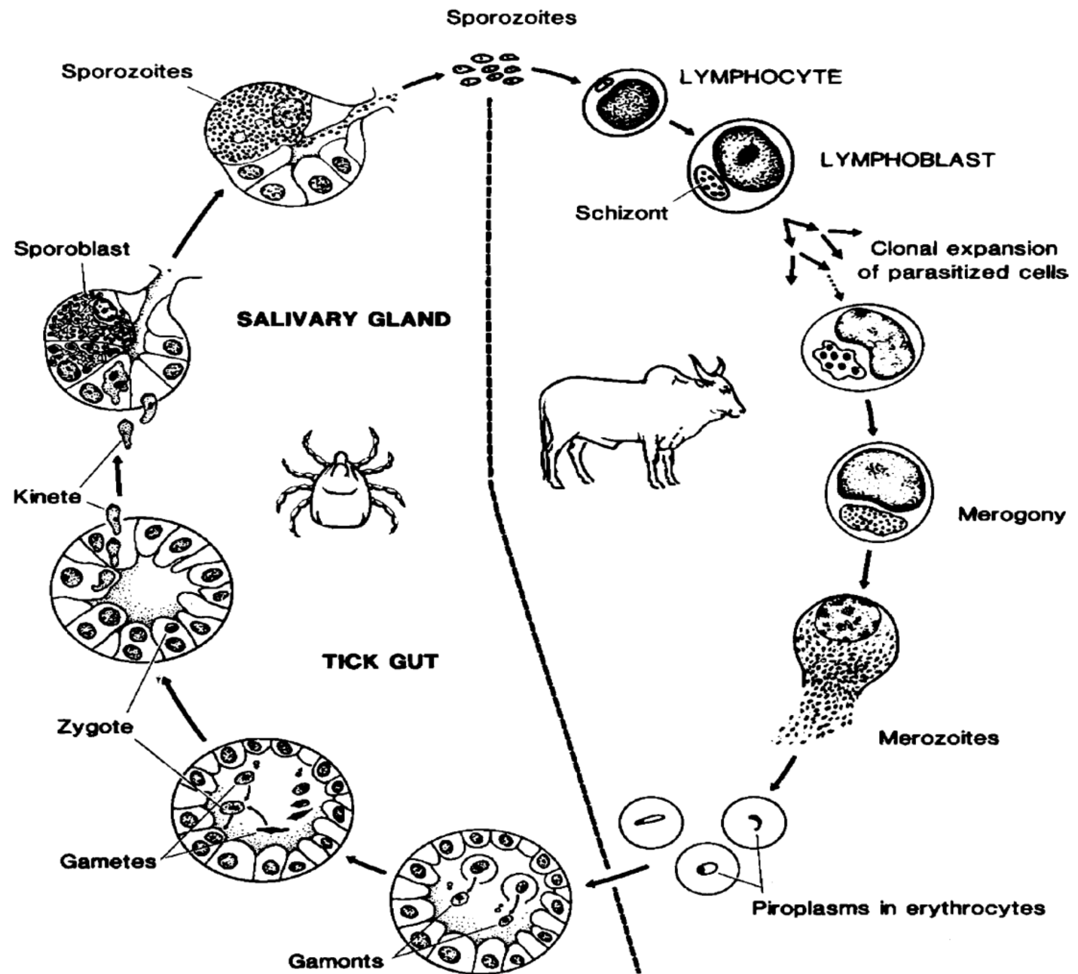

Figure 2.2: Life cycle of *Theileria*. Sporozoites are injected into the vertebrate host during tick feeding. In the host they invade the lymphocytes and monokines, whereby they are asexually morphed into merozoites through trophozoites. The merozoites multiply in the cells and finally the lymphocytes burst releasing the merozoites which invades the erythrocytes. Some trophozoites develop into gametocytes which can initiate infection in the tick vector (Bishop *et al*., 2004).

2.1.2.2 *Theileria* infection in wildlife

Theileria has been reported in domestic bovines, and in nearly all wild mammals (Uilenberg, 2006). In livestock *Theileria annulata* and *Theileria parva* cause

Tropical Theileriosis and East Coast Fever respectively (Bishop *et al*., 2004; Brown & Torres, 2008). East Coast Fever is characterized by generalized lymphadenopathy, fever, anorexia, and decreased milk production. Lacrimation, nasal discharge, corneal opacity and diarrhea can also be seen and terminally ill animals develop pulmonary edema. Tropical theileriosis generally resembles East Coast fever, but these parasites also destroy red blood cells, causing jaundice, anemia, ill thrift, dyspnea, hemorrhagic diarrhea, and in some cases, hemoglobinuria (Brown & Torres, 2008). *Theileria* is diagnosed in the laboratory by the same methods as those described for *Babesia*.

Theileria infection has been reported in many wild animals including the African buffalo (*Syncerus caffer),* Asian water buffalo (*Bubalus bubalis*), Chinese water deer (*Hydropotes inermis argyropus*), Eland (*Taurotragus oryx*), wildebeest (*Connochaetes taurinus*), hartebeest (*Alcelaphus buselaphus cokii*), lions (*Panther leo*), cheetah (*Acinonyx jubatus*), Pallas's cats (*Otocolobus manul*) without causing clinical disease (Grootenhuis *et al*., 1977; Cornelia *et al*., 2003; Penzhorn *et al*., 2006; Spitalska *et al*., 2005; Garcia *et al*., 2007; Jae *et al*., 2009; Yusufmia *et al*., 2010; Chaisi *et al*., 2011; Githaka *et al*., 2012) although there is a case whereby Theileriosis was linked to death of roan antelopes (*Hippotragus equinus*) translocated from Togo and Benin to South Africa, sable antelope (*Hippotragus niger*), greater kudu (*Tragelaphus strepsiceros*), and common gray duiker (*Sylvicapra grimmia*) in south Africa. On inspection after immobilization,

the study found the animals anemic, icteric, and pyrexic, with generalized lymph node enlargement and prolonged bleeding times (Nijhof *et al*., 2005).

The African buffalo (*Syncerus caffer)* is the primary host of *T. parva lawrensi* in which the parasite doesn't cause any disease, whereas the Asian water buffalo is the primary host of *T. annulata* (Burridge, 1975; Grootenhuis, 1989; Brown & Torres, 2008) and these two buffalo species play an important role in the epidemiology of cattle Theileriosis. *Theileria bicornis* and *Babesia bicornis* were initially thought to infect black rhinoceroses only since they were first reported in black rhinoceroses in South Africa and Tanzania, and were associated with mortalities in the rhinoceroses following stress related events (Nijhof *et al*., 2003). This was not the case because these species were later identified in the white rhinoceroses in South Africa (Govender *et al*., 2011). *Theileria bicornis* was also identified in nyala (*Tragelaphus angasii*) from KwaZulu-Natal, South Africa, and in cattle from different localities in Uganda which shows that this bicornis species can infect other animals (Muhanguzi *et al*., 2010; Pfitzer *et al*., 2011).

2.2 Population management of rhinoceroses in Kenya

Rhinoceros is one of the six surviving species of odd-toed ungulates in the family of Rhinocerotidae. It is one of the species listed by the IUCN as critically endangered. There are five species of rhinos, two African species, namely; the white (*Ceratotherium simum*) and black (*Diceros bicornis*) rhinosceros, and three

Asian species. The Asian species include; Indian (*Rhinoceros unicornis*), Javan (*Rhinoceros sondaicus*), and Sumatran (*Dicerorhinus sumatrensis*). There are four recognized Sub-species of the black rhino; *Diceros bicornis michaeli* (East Africa); *Diceros bicornis longipes* (West African); *Diceros bicornis minor* (South Central); *Diceros bicornis bicornis* (South –Western) (Emslie & Keryn, 1994; Okita *et al.,*2006). White rhino comprises of two sub-species; Southern white rhino (*Ceratotherium simum simum*) and Northern white rhino (*Caratotherium simum cottoni*). The white rhinos are not indigenous in Kenya but were introduced in Kenya from South Africa (Okita *et al*., 2007; Emslie *et al*., 2009).

The number of black rhinos in Kenya has declined mainly due to poaching and loss of habitat as a result of clearing land for settlement and agriculture (Brett, 1993; Okita *et al.,* 2006; Okita *et al*., 2007; Emslie *et al*., 2009). Most of them are fragmented over a wide range therefore reducing the opportunities of breeding and genetic interchange and hence accelerating the rate of extinction (Brett, 1990). Conditions have been worsened by breeding depression caused by environmental changes and demographic fluctuations such as biased sex ratio and genetic problems. The total number of black rhinos remaining in Kenya is around 670 animals (Emslie *et al*., 2009). The total number of white rhinos in the world is 20,170 with 370 individuals in Kenya on private, community or state land (Emslie *et al*., 2011). The surviving individuals are being intensively managed for

population recovery mainly through translocation into sanctuaries or parks where they are closely monitored (Emslie *et al*., 2009).

A rhino sanctuary is a small part of a State protected area, private land or communal land in which rhinoceroses are deliberately confined through perimeter fencing, the use of natural barriers or other methods of confinement and where law enforcement staff are deployed at high density to protect the rhinoceros population. The confinement of rhinos within a sanctuary permits close observation and relatively intense management and protection (Okita *et al*., 2007).

Translocation is the intentional movement of living organisms from one geographic area for free release into another area with the objective of establishing, re-establishing or augmenting a population and it is an important conservation tool being used to manage the species *in situ* (Woodford & Rossiter, 1993). It facilitates connectivity among the sub-populations thereby promoting gene flow (Muya & Oguge, 2010). In addition it is used to manage population density and balance sex ratio. Movement of individual rhinos from one area to another is done, either to improve chances of survival, to establish new populations, to keep established populations productive, or to enhance gene flow. Rhinos may be translocated to other areas of suitable habitat where they may be better protected from poachers (Okita *et al*., 2007). Translocation has disadvantages associated with it, such as the risk of introducing destructive

pathogens into naive wildlife population, exposure of translocated animals to pathogens in the new release site (Woodford & Rossiter, 1993; Leghton, 2002; Chapman *et al*., 2007).The process of capture and translocation is inherently a very stressful event for animals and most likely compromises the animal's immune defense (Woodford & Rossiter, 1993).

In natural systems, parasites co-evolve with their hosts and develop equilibrium or endemic stability in which infected hosts do not develop disease (Penzhorn *et al*., 1994; Penzhorn, 2006). In calves, passively acquired resistance from colostrum lasts about 2 months but is followed by innate immunity from 3 to 9 months of age (Mahoney & Ross, 1972). Therefore calves exposed to babesiosis during the first 6 to 9 months rarely show clinical symptoms and develop a solid long-lasting immunity (endemic stability). However, when the host-parasite equilibrium is altered by various stressor conditions, latent infections develop into disease (Penzhorn *et al*., 1994; Penzhorn, 2006).

Wild herbivores are known to be resistant to and are carriers of diseases which, while not harmful to them, can be transmitted to domestic animals in areas of mixed grazing with *Babesia*, *Theileria*, *Anaplasma*, and *Ehrlichia* having been shown to be parasites transmitted to domestic by wild ruminants, Transmission is either by intermediate hosts like ticks or directly through saliva or fecal material deposited onto the pastures and ingested by the domestic animal. Although they

seldom graze in areas where domestic animals wander around, tests have shown that rhinos are infected with many types of disease, most of which are suppressed except in times of stress. Such stress, be it due to capture, nutritional problems or even environmental change, can lower the resistance of the body so that the underlying diseases exhibit themselves as infections which can lead to death if untreated (Agarwal & Marshall, 2001). Stress leads to increase in cortisol levels which have immunosuppressive effects such as inhibition of inflammation, affect cytokine production, and increase in monocyte apoptosis.

2.3 Piroplasms in rhinoceros

Babesiosis was first reported in black rhinoceros (*Diceros bicornis*) during the late 1960s in Kenya (Brocklesby, 1967; Mugera & Wandera, 1967), Tanzania (McCullough & Achard, 1969) and also in white rhinoceros from South Africa in 1970 (Bigalke *et al*., 1970), while *Theileria* species were also found in South African rhinoceros imported from Dallas in 1989. Later in 2005 *Babesia* spp, (*B. bicornis*) was identified in three black rhinoceros in the Ngorongoro Conservation area in Tanzania and in nature reserves in South Africa. One of the black rhinoceros from South Africa had in addition to the *B. bicornis* a second species *T. bicornis* (Nijhof *et al*., 2003). Analysis showed two new species named *B. bicornis* and *T. bicornis*. In another study five of eleven blood samples collected from healthy black rhinoceros in South Africa were found to be infected with

Babesia bicornis. Seven of these animals were infected with *T. bicornis*, and one was co-infected with both *B. bicornis* and *T. bicornis* (Nijhof *et al*., 2003).

Between 2002 and 2006 a study was done on 46 black rhinos originating from various national parks and game reserves within South Africa. 12 of the 46 samples tested positive for presence of piroplasms and 9 of the 46 demonstrated presences of either *T. bicornis* or *B. bicornis* (Zimmerman, 2009). In another study carried on 195 white rhinos during an immobilization for sales to private owners between 2003 and 2006, none of the animals tested positive for *B. bicornis* but a prevalence of 36.41% of *T. bicornis* was seen in the population (Govender *et al* ., 2011). *Babesia* infected rhinoceros have limited successful translocations and clinical Babesiosis may be triggered in latent carrier animals by stress factors since in most cases animals die soon after capture, or during periods of nutritional or pregnancy related stress or during extreme climatic conditions (Penzhorn, 2006).

2.4 Diagnosis of piroplasms

Diagnosis of piroplasms is based on clinical manifestation, presence of tick vectors, the history of the area, and demonstration of either the parasites or the parasite's antibodies in the host (Homer *et al*., 2000; Brown & Torres, 2008; Marc *et al*., 2010). However for confirmation purposes the demonstration of parasites or the antibodies to the parasites is achieved by several laboratory methods. These

laboratory diagnosis involve; conventional methods, such as microscopy, and serology; molecular based techniques and; proteomics.

