EVALUATION OF PIROPLASM OCCURRENCE IN THE EASTERN BLACK (*DICEROS BICORNIS MICHAELI*) AND SOUTHERN WHITE (*CERATOTHERIUM SIMUM SIMUM*) RHINOCEROS METAPOPULATION IN KENYA

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Abstract
The eastern black and the white rhinoceros are endangered mainly due to poaching. Besides poaching diseases are another impediment to the species recovery. Infection with pirolasms has been linked to rhinoceros post-translocation mortality in South Africa and Tanzania. Presence of such piroplasms in the Kenyan rhinoceroses has not been investigated. This study aimedat evaluating occurrence of piroplasms in selected rhino sub-populations in Kenya. Blood samples were collected from 114 rhinoceroses, genomic DNA extracted, 18S rRNA genes of *Babesia* and *Theileria* amplified, and PCR products analyzed by gel electrophoresis. Fifty six samples tested positive for piroplasms. White rhinoceroses were significantly infected (65.6%) compared to black rhinoceroses (42.7%, p =0.028). Males were insignificantly highly infected compared to females (p = 0.353). Sub adults were insignificantly highly infected compared to adults and juveniles (p = 0.465). Infection rates among the sub-populations varied insignificantly (p = 0.140). The study concluded that Kenyan rhinoceroses are infected with piroplasms and recommends further studies to be carried out on piroplasm tick vectors, and piroplasm- vector- host interaction so as to understand the transmission dynamics, and possible disease-transmission in the human-wildlife-livestock ecosystem.

Key words: *Babesia bicornis*, *Theileria bicornis*, translocation, piroplasms

1.0 Introduction

Rhinoceros were abundant in Kenya in the early 20th Century (Brett, 1993) that they were viewed as agricultural pests that impeded human settlement in the eastern part of Kenya. The black rhino numbered around 20,000 individuals in 1970's and still had a wide 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 trans locating offsprings 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 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 rhino 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 *et al.,* 2011).

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 *et al*., 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 rhinoceroses in Tanzania, South Africa and Kenya (Nijhoh *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). There is no evidence whether these lethal species infect Kenyan rhinoceroses Moreover, it is not known which other species of piroplasms latently infect rhinos in the different sub-populations.The goal of this study was therefore to evaluate the occurrence, infection rates, and spatial distribution of piroplasms in selected rhinoceros meta- population in Kenya.

2.0 Materials and Methods

2.1 Study Animals and Ethical Consideration

Eastern black (*Diceros bicornis michaeli*) and southern white rhinoceroses (*Ceratotheriumsimum simum*) from Meru, Lake Nakuru, and Nairobi National Parks, Mugie Game Ranch, Solio and Ngulia Rhino Sanctuaries (Figure 1)were sampled during scheduled management activities by Kenya Wildlife Service between 2011 and 2012. A total of 114 (82 black and 32 white) rhinoceroses 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). 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.

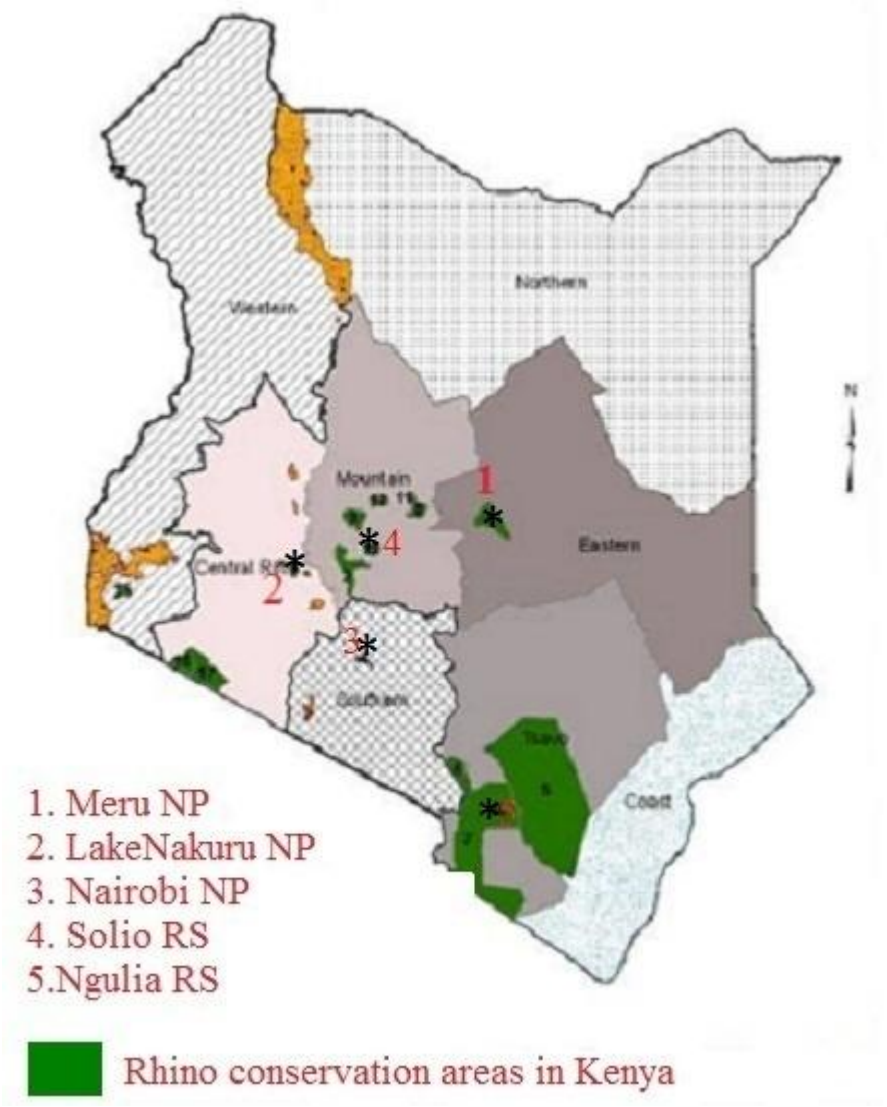

Figure 1: Rhinoceros conservation areas in Kenya with the areas from which blood samples were collected from both black and white rhinoceroses marked with an asterick and numbered in red

2.2 Collection, Handling and Processing of Blood Samples

Sampling was done opportunistically during scheduled immobilizations for population and health management as listed in table 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 (EDTA) 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 1: Rhinoceros conservation areas sampled event and the date of sampling

Area	Activity	Date
Meru National park	Ear notching	February 2011
Mugie Game Ranch	Translocation	December 2011-January2012
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

2.3 Extraction of DNA

DNA was extracted from 200 µl of Ethylene diamine tetra acetic acid (EDTA) anticoagulated blood using a DNA extraction kit (DNeasy blood &Tissue Kit, QIAGEN, Southern Cross Biotechnologies, South Africa) following manufacturers protocol. The stored blood was thawed and mixed 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 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.

2.4 Polymerase Chain Reaction

Genomic DNA extracted from 114 rhino 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') (Jeneby *et al*., 2011). 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).

2.5 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.

2.6 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.

2.7 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.

2.8 Statistical Analysis

The parasites infection rate differences between the rhinoceros species, sexes, age groups, and sampling locations was 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.

3.0 Results

3.1 Identification of Piroplasms using PCR, and Gel Electrophoresis

Out of the 114 rhinoceroses sampled 56 tested positive for piroplasms on PCR amplification and gel electrophoresis. The positive samples formed a band at approximately 400bp which was the expected fragment size (Figure 2).