2.4.1 Microscopy

For many years, microscopy has been the only tool available for the detection of protozoan parasites through inspection of blood smears. It was the first technique utilized to identify piroplasms and remains the most common diagnostic technique of *Babesia* for researchers and clinicians (Moody & Chiodini, 2000). Thin blood smears stained with Wright or Geimsa stain are examined for the presence of the protozoans (Homer *at al*., 2000; Moody & Chiodini, 2000). However, the occasional difficulty of identifying these parasite structures decreases the sensitivity of the method (Ambrosio & De Waal, 1990; Momar, 2009). In addition sample preparation for direct observation is time-consuming, labor intensive and proper diagnosis depends on qualified laboratory technicians. Definitive identification is particularly difficult when parasites are morphologically similar, very small in size or when parasitemia is low (Etkind *et al*., 1980).

2.4.2 Serology

This techniques rely both on the immunological factors induced by the parasites in the host and the parasite genome. These techniques can be divided into two

categories: antigen-detection assays and antibody-detection assays (Ambrosio & De Waal, 1990; Moody & Chiodini, 2000; Momar, 2009). These include the enzyme-linked immuno-sorbent assay (ELISA) and all its derived tests such as the Falcon assay screening test ELISA (FAST-ELISA) and the dot-ELISA. Falcon assay screening test ELISA (FAST-ELISA) consists of using synthetic and recombinant peptides to evaluate antibody responses to an antigen. The main difference between the regular ELISA and the dot-ELISA lies in the surface used to bind the antigen of choice. In the dot-ELISA, the plastic plate is replaced by a nitrocellulose or other paper membrane onto which a small amount of sample volume is applied. Other assays include; the hemagglutination (HA) test, indirect or direct immune-fluorescent antibody (IFA or DFA) tests, complement fixation test(CFT), immune-blotting and rapid diagnostic tests (RDTs) which consist of capturing soluble proteins by complexing them with capture antibodies embedded on a nitrocellulose strip (Ambrosio & De Waal, 1990; Momar, 2009).

Serological techniques such as immune-fluorescence antibody test (IFA) and enzyme-linked immune-sorbent assay (ELISA) have been a method of choice in epidemiology and diagnosis of many piroplasms (Homer *et al*., 2000; Rubaire *et al*., 2004; Brown & Torres, 2008). Specifically immune-fluorescence antibody test (IFA), enzyme-linked immune-sorbent assay (ELISA) and animal inoculation test which is impractical for routine diagnosis purposes but techniques have been

created to detect antibodies to *Babesia* species (Bose *et al.*, 1995: Moody & Chiodini, 2000; Irwin, 2010).

However, these techniques may pose a problem because of cross-reactions with other closely related organisms resulting to differences in interpretation. Although the ease of use and turnaround times for serologic assays are similar to microscopy, serology-based assays are more sensitive and specific (Ambrosio & De Waal, 1990). The use of immune diagnostic techniques has certain limitations since the demonstration of antibodies against parasites provides little information as to whether the parasite is still present in the animal's body or not (Ambrosio & De Waal, 1990; Marc *et al*., 2011). Moreover if the parasites are in the primary infection the disease will develop well before antibodies become detectable. To circumvent the limitations of conventional diagnostic tests, highly sensitive molecular tools such as polymerase chain reaction (PCR) tests are now preferred methods.

2.4.3 Molecular based techniques

The limitations of microscopy and serology-based assays have influenced parasitologists towards the use of molecular-based approaches based on genetic codes which offer greater sensitivity and specificity over the existing diagnostic tests. They permit the direct detection of infections from very low parasitized samples including those from asymptomatic individual's samples. Gene

amplification methods are made possible by the advent of the polymerase chain reaction (PCR) (Gasser, 2006; Tavares *et al*., 2011). The PCR makes it possible to perform selective amplification from complex genomes. This technique is based on the process of denaturing a double-stranded genomic DNA template using heat (Momar, 2009). Next, the temperature is lowered to ensure that primers can anneal to their complementary sequences in the template. Thus, the elongated DNA template follows in both directions from the primer site by means of enzymatic catalysis with a thermostable DNA polymerase, generating double-stranded products. Besides the traditional PCR, including nested and multiplexed PCR, real-time PCR (RT-PCR) has emerged which can detect several parasitic infections (Momar, 2009).

In recent years, PCR-based diagnostic methods have been used for Molecular characterization and phylogenetic analysis of piroplasms and other haemoparasites (Homer *et al*., 2000; Nijhof *et al*., 2005; Penzhorn *et al*., 2006; Oosthuizen *et al*., 2008; Zimmerman, 2009; Jeneby *et al*., 2011; Govender *et al* ., 2011). Primers used in the test can be designed to be genus specific or can amplify species-specific sequences of DNA, so that the technique can detect a single species (Moody & Chiodini, 2000). The majority of these tests have been based on 18S rRNA gene sequences, but other genes such as the internal transcribed spacer region, cytochrome b, and P50 have also been used as gene targets (Irwin, 2010). The first PCR test for *Babesia* was described by Fahrimal in

1992 (Fahrimal *et al*., 1992). Since then, a number of PCR based tests have been developed (Homer *et al*., 2000; Nijhof *et al*., 2005; Penzhorn *et al* ., 2006; Munson *et al*., 2008; Ferreli *et al*., 2008; Oosthuizen *et al*., 2008; Zimmerman, 2009; Irwin, 2010; Bosman *et al*., 2010; Govender *et al* ., 2011; Jeneby *et al*., 2011; Samson *et al*., 2012).

In many piroplasm characterization studies, three methods; hematology, serology and molecular techniques are used to determine morphological characteristics, identify, and determine the phylogenetic relationships between parasites (Homer *et al*., 2000; Munson *et al*., 2008; Bosman *et al*., 2010; Jeneby *et al*., 2011; Marc *et al*., 2011). Newer technologies such as loop-mediated isothermal amplification (LAMP) and Luminex-based assays have also emerged as possible new approaches for the diagnosis of parasitic diseases (Tavares *et al*., 2011). LAMP is a method of nucleic acid amplification with extremely high sensitivity and specificity to discriminate single nucleotide differences (Parida *et al*., 2008). It is characterized by the use of a DNA polymerase that has low sensitivity to inhibitors and a set of four primers specially designed to recognize six different sequences on the target gene (Paris *et al*., 2007). Amplification occurs only when all primers bind, thus forming a product. These tests are not influenced by environmental factors that usually can interfere with the results of a stool test, for example, thus ensuring highly reliable results. Recently, parasitologists have adapted the LAMP technique to detect several parasitic diseases, including animal

parasites such as *Theileria* and *Babesia* (Paris *et al*., 2007; Han *et al*., 2007; Nkouawa *et al*., 2009). Molecular assays have comprehensively assisted in the diagnosis, treatment and epidemiological studies of parasitic diseases that affect people and animals worldwide, helping to control parasitic disease mortality.

2.4.4 Proteomics

Since proteins are the main catalysts, structural elements, signalling messengers, and molecular machines of biological tissues, proteomic studies are able to provide substantial clinical relevance. Proteins can be utilized as biomarkers for tissues, cell types, developmental stages, and disease states as well as potential targets for drug discovery and interventional approaches.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study animals and ethical consideration

The eastern black rhino (*Diceros bicornis michaeli*) and the southern white rhino (*Ceratotherium simum simum*) were sampled during scheduled management activities by Kenya Wildlife Service between 2011 and 2012. The Committee of the Department of Veterinary and Capture Services of the Kenya Wildlife Service (KWS) approved the study including animal capturing, translocation and sample collection. KWS guidelines on Wildlife Veterinary Practice-2006 were followed. All KWS veterinarians were guided by the Veterinary Surgeons and Veterinary Para-Professionals Act, 2011 that regulates veterinary practice in Kenya.

3.2 Sample size

Since the population size of alternative hosts was infinite (over 10,000) the formula described by Dohoo (Dohoo *et al.*, 2003) was used. Assuming a diagnostic sensitivity of 50% and level of significance of 5% sample size was;

$n = Z^2 (P) (1-P)/ d^2$ Where: n = Sample size (infinite): Z = Z value (Confidence level, e.g.95%)

P = prevalence (e.g. 50%, p = 0.5): d = precision (e.g. 5%, d = 0.05)

Hence n= $(1.96)^2(0.5)(1-0.5)/0.05^2$ = 384 blood samples. With actual population size known a correction factor was used to adjust downward the sample size (*n*) obtained from infinite population. The adjusted sample size (*n'*) was calculated as follows:

$n' = 1/(1/n + 1/N)$

Where: *n' = adjusted* sample size N = population of rhinos (1040) n´=280

Two hundred and eighty was the required sample size from the rhino population in Kenya. However, this number of samples was technically and ethically difficult to achieve because blood sampling of a critically endangered species like the rhino is a very expensive exercise. In addition, the process of capture and immobilization has inherent risks on the life of the animal, hence considered unethical to just immobilize a rhino for sample collection. Minimal sample size is usually accepted for invasive studies such as this one on endangered species. A total of 114 (82 black and 32 white) rhinos were sampled and the subjects included males and females of all age ranges. The rhinoceroses were grouped into three age groups as follows; Juvenile 0-3.5years, sub adults above 3.5-7 years, and adults (above 7 years). In addition, the animals were sampled from different sub-populations located in various ecological habitats in Kenya. Blood samples were collected from immobilized rhinos by experienced KWS veterinarians.

3.3 Study sites

The study was conducted on rhinoceroses selected from six areas that hold the largest number of rhinoceros as shown in figure 3.1. All the rhinos in Mugie game ranch were removed from the ranch due to increased poaching and hence it's not included in the map.

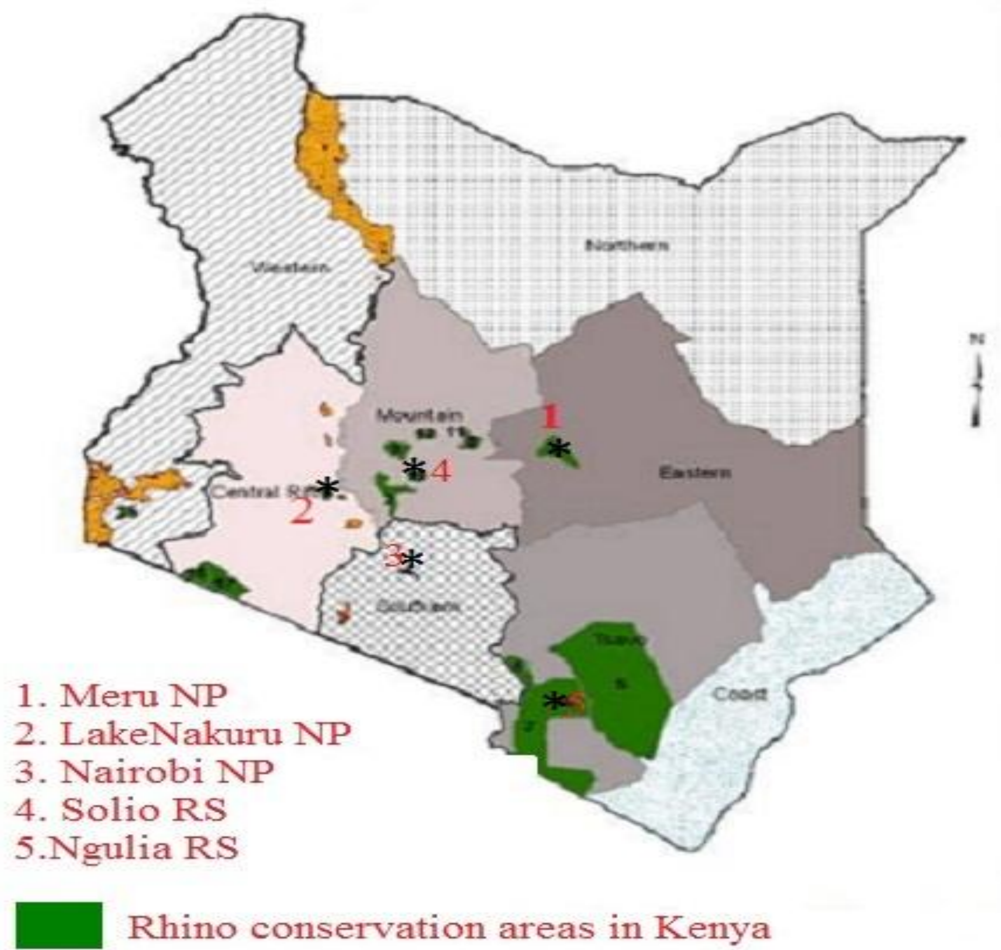

Figure 3.1: Rhinoceros conservation areas in Kenya with the areas from which blood samples were collected from both black and white rhinoceroses marked with an asterick and numbered in red.

3.3.1 Nairobi National Park

Nairobi National Park (NNP) was established in 1946 and it is the oldest national park in Kenya covering an area of approximately 117km². It is situated 10 km South of Nairobi city Centre and lies between 2° 18'-2° 20' South and 36° 23'-36° 28' East at an altitude of 1780m above the sea level. The area has a bi-modal rainfall pattern and receives a mean annual rainfall of between 762mm (East side) to 911mm (West side) in two rainy seasons. The major vegetation types in NNP are; Forests, open grasslands interspersed with acacia and bushland which dominates the valleys, gorges and slopes. The park has abundant animal species including mammals, reptiles, birds, amphibians, and numerous invertebrates. It° is home to black and white rhino, Cheetah, Leopard, Buffalo, Eland, Kongoni, Wildbeest, Zebra, Hyena, Jackal, Giraffe, Gazelles, Hippopotamus, Ostrich, Bush buck, among other fauna. The park is a rhino sanctuary for breeding and restocking other parks (KWS, 2003).

3.3.2 Meru National park

Meru National Park (MNP) is a one of the four protected areas along the Tana River comprising Meru Conservation Area (MCA). The other three are Bisanadi National Reserve, Kora National park, and Mwingi National Reserve. MCA is situated North-East of Mount Kenya and lies on the edge of the dry eastern Kenya plains. MNP was established in 1968 and is located in Meru North District and covers an area of 870 km². It receives an annual rainfall of 724mm. It has a

unique blend of arid and wetland ecosystem. It has a wide range of wild animals including elephant, Gravy Zebras, Beisa Oryx, Wild dogs, hippopotamus, lion, leopard, cheetah, and black and white rhinoceros, and a variety of bird species among others. On the Western boundary of the park there is a rhino sanctuary which is about 50 square kilometers devoted to the protection of rhinos and it is home to 22 black rhinos and 48 white rhino (KWS, 2005; KWS, 2007).

3.3.3 Ngulia Rhino Sanctuary

Ngulia rhino sanctuary is completely fenced with an area of 90 km^2 located within the central part of Tsavo West National Park (TWNP). TWNP is on South Eastern Kenya, 240km from Nairobi along the western side of Mombasa-Nairobi highway. The sanctuary was extended from 62 km^2 to its current size in 2007. The sanctuary has been one of the more successful areas for protection and breeding of black rhinos in Africa over the last 15 years. NRS was created in 1986 with the aim of protecting the black rhinos from poachers and to try and create a small breeding population. Altitude Ranges from 600m of low lands to 1800m of craggy hills, with average annual rainfall ranging from 150mm-760mm with a markedly seasonal pattern. The vegetation comprises of grassland, wooded grassland and bush lands (KWS, 1993).

3.3.4 Lake Nakuru National park

Lake Nakuru National Park (LNNP) lies between longitude 36° 05' East and 0° 24'South and its located in Nakuru municipality boundaries about 3Km South of Nakuru town centre, in Nakuru District, the Rift valley province of Kenya. It is completely fenced and covers a total area of 188 km². It was established in 1984 and is the first government managed rhino sanctuary. It has acted as a centre for biodiversity and conservation initiatives, Black rhino protection and breeding site, and for reintroduction to their former sites. Its vegetation is classified into four major habitats; open waters, woodlands, open grasslands and forests. The parks climate varies within the lake depending on altitude and topography. It ranges from cold and humid to arid and semi-arid. The region receives mean annual rainfall of about 750mm and evaporation, radiation, and temperature have a annual means of 1800mm, 1490 langrays and 27ºC respectively. Lake Nakuru National Park is home to 56 different species of mammals including more than 25 black and 70 white rhinos, Rothschild's giraffes, cape buffaloes, Deffassa waterbucks, Thomson's and grant's gazelles, impalas, lions, leopards, warthogs, olive baboons, black backed jackal, reedbucks, dik diks, elands among others (KWS, 2002).

3.3.5 Solio Rhino Sanctuary

Solio Ranch or Solio Game Reserve is a privately owned wildlife conservancy. The ranch is a fenced and protected area geared toward rhino conservation. The

17,500-acre reserve, is located 0° 16′ 12″ S, 37° 0′ 0″ E , 22 km north of Nyeri Town, plays a major part in the protection and breeding both white and black rhinos in Kenya. It is presently home to around 120 black and white rhinos which live in harmony with other wildlife including the buffalos, Zebra, Giraffe and plains game such as Eland, Oryx, Impala, Waterbucks, Thompson's gazelle and Warthog. Solio Rhino Sanctuary is one of the most successful rhino sanctuaries in Kenya; with a population of over 80 black rhinos it carries the largest single black rhino population in East Africa. This area has black rhino making up 36% of the total browser biomass (Okita *et al*., 2006)

3.3.6 Mugie Game Ranch

Mugie ranch is located at the end of the Laikipia Plateau with a temperate climate at 1,800m. Black rhinos were re-introduced to Mugie Sanctuary in 2004 which preserved and increased their population. The rhinos coexisted with other wildlife including Lion, Oryx, Elephant, Giraffe, Cape buffalo, Gravy zebra, Eland, over two hundred unique bird species among others until they were translocated to Meru National Park for security purposes.

3.4 Blood Sample collection, handling and processing

Sampling was done opportunistically during scheduled immobilizations for population and health management as listed in table 3.1. The rhinos were chemically immobilized using Etorphine and Hyaluronidase and were darted from

a helicopter. Upon recumbency, blood was drawn from the radial vein of the foreleg into ethylenediaminetetra-acetic acid tubes, mixed and labeled with animal's specific details as well as date and placed in cool box and transported to the laboratory in Nairobi.

Table 3.1: Table showing rhino conservation areas sampled event and the date of sampling.

Area	Activity	Date
Meru National park	Ear notching	February 2011
Mugie Game Ranch	Translocation	January 2012
Solio Rhino Sanctuary	Translocation	January 2012
Ngulia Rhino Sanctuary	Ear notching	March 2012
Nairobi National Park	Ear notching	June 2012
Lake Nakuru National Park	Ear notching	July 2012

3.5 Extraction of DNA

DNA was extracted from 200µl of EDTA-anticoagulated blood using a DNA extraction kit (DNeasy blood &Tissue Kit, QIAGEN, Southern Cross Biotechnologies, South Africa) following manufacturer's protocol. The stored blood was thawed and shaken at room temperature, then, 20µl of proteinase K was pipeted into a 2ml microcetrifuge tube and 200µl of anticoagulated blood added. AL Buffer (200 µl) was added and mixed by vortexing and then incubated at 56ºC for 10 min. Ethanol (200µl) was added and mixed by vortexing and afterwards the mixture was pipeted into a DNeasy mini spin column in a 2ml collection tube and centrifuged at 8000 rpm for one minute. The flow-through and

collection tube were discarded, the spin column was placed in a new 2ml collection tube and 500µl AW1 washing buffer added and centrifuged at 8000rpm for one minute after which the flow-through and collection tube were discarded. This washing step was repeated by adding 500µl of AW2 washing buffer and centrifuged at full speed for three minutes. The spin column was then transferred to a new 2ml microcentrifuge tube and 200µl of elution buffer AE added and incubated for 1 minute at room temperature then centrifuged at 8000rpm for one minute to elute the DNA. The last step was repeated for maximum yield. The DNA samples were stored at -20ºC for further analysis.

3.6 Polymerase Chain Reaction

Genomic DNA extracted from 114 rhinoceros whole blood was subjected to a nested amplification of the 18 Small Subunit ribosomal RNA (18S rRNA) genes of *Babesia* and *Theileria* using two newly designed set of primers (BecA-Hub, International Livestock Research Institute, Kenya). Primers used for primary amplification were ILO-9029 (Forward) (5'CGGTAATTCCAGCTCCAATAGCGT-3') and ILO-9030 (Reverse) (5'-TTTCTCTCAAAGGTGCTGAAGGAGT-3'). For The Secondary amplification the primers used were, MWG4/70 (forward) (5'-AGCTCGTAGTTGAATTTCTGCTGC-3') and ILO-7782 (Reverse) (5'-AACTGACGACCTCCAATCTCTAGTC-3') (Jeneby *et al*., 2011).

3.6.1 Primary Amplification

Primary amplification was performed in a final volume of 50µl containing 45µl of 1.1X Platinum blue supermix, 1.0µl of each primer (forward and reverse), and 3µl of the genomic DNA. The tubes were put directly into a programmed Applied Biosystems Veriti 96 well thermocycler. The primary PCR comprised 30 cycles, where the denaturation step was done at 95°C for 30s, the annealing of primers at 53°C for 30s and the extension of strands for 1minute at 72°C. The PCR was completed with a final extension cycle of 9 minutes at 72°C, and left at 4°C.

3.6.2 Secondary Amplification

Secondary amplification was performed in a final reaction volume of 50µl containing, containing 45µl of 1.1X Platinum blue supermix, 1.5µl of each primer (forward and reverse), and 2µl of the primary amplification product. The tubes were put directly into a programmed Applied Biosystems Veriti 96 well thermocycler. The secondary PCR comprised 30 cycles, where the denaturation step was done at 95°C for 30 s, the annealing of primers at 55°C for 30 s and the extension of strands for 1minute at 72°C. The PCR was completed with a final extension cycle of 9 minutes at 72°C, and left at 4°C.

3.7 Electrophoresis

PCR amplification products were analyzed by running them on electrophoresis gel. 5µl aliquots of PCR product and DNA ladder of 100bp were run on a 1% agarose gel stained with ethidium bromide at 80volts for 50 minutes.

3.8 Sequencing

A subset of the PCR products showing successful amplification on agarose gel analysis was sequenced for both forward and reverse strands. The subset was a representative of both rhino species and all the subpopulations sampled. The PCR products were purified using Thermo Scientific GeneJet PCR Purification Kit (Thermo Scientific) prior to sequencing.

3.8.1 Purification of PCR Product

The PCR products were purified using Thermo Scientific GeneJet PCR Purification Kit (Thermo Scientific) following manufacturers protocols. The stored PCR product was thawed and mixed at room temperature. In PCR tubes containing 46µl of PCR product, 46µl of binding buffer were added and mixed thoroughly until the colour of the solutions turned yellow. The solutions were then transferred to labelled GeneJET purification columns, centrifuged for 60 seconds, and the flow-through discarded.700µl of wash buffer was added to the GeneJET purification columns, centrifuged for 60 seconds, and the flow-through

discarded. The empty GeneJET columns were centrifuged for an additional 1minute to completely remove any residual wash buffer, and then transferred to a clean and labeled 1.5ml microcentrifuge tubes. 50µl of elution buffer were added to the centre of the GeneJET purification columns membrane and centrifuged for 1 minute after which the columns were discarded and the eluted purified DNA stored at -20°C awaiting sequencing.

3.8.2 Sequencing and analysis of sequences.

Eluted purified DNA samples were sent to Biosciences eastern and central Africa-International Livestock Research Institute (BecA-ILRI) for sequencing. Sequencing was carried out on an ABI 3700 (Applied Biosystems). The complementary reads were used to resolve rare, ambiguous base-calls in Sequencher v.4.9. Sequences were aligned in Seaview v.4.2.12 (Gouy *et al*., 2010) under ClustalW (Larkin *et al*., 2007) default settings. Incomplete terminal sequences were removed from the alignment. Nucleotide substitutions and *p*-uncorrected distances were performed in MEGA v5 (Tamura *et al*., 2011) and phylogenetic analyses were performed with Mr Bayes v.3.1.2 (Huelsenbeck & Ronquist, 2001). Sequence BLAST (Basic Local Alignment Search Tool) searches were conducted in Genbank to identify closest match to the sequences. Sequences with 98% or more similarity to the target sequences from the blast searches and others belonging to the same genus (as suggested from the blast

searches) of African origin were included in the alignment. The designated outgroup was *T. gondii* following Nijhof *et al*. (2003).

The most appropriate substitution model for the Bayesian Inference was determined by the Bayesian Information Criterion (BIC) in Model test v.0.1.1 (Posada, 2008). Mr Bayes was used with default priors and Markov chain settings and with random starting trees. The gamma shape parameter and proportion of invariant sites were estimated from the data. Each run consisted of four chains of 10,000,000 generations, sampled each 10,000 generations for a total of 1,000 trees. A plateau was reached after few generations with 25% (250 trees) of the trees resulting from the analyses discarded as "burn in".

3.9 Statistical analysis

The parasites infection rate differences between the rhinoceros species, sexes, age groups, and sampling locations were assessed using the Pearson Chi-square test (χ^2). Statistical significance was accepted at $p < 0.05$ with confidence interval, (CI) of 95%. SPSS version 18.0 (Chicago, IL, USA) for windows was used for data processing.

CHAPTER FOUR

RESULTS

4.1 Identification of piroplasms using PCR, and gel electrophoresis.

Between June 2011 and August 2012 a total of 114 blood samples of black (n = 82) and white (n = 32) rhinoceroses were sampled from different rhino sub-populations in Kenya. Out of the 114 rhinoceroses sampled 56 tested positive for piroplasms on PCR amplification and gel electrophoresis as shown in Plate 4.1. The positive samples formed a band at approximately 400bp as shown in the diagram which was the expected fragment size.

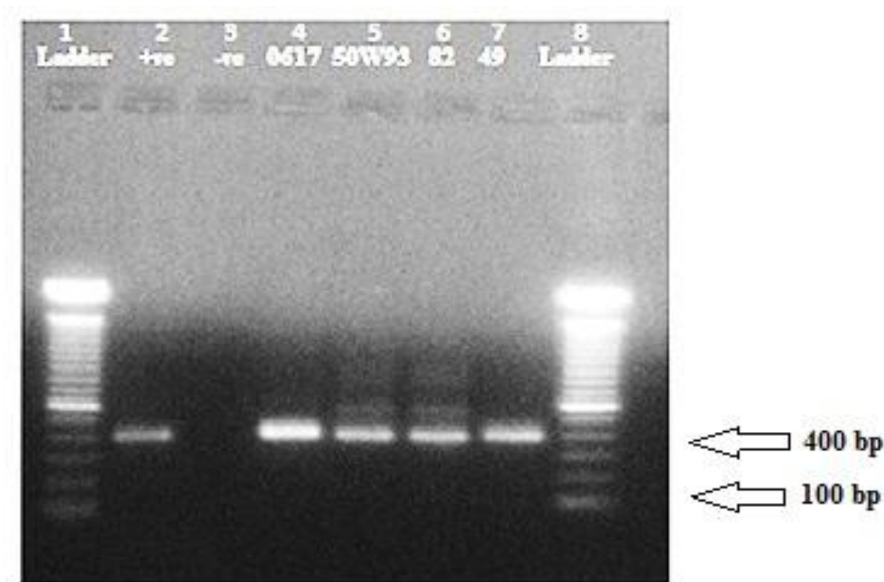

Plate 4.1: Gel electrophoresis PCR amplification of a partial sequence of 18S rRNA of *Babesia* spp./*Theileria* spp. gene, product size approximately 400 bp. Lanes (From Left): (1) Molecular weight marker = 100bp; (2) Positive control; (3) Negative control; (4-7) Products of the amplified PCR product obtained from secondary PCR; (8) Molecular weight marker = 100bp.

4.1.1 Piroplasm Infection rates in black and white rhinoceroses

Table 4.1 shows piroplasm infection rates in the two rhinoceros species (*C.simum, and D. bicornis*) sampled from six conservation areas in Kenya. Ngulia and Solio Rhino Sanctuaries did not have white rhinoceros representatives because they are purely black rhinoceros conservation areas. In all the areas having both species white rhinoceroses were highly infected than the black rhinoceroses. Highest infection rate in white and black rhinoceroses was observed in Nairobi (100%) and Lake Nakuru National Park (53.3%) respectively. The overall infection rate of the white rhinoceroses (65.6%) was significantly higher than that of the black rhinoceroses (42.7%, χ^2=0.028, Figure 4.1). All the white rhinoceroses from Nairobi National Park were infected whereas none from Mugie Game Ranch was infected. The lowest infection rate was observed in Solio Rhino Sanctuary black rhinoceroses (12.5%).

Table 4.1: Piroplasm infection rates in *D. bicornis* and *C. simum* from different conservation areasin Kenya.

Species	*C. simum*	*D. bicornis*
Conservation area	**Positive (%)**	**Positive (%)**
Lake Nakuru National Park	66	53.3
Nairobi National Park	100	44.4
Ngulia Rhino Sanctuary	-	44.8
Meru National Park	70	50
Solio Rhino Sanctuary	-	12.5
Mugie Game Ranch	0	42.1

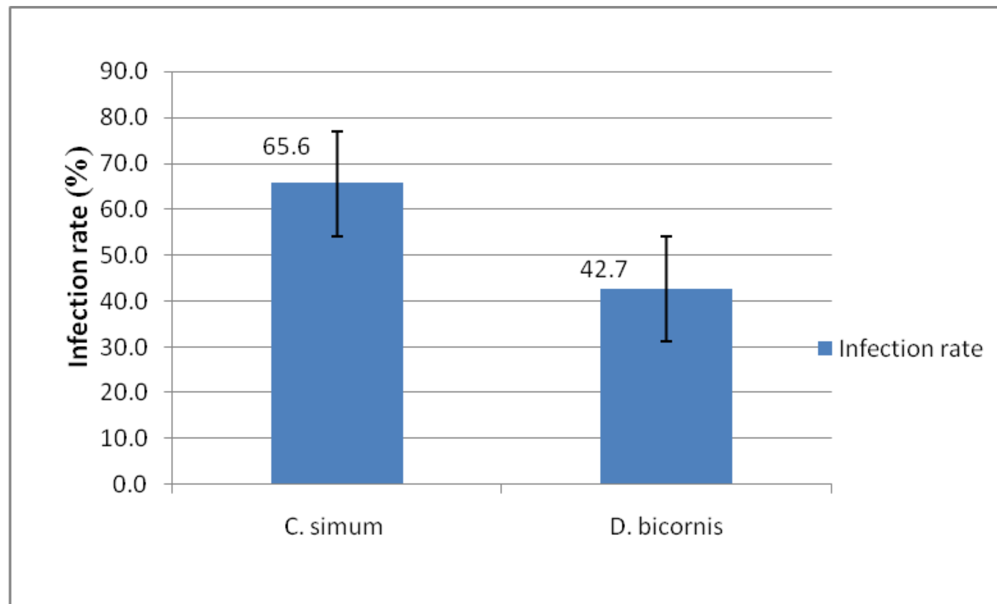

Figure 4.1: Overall piroplasm infection rates in *C. simum* and *D. bicornis* from different rhinoceros conservation areas in Kenya.

4.1.2 Piroplasm Infection rates in male and female rhicoceroses

Table 4.2 shows piroplasm infection rates in the female and male rhinoceroses from the six sampled conservation areas in Kenya. More males from Lake Nakuru and Meru National Parks, and Mugie Game Ranch were infected than the females. In Nairobi National Park males and females were equally infected, whereas in Solio Rhino Sanctuary males were not infected. The highest infection rate was observed in Meru National Park males (75%). Overall infection rate in the males (53.7%) was higher compared to that of females (45%) in both rhinoceros species (Figure 4.2), although the difference was not statistically significant ($\chi^2 = 0.353$).

Table 4.2: Piroplasm infection in male and female rhinoceroses of both species in different conservation areas in Kenya

Sex	**Male**	**Female**
Conservation area	**Positive (%)**	**Positive (%)**
Lake Nakuru National Park	65	53.3
Nairobi National Park	50	50
Ngulia Rhino Sanctuary	30	52.6
Meru National Park	75	50
Solio Rhino Sanctuary	0	20
Mugie Game Ranch	55.6	27.3

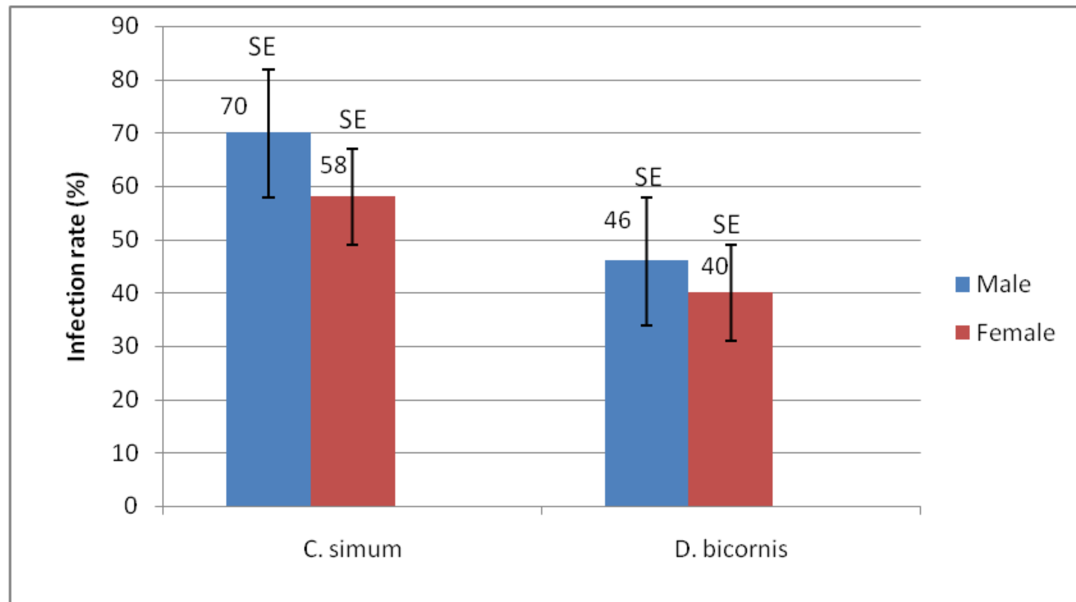

Figure 4.2: Overall piroplasm infection rate in males and females in both rhinoceros species from different conservation areas in Kenya.

4.1.3 Infection rates in the juvenile, adult and sub-adult rhinoceroses

Table 4.3 shows piroplasm infection rates in adults, sub-adults, and juveniles of both rhinoceros species from the six sampled rhinoceros conservation areas in Kenya. No juveniles were sampled from Lake Nakuru and Meru National Parks, and Solio Rhino Sanctuary. All adults from Nairobi National Park were infected, whereas none from Solio Rhino Sanctuary was infected. Highest sub-adult infection was observed in Lake Nakuru National Park while in the juveniles it was observed in Mugie Game Ranch where all were infected. Figure 4.3 shows the overall infection rates in the age groups. Juveniles had lower infection rate (35.3%) compared to adults (51.2%) and sub-adults with sub adults having the highest infection rate (51.8%), but the difference was. not statistically significant ($\chi^2 = 0.465$).

Table 4.3: Piroplasm infection rates in adults, sub-adults and juveniles of both rhinoceros species sampled from different rhinoceros sub-populations in Kenya.

Age group	**Adults**	**Sub Adults**	**Juveniles**
Conservation area	**Positive (%)**	**Positive (%)**	**Positive (%)**
Lake Nakuru NP	57.1	60.7	-
Nairobi National Park	100	50	42.9
Ngulia Rhino Sanctuary	53.6	57.1	22.2
Meru Naional Park	75	62.5	-
Solio Rhino Sanctuary	0	16.7	-
Mugie Game Ranch	42.9	25	100

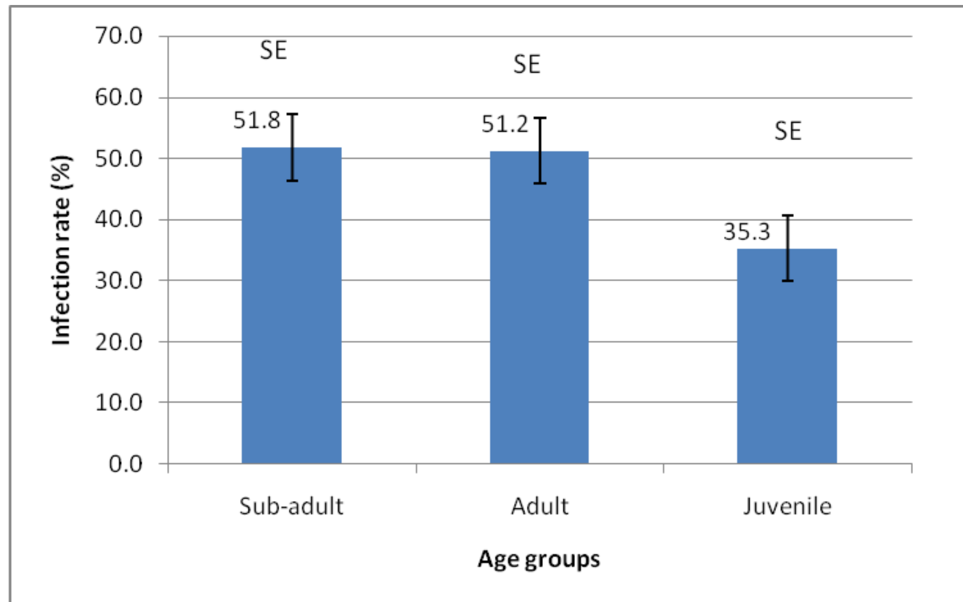

Figure 4.3: Overall piroplasm infection rates in the juveniles, sub-adults and adults in both rhinoceros species from six rhinoceros conservation areas in Kenya.

4.1.4 Infection rates in the different rhinoceros conservation areas sampled

Figure 4.4 shows piroplasm infection rates from the six rhinoceros conservation areas in Kenya. Among the sub populations Meru National Park had the highest infection rate (66.7%) compared to the rest, while Solio Rhino Sanctuary had the least (12.5%). Although the infection rate varied among the six sub-populations, the differences were not significant ($\chi^2 = 0.140$).

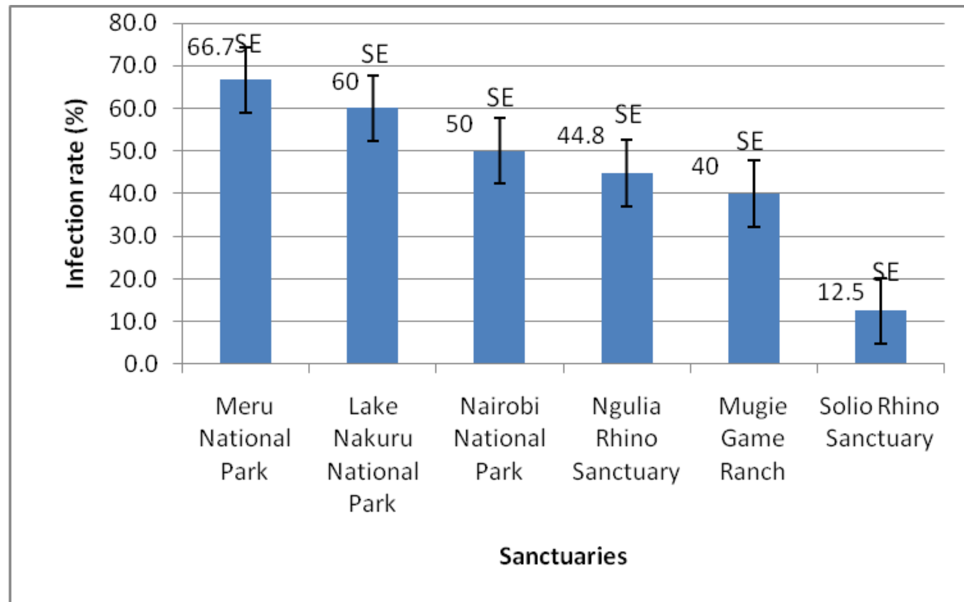

Figure 4.4: Rhinoceros infection rates in the six conservation areas in Kenya.

4.2 Species identification by sequencing

Out of the 20 sequenced samples, five gave noisy sequences and were discarded. After blasting the edited sequences in Genbank, three haplotypes of *Theileria bicornis* were identified which were named H1, H2, and H3 as shown in figure 4.5, 4.6 and 4.7.

```
QUERY  1     CCAATCTCTAGTCGGCATAGTTTATGGTTAGGACTACGACGGTATCTGATCGTCTTCGAT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  1006  CCAATCTCTAGTCGGCATAGTTTATGGTTAGGACTACGACGGTATCTGATCGTCTTCGAT  947

QUERY  61    CCCCTAACTTTCGTTCTTGATTAATGAAAACATCCTTGGCAAATGCTTTCGCAGTAGTTC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  946   CCCCTAACTTTCGTTCTTGATTAATGAAAACATCCTTGGCAAATGCTTTCGCAGTAGTTC  887

QUERY  121   GTCTTTAACAAATCTAAGAATTTCACCTCTGACAGTTAAATACGAATGCCCCCAACTGTT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  886   GTCTTTAACAAATCTAAGAATTTCACCTCTGACAGTTAAATACGAATGCCCCCAACTGTT  827

QUERY  181   CCTATTAACCATTACTTTGGT-TCGAAAACCAAC-AA-AATAGA-ACC-AAAGTCCTACT  235
             ||||||||||||||||||||| || ||||||||| || |||| | ||| |||||||||||
SBJCT  826   CCTATTAACCATTACTTTGGTCTCAAAAACCAACCAACAATATAGACCAAAAGTCCTACT  767

QUERY  236   TTATTATTCCATGCTAAAGTATTCAAGGCAAAAGCCTGCTTGAAGCACTCTAATTTTCTC  295
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  766   TTATTATTCCATGCTAAAGTATTCAAGGCAAAAGCCTGCTTGAAGCACTCTAATTTTCTC  707

QUERY  296   AAAGTAAAAAAATCTGGAAAAAAAG-CCGAAGCC-AATCAACCAGAAAAAAGCCACAACG  353
             ||||||||||| ||||||||||||| || ||||| |||||||||||||||||||||||||
SBJCT  706   AAAGTAAAAAA-TCTGGAAAAAAAGGCCCAAGCCAAATCAACCAGAAAAAAGCCACAACG  648

QUERY  354   CAAACGAAACCAATAAAGGGACGAAAGCGAAGCAGCAGAAATT  396
             ||||||||||||| ||||| |||||||||||||||||||||||
SBJCT  647   CAAACGAAACCAA-AAAGGAACGAAAGCGAAGCAGCAGAAATT  606
```

Description	Max score	Total score	Query cover	E value	Identity	Accession
	664	664	23%	0.0	97%	30151

Figure 4.5 Genbank blast search for haplotype 1. QUERY represents haplotype 1 while SBJCT represents *T. bicornis*

```
QUERY  1     CCAATCTCTAGTCGGCATAGTTTATGGTTAGGACTACGACGGTATCTGATCGTCTTCGAT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  1006  CCAATCTCTAGTCGGCATAGTTTATGGTTAGGACTACGACGGTATCTGATCGTCTTCGAT  947

QUERY  61    CCCCTAACTTTCGTTCTTGATTAATGAAAACATCCTTGGCAAATGCTTTCGCAGTAGTTC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  946   CCCCTAACTTTCGTTCTTGATTAATGAAAACATCCTTGGCAAATGCTTTCGCAGTAGTTC  887

QUERY  121   GTCTTTAACAAATCTAAGAATTTCACCTCTGACAGTTAAATACGAATGCCCCCAACTGTT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  886   GTCTTTAACAAATCTAAGAATTTCACCTCTGACAGTTAAATACGAATGCCCCCAACTGTT  827

QUERY  181   CCTATTAACCATTACTTTGGT-TCAAAAACCAAC-AA-AATAGA-ACC-AAAGTCCTACT  235
             ||||||||||||||||||||| |||||||||||| || |||| | ||| |||||||||||
SBJCT  826   CCTATTAACCATTACTTTGGTCTCAAAAACCAACCAACAATATAGACCAAAAGTCCTACT  767

QUERY  236   TTATTATTCCATGCTAAAGTATTCAAGGCAAAAGCCTGCTTGAAGCACTCTAATTTTCTC  295
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  766   TTATTATTCCATGCTAAAGTATTCAAGGCAAAAGCCTGCTTGAAGCACTCTAATTTTCTC  707

QUERY  296   AAAGTAAAAAATCTGGAAAAAAAGGCCGAAGCCAAATCAACCAGAAAAAAGCCACAACGC  355
             ||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
SBJCT  706   AAAGTAAAAAATCTGGAAAAAAAGGCCCAAGCCAAATCAACCAGAAAAAAGCCACAACGC  647

QUERY  356   AAACGAAACCAAAAAGGAACGAATGCGGAGCAGCAGAAATT  396
             ||||||||||||||||||||||| ||| |||||||||||||
SBJCT  646   AAACGAAACCAAAAAGGAACGAAAGCGAAGCAGCAGAAATT  606
```

Description	Max score	Total score	Query cover	E value	Identity	Accession
	686	686	23%	0.0	98%	32977

Figure 4.6 Genbank blast search for haplotype 2. QUERY represents haplotype 2 while SBJCT represents *T. bicornis*

```
QUERY  1     CCAATCTCTAGTCGGCATAGTTTATGGTTAGGACTACGACGGTATCTGATCGTCTTCGAT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  1006  CCAATCTCTAGTCGGCATAGTTTATGGTTAGGACTACGACGGTATCTGATCGTCTTCGAT  947

QUERY  61    CCCCTAACTTTCGTTCTTGATTAATGAAAACATCCTTGGCAAATGCTTTCGCAGTAGTTC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  946   CCCCTAACTTTCGTTCTTGATTAATGAAAACATCCTTGGCAAATGCTTTCGCAGTAGTTC  887

QUERY  121   GTCTTTAACAAATCTAAGAATTTCACCTCTGACAGTTAAATACGAATGCCCCCAACTGTT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  886   GTCTTTAACAAATCTAAGAATTTCACCTCTGACAGTTAAATACGAATGCCCCCAACTGTT  827

QUERY  181   CCTATTAACCATTACTTTGGT-TCAAAAACCAAC-AA-AATAGA-ACC-AAAGTCCTACT  235
             ||||||||||||||||||||| |||||||||||| || |||| | ||| |||||||||||
SBJCT  826   CCTATTAACCATTACTTTGGTCTCAAAAACCAACCAACAATATAGACCAAAAGTCCTACT  767

QUERY  236   TTATTATTCCATGCTAAAGTATTCAAGGCAAAAGCCTGCTTGAAGCACTCTAATTTTCTC  295
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  766   TTATTATTCCATGCTAAAGTATTCAAGGCAAAAGCCTGCTTGAAGCACTCTAATTTTCTC  707

QUERY  296   AAAGTAAAAAATCTGGAAAAAAAGGCCGAAGCCAAATCAACCAGAAAAAAGCCACAACGC  355
             ||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
SBJCT  706   AAAGTAAAAAATCTGGAAAAAAAGGCCCAAGCCAAATCAACCAGAAAAAAGCCACAACGC  647

QUERY  356   AAACGAAACCAAAAAGGAACGAAAGCGAAGCAGCAGAAATT  396
             |||||||||||||||||||||||||||||||||||||||||
SBJCT  646   AAACGAAACCAAAAAGGAACGAAAGCGAAGCAGCAGAAATT  606
```

Description	Max score	Total score	Query cover	E value	Identity	Accession
	697	697	23%	0.0	98%	62853

Figure 4.7 Genbank blast search for haplotype 3. QUERY represents haplotype 3 while SBJCT represents *T. bicornis*

ClaustalW alignment of this three new haplotype sequences and other *Theileria* species of African origin resulted in 385 bp in length. Bayesian statistics gave the best-fitting model for the BML tree as TIM3+I+G ($-\ln L = -1584.9442$,

BIC=3396.1116). The Bayesian Inference 50% consensus phylogram recovered all the three haplotypes monophyletic with *T. bicornis* with a very high Bayesian Posterior Propability (0.98) as shown in figure 4.8. No animal tested positive for *Babesia* species. The sequences were deposited in the Genbank under the accession numbers H1-KC771140, H2- KC771141, and H3-KC771142.

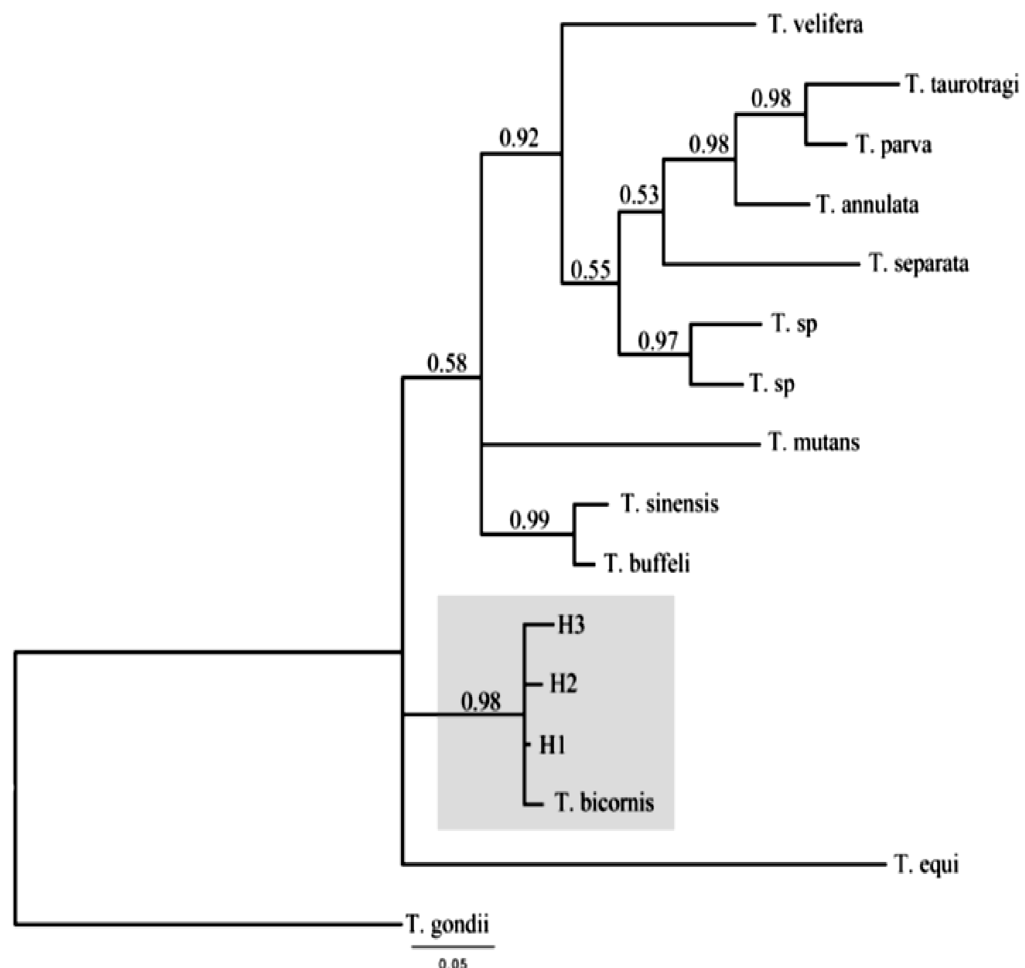

Figure 4.8: Bayesian Inference 50% consensus phylogram of *Theileria* species (18S rRNA partial sequence). Values by nodes are the posterior probabilities recovered from the Bayesian analysis. All three recovered haplotypes (H1, H2, and H3) and *T. bicornis* are shown in a shaded background.

4.2.1 Nucleotide substitution and *p*- uncorrected distances

Table 4.4 shows nucleotide substitution and genetic *p*-uncorrected distances between *Theileria* species included in the alignment. As shown in the shaded background (Table 4.4), genetic *p*-uncorrected distances between *T. bicornis* and the three haplotypes were very small (*T. bicornis*: H1= 0.016, *T. bicornis*: H2= 0.011, and *T. bicornis*: H3= 0.005) compared to that of the other *Theileria* species indicating a very close genetic affinity between *T. bicronis* and the three haplotypes. Simmilary nucleotide substitutions between *T. bicornis* and the three haplotyes were also small (*T. bicornis*: H1= 6, *T. bicornis*: H2= 4, and *T. bicornis*: H3= 2) compared to those of the other *Theileria* species. Between the haplotypes, haplotype 3 and 2 had the least nucleotide substitution (2), whereas Haplotype 1 and 2 had the largest nucleotide substitution (6).

Table 4.4: Nucleotide substitutions (above diagonal) and *p*-uncorrected distances (below diagonal) for each pair wise comparison between *Theileria* species. All three recovered haplotypes (H1, 2, 3) and *T. bicornis* are shown in a shaded background. Abbreviations on the top of the table follow the three first letters of the species names.

	H1	*H2*	*H3*	*T.bic*	*T.vel*	*T.tau*	*T.par*	*T.ann*	*T.mut*	*T.sep*	*T.buf*	*T.sin*	*T.sp1*	*Tsp2*	*T.equ*	*T.gon*
H1	-	6	4	6	31	32	26	25	36	33	23	22	29	30	39	52
H2	0.016	-	2	4	30	31	27	28	37	33	23	22	26	29	41	50
H3	0.011	0.005	-	2	29	30	25	26	36	31	21	20	28	29	39	50
T. bicornis	0.016	0.011	0.005	-	31	32	27	28	38	33	23	22	30	31	41	50
T. velifera	0.087	0.085	0.082	0.087	-	31	30	29	37	30	26	29	26	23	44	53
T. taurotragi	0.091	0.089	0.086	0.091	0.088	-	12	17	25	30	29	28	23	25	48	56
T. parva	0.074	0.076	0.071	0.076	0.085	0.034	-	14	26	28	27	27	24	22	46	54
T. annulata	0.071	0.079	0.073	0.079	0.082	0.049	0.040	-	33	27	26	27	23	24	49	52
T. mutans	0.103	0.105	0.103	0.108	0.107	0.073	0.075	0.095	-	40	30	31	35	31	42	56
T. separata	0.093	0.093	0.087	0.093	0.085	0.085	0.079	0.076	0.114	-	31	31	28	26	52	59
T. buffeli	0.065	0.065	0.059	0.065	0.074	0.083	0.077	0.074	0.086	0.087	-	4	25	21	38	45
T. sinensis	0.062	0.062	0.056	0.062	0.082	0.080	0.076	0.076	0.089	0.087	0.011	-	27	24	36	46
T. sp1	0.081	0.072	0.078	0.084	0.073	0.065	0.067	0.065	0.100	0.078	0.070	0.076	-	12	49	53
T. sp2	0.084	0.081	0.081	0.087	0.065	0.071	0.062	0.067	0.088	0.072	0.059	0.067	0.033	-	45	50
T. equi	0.109	0.115	0.109	0.115	0.124	0.136	0.129	0.138	0.120	0.145	0.107	0.101	0.136	0.125	-	57
T. gondii	0.144	0.139	0.139	0.139	0.151	0.161	0.154	0.149	0.162	0.167	0.129	0.131	0.149	0.141	0.161	-

4.2.2 Haplotype distribution

Table 4.5 shows distribution of the three recovered *T.bicornis* haplotypes, Haplotype 1, 2 and 3 in the six rhinoceros conservation areas sampled in Kenya. Haplotype 3 was the most dominant (66.7%) and was identified in all the sampled rhinoceros subpopulations and had infected both black and white rhinoceroses. Haplotype 1 was identified only in Lake Nakuru National Park's black rhinoceros whereas Haplotype 2 had infected both black and white rhinoceros and was

identified in Lake Nakuru and Meru National Parks, and Ngulia rhino sanctuary as shown in table 4.5.

Table 4.5: Rhinoceroses whose samples were subjected to sequencing, species, geographical location, and *T. bicornis* haplotype identified.

	Rhino Species	Location	Haplotype
1	*Ceratotherium simum simum*	Lake Nakuru National Park	H2
2	*Ceratotherium simum simum*	Lake Nakuru National Park	H3
3	*Diceros bicornis michaeli*	Lake Nakuru National Park	H1
4	*Diceros bicornis mickeali*	Lake Nakuru National Park	H3
5	*Diceros bicornis michaeli*	Nairobi National Park	H3
6	*Diceros bicornis michaeli*	Nairobi National Park	H3
7	*Ceratotherium simum simum*	Nairobi National park	H3
8	*Ceratotherium simum simum*	Meru National park	H2
9	*Ceratotherium simum simum*	Meru National park	H2
10	*Diceros bicornis michaeli*	Meru National park	H3
11	*Diceros bicornis michaeli*	Ngulia Rhino Sanctuary	H2
12	*Diceros bicornis michaeli*	Ngulia Rhino Sanctuary	H3
13	*Diceros bicornis michaeli*	Mugie Game Ranch	H3
14	*Diceros bicornis michaeli*	Mugie Game Ranch	H3
15	*Diceros bicornis michaeli*	Solio Rhino Sanctuary	H3

4.3 Prevalence

A population prevalence of infection with *T. bicornis* of 49.1% (56/114) was observed, with white rhinoceroses having significantly (χ^2 = 0.028) higher infection rate (65.6%) compared to the black rhinoceroses (42.7%, Figure 4.1).

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Rhinoceros being endangered animals need protection and appropriate conservation measures to prevent their extinction. Here in Kenya the 670 and 370 black and white rhinoceros respectively (Brett, 1990; Emslie *et al*., 2009; Emslie *et al*., 2011) are being intensively managed for population recovery mainly through translocation into sanctuaries or parks where they are closely monitored (Emslie *et al*., 2009). Other than poaching which is main threat facing the rhinoceroses, diseases have emerged as another threat of great concern (Nijhoh *et al*., 2003). To conserve the remaining individuals, several strategies are being applied among them translocation of rhinoceroses from one conservational area to another. This is done to establish new populations, re-establish a population in a place where it existed before (Woodford & Rossiter, 1993), to keep established populations productive, manage population density and balance sex ratio (Muya & Oguge, 2011). In addition it is a protective measure whereby the rhinoceroses are translocated to other areas of suitable habitat and where they may be better protected from poachers (Okita *et al*., 2007).

Translocation has disadvantages associated with it such as the risk of introducing destructive pathogens into naive wildlife population, exposure of translocated animals to pathogens in the new release site (Woodford & Rossiter, 1993;

Chapman *et al*., 2008). The process of capture and translocation is inherently a stressful event for animals and most likely compromises the animal's immune defense (Woodford & Rossiter, 1993; Cross *et al*., 2009). The effect of stress on rhinoceroses' immunity following translocation is of concern. This study focused on piroplasms, which are hemoprotozoan parasites naturally known to coexist with the rhinoceros host without causing disease, but when the rhinoceroses are exposed to stress such as following translocation, during pregnancy, or during unfavorable climatic conditions clinical disease develops which when not treated can cause death. Death of rhinoceroses due to babesiosis following stressful conditions has been reported in Tanzania (Nijhoh *et al*., 2003) and in addition piroplasms have been reported in South Africa's black and white rhinoceroses (Nijhof *et al*., 2003; Zimmerman, 2009; Govender *et al*., 2011). Here in Kenya, piroplasms presence in rhinoceros meta-population is unresolved and this study aimed at evaluating the diversity, prevalence and spatial distribution of piroplasms in the Kenyan rhinoceros meta-population by using molecular based techniques.

In this study piroplasms were isolated and identified among selected rhinoceros sub-populations in Kenya. The major identified species was *T. bicornis* with three new haplotypes H1, H2 and H3 isolated in the Kenyan black and white rhinoceros. This species was isolated in all the rhino sub-populations sampled and infected individuals comprised both black and white rhinoceros of both sexes and

age groups. The occurrence of *T. bicornis* in these sub-populations suggests that both species in Kenya are susceptible. Further, it suggests that Kenyan rhinoceroses are sub-clinically infected with *T. bicornis*, which makes them reservoirs for piroplasms which may be transmitted by tick vectors.

These findings are similar to the results of studies carried out in Tanzania and South Africa's black and white rhinoceroses. In Tanzania the study identified new *Babesia* and *Theileria* species in black rhinoceroses which were named *B. bicornis*, and *T. bicornis* respectively (Nijhof *et al*., 2003) in Ngorongoro Conservation Area in northern Tanzania. The same two parasites were identified in black rhinoceroses from the Great Fish River Reserve Complex in Eastern Cape Province, South Africa, which originated from a population in the KwaZulu-Natal Province of South Africa (Nijhof *et al*., 2003), and from other several localities in South Africa (Zimmermann, 2009). Later *T. bicornis* was identified from South Africas white rhinoceroses in 2009, in addition to *T. equi* (Govender *et al*., 2011).

This study did not isolate any *Babesia* species. This could be due to low infection rate of *Babesia* in the wild, and the small sample size used in this study. There are similar findings where *Theileria* species were found to be the only infecting species in South Africa (Govender *et al*., 2011), and Namibia's (Penzhorn *et al*., 2008) black and white rhinoceroses. In South Africa the white rhinoceroses were

infected with *T. bicornis* and *T. equi*, whereas in Namibia the rhinoceroses were not infected with either *Babesia* or *Theileria* species.

The infection rate in white rhinoceroses was significantly higher than that of the black rhinoceroses, which suggest that although both species are susceptible to piroplasm infection, the white rhinoceroses are more susceptible than the black rhinoceroses, they are important in the epidemiology of rhinocerotid-specific piroplasms, and that they may influence the overall endemic stability in the rhinoceros population.

Sex-biased prevalence is observed in many parasitic infections with males having higher prevalence and intensity of infections than their con-specific females (Klein, 2004; Cross *et al*., 2009). In many species males have larger home ranges, which may also lead to increased exposure to parasites (Miller & Conner., 2005). In addition territorial males have been shown to be exposed and susceptible to parasitic infections (Ezenwa, 2003; Cross *et al*., 2009), because territorial defense often involves aggressive encounters that may increase exposure to parasites and because defensive behaviors are energetically costly and may increase stress and testosterone levels, which has immune suppressive effects (Zuk & McKean, 1996). Although in this study there was no significant sex-biased difference in piroplasms infection rates, males of both species were seen to have a higher infection rate than the females which corresponds to the above statements.

Age is an important factor that may influence infection rate in chronic diseases. In this study, infection rate was seen to increase with age with highest infection rate observed among sub-adults. Rhinoceros sub-adults are usually subjected to variable stressful conditions, such as reproductive maturity, courtship, territorial defense (Brett, 1998; Govender *et al.*, 2011) or conquest, mating, and pregnancy, which may lead to immune suppression and trigger infection (Glacer & Kiecolt-Glacer, 2005; Meulenbein, 2006). The findings of this study therefore confirm that rhinoceroses in the sub-adult age-group are highly susceptible to piroplasms infection since they had a higher infection rate compared to the adults and juveniles. Although there was no statistical difference in infection rates among age groups, the findings are similar to those of white rhinoceros population in South Africa (Govender *et al.*, 2011) where sub-adult cows were significantly infected than the other age groups. On the other hand juveniles may initially be protected by maternal antibodies, but once passive immunity wane they may become susceptible to parasitic infections (Cross *et al.*, 2009). The findings of this study support the above statement since juveniles had the least infection rate.

Spatially structured populations, weather natural or artificially created like the rhinoceros meta-population and host migration can modify host-parasite interactions (Thrall & Burdon, 1997), and cause changes in infection rates (Grosholz, 1993). In rhinoceros meta-population, host migration is by host translocation, which ensures spread of both vector and parasites across

habitat/populations. This study showed universal infection with *T. bicornis* in all the sampled rhinoceros meta-populations. These findings suggest that these translocations could have exposed the naive rhino population in the endemic areas, which may account for the circulation of *T. bicornis* haplotypes in the six sub-populations. These parasites have co-evolved with their hosts and have developed an equilibrium or endemic stability in which infected hosts do not develop disease (Pezhorn *et al*., 1994; Penzhorn, 2006).

Although results showed variations in the infection rates among the sub-populations in this study which could be influenced by multiple localized factors, the difference was not significant (χ^2 = 0.140). Given the fact that healthy and nutritionally unstressed rhinos were chosen for translocation could also have contributed to the low infection rates observed in Mugie Game Ranch and Solio Rhino sanctuary. According to Lopez *et al.* (2005), frequent introduction of parasites into a population via host migration contributes to local population prevalence. This implies that rhino sanctuaries that frequently receive new individuals are likely to harbor higher parasite infection. Findings of this study does not support the above statement even though there were differences in infection rates in the different conservation area, they were not significant.

In natural meta-population, host migration between patches increases connectivity and biodiversity (Martensen *et al*., 2008), and re-colonization of extinct patches.

However, with increased migration, transmission of parasites among patches (Lopez *et al*., 2005) and the exposure to a pathogen also increase, for example due to increased contact rate with other host species (Harding *et al*., 2012). Host migration in the artificially established rhino meta-population in Kenya is achieved by frequent capture and translocation, an exercise that is inherently stressful and suggested to trigger clinical or fatal piroplasmosis in the species. In this study, all the sampled sub-populations of rhinoceroses were infected with *T. bicornis*, which mean that the populations were chronically infected and immunologically challenged. It is known that acute or chronic stress is able to cause such latent infections to progress to clinical and fatal piroplasmosis (Brocklesby, 1967; Mugera & Wandera, 1967; Nijhof *et al*., 2003; Nijhof *et al*., 2005). This is because it is able to physiologically suppress host immune competence (Agarwal & Marshall, 2001; Muehlenbein, 2006), allowing proliferation of parasites in the host to a threshold that leads to clinical state which may become fatal (Nijhof *et al*., 2003).

Piroplasms especially in the genus *Theileria* are known to be non-pathogenic in most cases (Fuente *et al*., 2008) and for the pathogenic species for instance *T. annulata* and *T. parva* which cause tropical Theileriosis and East Cost Fever respectively, the virulence varies depending on the stock of the parasite, the dose of parasite and the type of mammalian host (Norval *et al*., 1992). *T. bicornis* which has been identified in this study, has not yet been directly linked to clinical

disease although there are limited studies that have been carried out in the wildlife to assess their involvement in wildlife mortalities (Nijhof *et al*., 2003; Fuente *et al*., 2008). In cattle, high prevalence of piroplasm without clinical disease is referred to as endemic stability. It is probable that the high prevalence of infection with low incidence of clinical disease in the Kenyan rhinoceros meta-population indicate a state of endemic stability (Penzhorn, 2006) that may accord populations or individuals' sufficient immune defense.

Although Kenyan rhinoceroses could be endemically stable, the impact of chronic infection may be diverse and deleterious (Chapman *et al*., 2007; Fuente *et al*., 2008). However in terms of translocation-based population management, a state of universal piroplasm infection may be beneficial since disease risks associated with introduction of novel pathogens into a naive population or naive individuals into endemic areas is reduced. In Namibia, some rhino populations were completely not infected with piroplasms, which means they were immunologically naive, hence when released into piroplasms infected areas, they are vulnerable to clinical disease (Penzhorn *et al*., 2008). Therefore the widespread of piroplasm infection in Kenyan rhinoceroses maybe of health benefit. The results of this study showed that in spite of difference in geographical location, the variations in infection rates though not statistically significant may imply that variations in environmental conditions or sanctuary size may significantly influence the infection rates with piroplasms.

In this study the 18S small subunit ribosomal RNA (18S ssrRNA) gene was partially amplified and the molecular techniques used were able to identify three new *T. bicornis* haplotypes, and create phylogenetic relationship between the haplotypes and other *Theileria* species of African origin. This study therefore confirms that 18S small subunit ribosomal RNA gene is an important marker for piroplasm characterization. This gene has been used widely in molecular analysis to reconstruct the evolutionary history of organisms, and it has been successfully used in many studies to identify and establish piroplasm phylogenetic relationships (Homer *et al*., 2000; Nijhof *et al*., 2005; Penzhorn *et al*., 2006;.Oosthuizen *et al*., 2008; Zimmerman, 2009; Jeneby *et al*., 2011; Govender *et al*., 2011). It is an important marker for random target polymerase chain reaction (PCR) because of two reasons. First the rRNA gene sequences are easily accessed due to highly conserved flanking regions which allow the use of universal primers, and secondly their repetitive arrangement within the genome provides excessive amounts of template DNA for PCR, even in smallest organisms.

5.2 Conclusions

From the findings of this study it was concluded that;

1. Rhinoceroses from the six conservational areas were infected with *T. bicornis*.
2. Three new *T. bicornis* haplotypes, H1-KC771140, H2- KC771141, and H3-KC771142 were found to infect black and white rhinoceroses in these areas.
3. Haplotype three (H3) was the most dominant; it was distributed in all the six conservational areas and had infected both rhinoceros species, whereas H1 was the least dominant and had only infected black rhinoceroses.
4. White rhinoceroses are more susceptible to piroplasm infection than the black rhinoceroses.
5. Male rhinoceroses of both species were highly infected with piroplasms than females.
6. Rhinoceroses in the sub-adult age group are more susceptible to piroplasm infection than juveniles and adults.

From the data generated in this study, the null hypothesis which stated that; there is no host species and rhinoceros sub-population difference in the infection rates of piroplasms in Kenya was rejected, and the alternative hypothesis accepted.

5.3 Recommendations

Following the findings of this study it is recommended that;

1. A larger epidemiological study be carried out to establish distribution of *Babesia* species.
2. Further study be carried out to determine the pathological effects of *T. bicornis* on rhinoceroses
3. Kenya Wildlife Service to consider *Theileria* prophylactic measures during rhino translocation to reduce possible mortalities.

REFRENCES

Agarwal, S.K., and Marshall, G.D. (2001). Stress effect on immunity and its application to clinical immunology. *Clinical and Experimental Allergy*, **32**: 25-31.

Ambrosio, R.E., and De Waal, D.T. (1990). Diagnosis of parasitic disease. *Scientific and Technical Review*, **9**(3); 759-778.

Bishop, R., Musoke, A., Morzaria, S., Gardner, M., and Nene, V. (2004). Theileria: intracellular protozoan parasites of wild and domestic ruminants transmitted by ixodid ticks. *Journal of Parasitology*, **129**: S271-S278.

Bigalke, R.D., Keep, M.E., Keep, P.J., and Schoeman, J. H. (1970.). A large *Babesia* sp and a *Theileria* like piroplasm of the squire-lipped rhinoceroses. *Journal of South Africa Veterinary Medicine*, **41** (4): 292-294.

Bock, R., Jackson, L., De VOS, A., and Jorgensen, W. (2004). Babesiosis of Cattle. *Parasitology Journal*, **129**: S247-S269.

Bose, R., Jorgensen, W.K., Dalgliesh, R.J., Friedhoff, K.T., and De Vos, A.J. (1995). Current state and future trends in the diagnosis of babesiosis. *Veterinary Parasitology*, **57**: 61-74.

Bosman, A.M. (2010). Detection of Babesia Species in Domestic and Wild Southern African Felids by Means of DNA Probes. University of Pretoria. South Africa.

Brett, R. (1990). The Black Rhinoceros Sanctuaries in Kenya. *Pachyderm*, **13**: 31-34.

Brett, R. (1993). Conservation strategy and management plan for black rhinoceros (*Diceros bicornis*) in Kenya. *Kenya Wildlife Service*. Pp 1-105.

Brett, R.(1998). Mortality factors and breeding perfomance of translocated black rhinos in Kenya: 1984-1995. *Pachyderm*, **26**: 69-82.

Brocklesby, D. W. (1967). A Babesia species of the black rhinoceros. *Veterinary Record Journal*, **80**(15): 484.

Brown, C., and Torres, A., Eds. (2008). USAHA Foreign Animal Diseases, 7th Ed.Commitee of Foreign and Emerging Diseases of the US Animal Health Association. Boca Publications Group, Pp 147-157,401-404.

Burridge, M.J. (1975). The Role of Wild Mammals in The Epidemiology of Bovine Theileriosis in East Africa. *Journal of Wildlife Diseases*, **11**: 68.

Chaisi, M.E., Sibeko, K.P., Collins, N.E., Potgieter, F.T., and Oosthuizen, M.C. (2011). Identification of *Theileria parva* and *Theileria sp*. (buffalo) 18S rRNA gene sequence variants in the African Buffalo (*Syncerus caffer*) in Southern Africa. *Veterinary Parasitology*, **182**(2-4): 150-162.

Chapman, A.C., Saj, L.T. and Snaith,V.T. (2007). Temporal dynamics of nutrition, parasitism, and stress in Colobus monkeys: implications for regulation and conervation. *American Journal of Physical Anthropology*, **134**: 240-250.

Claive, A.S. (2012). African ecology- Benchmark and historical perspectives. Springer Heidelberg Dordrecht, London, New Yolk. Pp 1147-1148.

Coleman, P.G., Perry, B.D., and woolhouse, M.E.J. (2001). Endemic stability- a veterinary idea applied to human public health. *Lancet*, **357**: 1284-1286.

Cornelia, J.K., Mason, V.R., Rolnald, V.D., John, P.H., James, M., and Kocan, A.A. (2003).An Intraerythrocytic Small Piroplasm In Wild-caught Pallas's Cats (*Otocolobus manul*) from Mongolia. *Journal of Wildlife Diseases*, **39**(2): 424-430.

Cross, P.C., Drewe, J., Patrek, V., Pearce, G., Samuel, M.D., and. Delahay. R.J. (2009).Wildlife Population Structure and Parasite Transmission: Implications for Disease Management. In Management of Disease in Wild Mammals (eds Delahay, R.J., Smith, G.C., and Hutchings, M.R). Springer, Tokyo. Pp 9-30.

De Vos, A.J., De Waal, D.T., and Jackson, L.A. (2004). Bovine Babesiosis In: Infectious Diseases of Livestock. Coetzer, J.A.W. & Tustin, R.C (eds). 2nd edition.Cape Town: oxford University Press. Pp 406-424.

Dohoo, I., Martin, W. & Stryhn, H., (2003), Veterinary Epidemiologic Research, Atlantic Veterinary College, Charlottetown.

Emslie, R.H., Amin, R., and Kock. R. (2009). Guidelines for the in situ Reintroduction and translocation of African and Asian rhinoceroses. Gland, Switzerland; Occasional Paper of the IUCN Species Survival Commision No.39.

Emslie, R. (2011). *Diceros bicornis longipes*. IUCN Red List of Threatened Species. Version 2. International Union for Conservation of Nature.

Emslie, R., and Keryn, A. (1994). Managing black rhinoceros. Proceedings of a symposium on rhinos as game ranch animals. Onderstepoort:Wildlife Group of the South Africa Veterinary Association. Pp 100-107.

Etkind, P., Piesman, J., Ruebush II, T.K., Spielman, A., and Juranek. D.D. (1980). Methods for detection of *Babesia microti* infection in wild rodents. *Journal of Parasitology*, **66**: 107-110.

Ezenwa, V.O. (2003). Host social behavior and parasitic infection: a multi-factorial approach. *Behavioral Ecology*, **15**(3): 446-454.

Fahrimal, Y., Goff, W.L. and Jasmer, D.P. (1992). Detection of *Babesia bovis* carrier cattle by using polymerase chain reaction amplification of parasite DNA. *Journal of Clinical Microbiology*, **30**:1374-1379.

Ferreri, L., Benitez, D., Dominguez, M., Rodriguez,A., Asenzo, G., Mesplet, M., Florin, C.M., and Schnittger, L. (2008). Water Buffalos as Carriers of *Babesia bovis* in Argentina. Animal Annals of the New York Academy of Sciences, 1149: 149-151.

Fuente, J., Estrada-Pena, A., Venzal, J.M., Kocan, K.M., and Sonenshine, D.E. (2008). Overview: Ticks as vectors of pathogens that cause disease in humans and animals. *Frontiers in Bioscience*, **13**: 6938-6946.

Garci´a, S.J., Aurtenetxe, O., Barral, M., Marco, I., Lavin, S., Garci´a, A.P., and Hurtado, A. (2007). Molecular detection and characterization of piroplasms infecting cervids and chamois in northern spain. *Parasitology*, **134**: 391-398.

Gasser, R.B. (2006). Molecular tools–advances, opportunities and prospects. *Veterinary Parasitology*, **136**(2): 69-89.

Githaka, N., Konnai, S., Kariuki, E., Kanduma, E., Murata, S., and Ohashi, K. (2012). Molecular detection and characterization of potentially new Babesia and Theileria species/variants in wild felids from Kenya. *Journal of Clinical Microbiology*, **48**(8): 2703-2708.

Glacer, B., and Kiecolt-Glacer, J.K. (2005). Stress-induced immune dysfunction:implications for health.*Nature Reviews, Immunology*, **5**: 243-251.

Goul, M Ste´phane, G., and Olivier, G. (2010). SeaView Version 4: A

Multiplatform Graphical User Interface for Sequence Alignment and Phylogenetic Tree Building. *Molecular Biology and Evolution*, **27**(2): 221-224.

Govender, D., Oosthuisen, M.C., and Penzhorn, B.L. (2011). Piroplasm parasites of white rhinoceroses (*Ceratotherium simum*) in the Kruger National Park, and their relation to anaemia. *Journal of South Africa Veterinary Association*, **82**: 36-40.

Grootenhuis, J.G., Young, A.S., Kanhai, G.K., and Paling, R.W. (1977). Experimental Tick Transmission of Theileria Species between Eland. *The Journal of Parasitology*, **63**(6): 1127-1129.

Grosholz, E.D.(1993). The influence of habitat heterogenicity on host-pathogen population dynamics. *Oecologia*, **96**: 347-353.

Han, E.T., Watanabe, R., Sattabongkot, J., Khuntirat, B., Sirichaisinthop, J., Iriko, H. (2007). Detection of four Plasmodium species by genus and species-specific loop-mediated isothermal amplification for clinical diagnosis. *Journal of Clinical Microbiology*, **45**(8):2521-2528.

Harding, K.C., Begon, M., Eriksson, A., and Wennberg, B. (2012). Increased migration in host–pathogen metapopulations can cause host extinction. *Journal of Theoretical Biology*, **298**: 1-7.

Homer, M.J., Aguiler-Delfin, I., Telford, S.R., Krause, P.J.,and Persing, D.H. (2000). Babesiosis. *Journal of clinical Microbiology*, **13**(3): 451-469.

Huelsenbeck, J.P., Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, **17**(8): 754-755.

Hunfeld, K.P., Hildebrandt, A., and Gray, J.S. (2008). Babesiosis: Recent insights into an ancient disease. *International Journal for Parasitology*, **38**: 1219-1237.

Irwin, P.J. (2010). Canine Babesiosis. *Veterinary Clinics of North America*, **40**: 1141-1156.

Jae, I.H., Hye, J.J., and Ki-Jeong, N. (2009). Molecular Detection of *Theileria sp*. in Wild Chinese Water Deer (*Hydropotes inermis argyropus*). *Journal of Wildlife Diseases*, **45**(4): 1213-1216.

Jeneby, M.M., Mbaruk, A.S., Mercy, A., Hastings, O., Thomas, K., and Hans-Erik, C. (2011). Prevalence of *Babesia microti* in free ranging Kenyan

baboons and African green monkeys. *Journal of Parasitology*, **97**(1): 63-67.

Karbe, E., Grootenhuis, J.G., Kelley, S., and Karstad, L. (1979). Experiments on the *Babesia bigemina* carrier state in East African buffalo and eland. *Tropenmed Parasitology*, **30** (3): 313-317.

Klein, S.L. (2004). Hormonal and immunological mechanisms mediating sex differences in parasite infection. *Parasite Immunology*, **26**: 247-264.

KWS, (2007) Meru Conservation Area Management Plan 2007-2017.

KWS, (2005) Meru Conservation Area development project 2005.

KWS, (2003) Nairobi National Park Ecosystem Management Plan 2003-2007.

KWS, (2002) Lake Nakuru National Park integrated ecosystem management plan 2002-2012.

KWS, (1993) Tsavo West management plan 1993-1997.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., and Higgins, D.G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*, **23**(21): 2947-2948.

Leighton, F.A. (2002). Health Risk Assessement of the Translocation of Wild animals. *Scientific and Technical Review*, **21**(1): 187-195.

Levine, N. D. (1971). Taxonomy of the piroplasms. Transactions of the American Microscopical Society, **90**: 2-33.

Lopez, J.E., Gallinot, L.P., and Wade, M.J. (2005). Spread of parasites in metapopulation: an experimental study of the effects of host migration rate and local host population size. *Parasitology*, **130**: 323-332.

Mahoney, D.F. and Ross, D.R. (1972). Epizootiological factors in the control of bovine babesiosis. *Australian Veterinary Journal*, **4**: 292-298.

Marc, K.K., Vaia, K., Alvin, A.G., Jerold, H.T., Elias, P., and Georgios, T. (2010). Seroprevalence of equine piroplasmosis and host related factors associated with infection in Greece. *Veterinary Parasitology*, **169**: 273-278.

Maria, R.M., and Jeffrey, A.G. (1996). Babesiosis. *Clinical Infectious Diseases*, **22**: 611-5.

Martensen, A.C., Pimentel, R.G., and Metzger, J.P. (2008). Relative effects of fragment size and connectivity on bird community in the Atlantic Rain Forest: implications for conservation. *Biological Conservation*, **141**: 2184-2192.

McCulloch, B., and Achard, P.L.(1969). Mortalities associated with capture, translocation, trade and exhibition of black rhinoceroses. *International Zoo yearbook*, **9**: 184-195.

Meltizer, D.G.A. (1994). Diseases in free ranging black and white rhinoceroses. Proceedings of a symposium on rhinos as game ranch animals. Onderstepoort:Wildlife Group of the South Africa Veterinary Association. Pp176.

Merz, A. (1994). Lewa Updates. *H.O.R.N Lewa*. **4**(4): 4.

Miller, M.W., and Conner, M.M. (2005). Epidemiology of chronic wasting disease in free ranging mule deer: Spatial, temporal, and demographic influences on observed prevalence patterns. *Journal of Wildlife Diseases*, **41**: 275-290.

Mohamad, A.T., Nguyen, X.H., Cao, S., Tawin, I., Khuanwalai, M., Mahmoud, A., Akio, U., Youn, K.G., Naoaki, Y., Sathaporn, J., Xuenan, X., and Ikuo, I. (2011). Molecular and serological prevalence of *Babesia bovis* and *Babesia bigemina* in water buffaloes in the Northeast region of Thailand. *Veterinary Parasitology*, **178**: 201-207.

Momar. N. (2009). Diagnosis of Parasitic Diseases: Old and New Approaches. *Interdisciplinary Perspectives on Infectious Diseases*, **2009**, Article 278246.

Moody, A.L., and Chiodini, P.L. (2000). Methods for the detection of blood parasites. *Clinical and Labatory Haematology*, **22**: 189-202.

Mugera, G.M., and Wandera J.G. (1967). Degenerative polymyopathies in East African Domestic and Wild Animals. *Veterinary Record*, **80**(13): 410-413.

Muehlenbein, P.M. (2006). Intestinal Parasite Infections and Fecal Steroid Levels in Wild Chimpanzees. *American Journal of Physical Anthropology*, **130**: 546-550.

Muhanguzi, D., Matovu, E., and Waiswa, C. (2010). Prevalence and Characterization of *Theileria* and *Babesia* Species in Cattle under Different Husbandry Systems in Western Uganda. *International Journal of Animal*

and Veterinary Advances, **2**(2): 51-58.

Munson, L., Terio, K.A., Kock, R., Mlengeya, T., Roelke, M.E., Edward, D., Brian, S., Anthony, R.E.S., and Craig, P. (2008). Climate Extremes Promote Fatal Co-Infections during Canine Distemper Epidemics in African Lions. *PLoS ONE*, **3**(6): e2545.

Muya, S.M., and Oguge, N.O. (2000). Effects of Browse Availability and Quality on Black Rhino (*Diceros bicornis michaeli*) diet in Nairobi National Park, Kenya. *African Journal of Ecology*, **38**: 62-71.

Nijhof, A.M., Pillay, V., Steyl, J., Prozesky, L., Stoltsz, W.H., Lawrence, J.A., Penzhorn, B.L., and Jongejan, F. (2005). Molecular characterization of *Theileria* species associated with mortality in four species of African antelopes. *Journal of Clinical Microbiology*, **43**: 5907-5911.

Nijhof, A.M., Penzhorn, B.L., Lyenn, G., Mollel, J.O., Morkel, P., Bekker, C.D.J., and Jongejan, F. (2003). *Babesia bicornis* sp. nov. And *Theileria bicornis* sp. nov: tick-borne parasites associated with mortality in the black rhinoceros (*Diceros bicornis*). *Journal of Clinical microbiology*, **203**: 2249-2254.

Nkouawa, A., Sako, Y., Nakao, M., Nakaya, K., and Ito, A. (2009). Loop-mediated isothermal amplification method for differentiation and rapid detection of Taenia species. *Journal of Clinical Microbiology*, **47**(1): 168-174.

Norval, R.A.I., Perry, B.D., and Young, A.S. (1992). The Epidemiology of Theileriosis in Africa. London, San Diego, New York, Boston, Sydney, Tokyo, Toronto. Academic Press. Pp63-279.

Obanda, V., Kagira, J., Chege, S., Okita-Ouma, B., and Gakuya, F. (2011). Trypanosomiasis and other co-infections in translocated black (*Diceros bicornis michaeli*) and white (*Ceratotherium simum simum*) rhinoceroses in Kenya. *Scientia Parasitologica*, **12**(2): 103-107.

Okita, B., Amin, R., and Kock, R. (2007). Conservation and Management Strategy for the Black Rhinoceros (*Diceros bicornis*) and Management Giudelines for the White Rhino (*Ceratotherium simum simum*) in Kenya. 3rd Ed.

Okita, B., Amin, R., Adcock, K., Kock, R., A., Keryn, A., Richard, E., and Richard, K. (2006). A Positive Turning Point in Black Rhino Conservation in Kenya.

Oosthuizen, M.C., Zweygarth, E., Collins, N.E., Troskie, M., and Penzhorn, B.L. (2008). Identification of a Novel Babesia sp. from a Sable Antelope (*Hippotragus niger Harris*, 1838). *Journal of Clinical Microbiology*, **46**(7): 2247-2251.

Parida, M., Sannarangaiah, S., Dash, P.K., Rao, P.V., and Morita, K. (2008). Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. *Review in medical virology*, **18**(6): 407-421.

Paris, D.H., Imwong, M., Faiz, A.M., Hasan, M., Yunus, E.B., and Silamut, K., Lee, S.J., Day, N.P., and Dondorp, A.M. (2007). Loop-mediated isothermal PCR (LAMP) for the diagnosis of falciparum malaria. *American Journal of Tropical Medicine and Hygiene*, **77**(5): 972-976.

Peirce, M.A. (2000). A taxonomic review of avian piroplasms of the genus *Babesia Starcovici*, 1893 (Apicomplexa: Piroplasmorida: Babesiidae). *Journal of Natural History*, **34**(3): 317-332.

Penzhorn, B.L. (2006). Babesiosis of Wild Carnivores and Ungulates. *Journal of Veterinary Parasitology*, **138**: 11- 21.

Penzhorn, B.L., Oosthuizen, C.M., Bosman A.M., Werner, J.K., and Ivan, G.H. (2008). Black rhinoceros (*Diceros bicornis*) population in Northwestern Namibia Are apparently not infected with Piroplasms. *Journal of Wildlife Diseases*, **44**(4): 1032-1035.

Penzhorn, B. L., Krecek, R.C., Horak, J.G., Verster, A.J.M., Walker, J.B., Boomk, J.D., Knapp, S.E., and Quandt, K.F.(1994). Parasites of African Rhinos. Proceedings of a symposium on rhinos as game ranch animals.Onderstepoort:*Wildlife Group of the South Africa Veterinary Association*. Pp 168-170.

Pfitzer, S., Oosthuizen, M.C., Bosman, A.M., Vorster, I., and Penzhorn, B.L. (2011).Tick-borne blood parasites in nyala (*Tragelaphus angasii*, Gray 1849) from KwaZulu-Natal, South Africa. *Veterinary Parasitology*, **176** (2-3): 126-131.

Posada, D. (2008). jModelTest: Phylogenetic Model Averaging. *Molecular Biology and Evolution*, in press.

Ramsey, E.C.and Zainuddin, Z.Z.(1993). Infectios Diseases of The Rhinoceros and Tapir.In: Fowler M.E. (ed) Zoo znd Wild Animal Medicine.

Philadelphia: WB Saunders Co. Pp 458-468.

Rubaire, A.C., Okello, O.J., Nasinyama, G.W., Vaarst, M., Kabagambe, E.K., Mwayi, W., Musunga, D., and Wandukwa, W. (2004). The prevalence of serum antibodies to tick-borne infections in Mbale District, Uganda: The effect of agro-ecological zone, grazing management and age of cattle. *Journal of Insect Science*, **4**: 8.

Samson, S.Y.W., Rosana W.S.P., Janet, J.Y.H., and Kwok,Y.Y. (2012). Detection of *Babesia hongkongensis sp.* nov. in a Free-Roaming Felis catus Cat in Hong Kong. *Journal of Clinical Microbiology*, **50**(8): 2799-2803.

Spitalska, E., Riddell, M., Heyne, H., Olivier, A., Sparagano, E. (2005). Prevalence of Theileriosis in Red Hartebeest (*Alcelaphus buselaphus caama*) in Namibia. *Parasitology Research*, **97**: 77-79.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution*, **28**: 2731-2739.

Telford, S.R., Gorenflot, A., Brasseur, P., and Splelma, A. (1993). Babesial infection in human and wildlife. In Kreier, J.P. (ed). Parasitic protozoa, Second edition, Vol 5. Academic press, San Diego, Califonia, Pp 1-47.

Thrall, P.H., and Burdon, J.J. (1997). Host-pathogen dynamics in a metapopulation context:the ecological and evolutionary consequences of being spatial. *Journal of Ecology*, **85**: 743-753.

Tavares, R.G., Staggemeier, R., Borges, A.L.P., Rodrigues, M.T., Castelan, L.A., Vasconcelos, J., Anschau, M.E., and Spalding, S.M. (2011). Molecular techniques for the study and diagnosis of parasite infection. *The Journal of Venomous Animals and Toxins including Tropical Diseases*, **17**(3): 239-248.

Uilenberg, G. (2006). Babesia-A historical overview. *Veterinary Parasitology*, **138**(1-2): 3-10.

Woodford, M.H., and Rossiter P.B. (1993). Disease risks associated with wildlife translocation projects. *Scientific and Technical Review*, **12**(1): 115-135.

Yasser, S.M. (2012). Molecular Detection of Natural *Babesia bovis* Infection from Clinically Infected and Apparently Healthy Water Buffaloes (*Bubalus bubalis*) and Crossbred Cattle. *Journal of Buffalo Science*, **1**: 55-60.

Yusufmia, S.B., Collins, N.E., Nkuna, R., Troskie, R., Van, D.P.B., and Penzhorn, B.L. (2010). Occurrence of Theileria parva and other haemoprotozoa in cattle at the edge of Hluhluwe-iMfolozi Park, KwaZulu-Natal, South Africa. *Journal of the South African Veterinary Association*, **81**(1): 45-49.

Zimmerman, D.E., (2009). The occurrence of piroplasms in various South African black rhinoceros (*Diceros bicornis*) populations, MSc (Veterinary Tropical Diseases) Dissertation, University of Pretoria, Pretoria, viewed 2012/04.01.

Zuk, M., and McKean, K.A. (1996) Sex differences in parasite infections: Patterns and processes. *International Journal for Parasitology* **26**: 1009-1023.

APPENDICES

APPENDIX 1: Worksheet for analysis of *Babesia* and *Theileria* 18S rRNA genes by Nested PCR

Reagents

Platinum® Blue Supermix- Invitrogen

10pmol/µl ILO-9029

10pmol/µl ILO-9030 BecA-Hub, International Livestock Research Institute, Kenya

10pmol/µl MWG4/70

10pmol/µl ILO-7782

Genomic DNA- extracted from Rhino anticoagulated whole blood

Equipment

0.2 ml PCR tubes- Applied Biosystems

1.5ml microcentrifuge tubes

Thermocycler (Applied Biosystem verity 96 well thermal cycler)

Primary amplification reaction mix

Reagent	Volume
Platinum® Blue PCR Supermix	45µl
ILO-9029 (Forward Primer)	1µl
ILO-9030 (Reverse Primer)	1µl
Genomic DNA	3µl
Total	50µl

Primary PCR Programme

Temperature	**Time**		Function
95°C	5 Min		Initial denaturation
95°C	30 Sec	× 30 cycles	DNA denaturation
53°C	30Sec		Annealing of primers
72°C	1 Min		Exention
72°C	9 Min		Final extension
4°C	∞		Storage

Secondary amplification reaction mix

Reagent	**Volume**
Platinum® Blue PCR Supermix	45µl
MWG4/70 (Forward Primer)	1.5µl
ILO-7782 (Reverse Primer)	1.5µl
Primary PCR product	2µl
Total	50µl

Secondary PCR Programme

Temperature	Time		Function
95°C	5 Min		Initial DNA denaturation
95°C	30 Sec	×30 cycles	DNA denaturation
55°C	30Sec		Annealing of primers
72°C	1 Min		Extension
72°C	9 Min		Final extension
4°C	∞		Storage

APPENDIX 2: Sequences of the three haplotypes recovered

H1
CCAATCTCTAGTCGGCATAGTTTATGGTTAGGACTACGACGGTATCTG
ATCGTCTTCGATCCCCTAACTTTCGTTCTTGATTAATGAAAACATCCTT
GGCAAATGCTTTCGCAGTAGTTCGTCTTTAACAAATCTAAGAATTTCA
CCTCTGACAGTTAAATACGAATGCCCCCAACTGTTCCTATTAACCATTA
CTTTGGTTCGAAAACCAACAAAATAGAACCAAAGTCCTACTTTATTAT
TCCATGCTAAAGTATTCAAGGCAAAAGCCTGCTTGAAGCACTCTAATT
TTCTCAAAGTAAAAAAATCTGGAAAAAAAGCCGAAGCCAATCAACCA
GAAAAAAGCCACAACGCAAACGAAACCAATAAAGGGACGAAAGCGA
AGCAGCAGAAATT

H2
CCAATCTCTAGTCGGCATAGTTTATGGTTAGGACTACGACGGTATCTG
ATCGTCTTCGATCCCCTAACTTTCGTTCTTGATTAATGAAAACATCCTT
GGCAAATGCTTTCGCAGTAGTTCGTCTTTAACAAATCTAAGAATTTCA
CCTCTGACAGTTAAATACGAATGCCCCCAACTGTTCCTATTAACCATTA
CTTTGGTTCAAAAACCAACAAAATAGAACCAAAGTCCTACTTTATTAT
TCCATGCTAAAGTATTCAAGGCAAAAGCCTGCTTGAAGCACTCTAATT
TTCTCAAAGTAAAAAATCTGGAAAAAAAGGCCGAAGCCAAATCAACC
AGAAAAAAGCCACAACGCAAACGAAACCAAAAAGGAACGAATGCGG
AGCAGCAGAAATT

H3
CCAATCTCTAGTCGGCATAGTTTATGGTTAGGACTACGACGGTATCTG
ATCGTCTTCGATCCCCTAACTTTCGTTCTTGATTAATGAAAACATCCTT
GGCAAATGCTTTCGCAGTAGTTCGTCTTTAACAAATCTAAGAATTTCA
CCTCTGACAGTTAAATACGAATGCCCCCAACTGTTCCTATTAACCATTA
CTTTGGTTCAAAAACCAACAAAATAGAACCAAAGTCCTACTTTATTAT
TCCATGCTAAAGTATTCAAGGCAAAAGCCTGCTTGAAGCACTCTAATT
TTCTCAAAGTAAAAAATCTGGAAAAAAAGGCCGAAGCCAAATCAACC
AGAAAAAAGCCACAACGCAAACGAAACCAAAAAGGAACGAAAGCGA
AGCAGCAGAAATT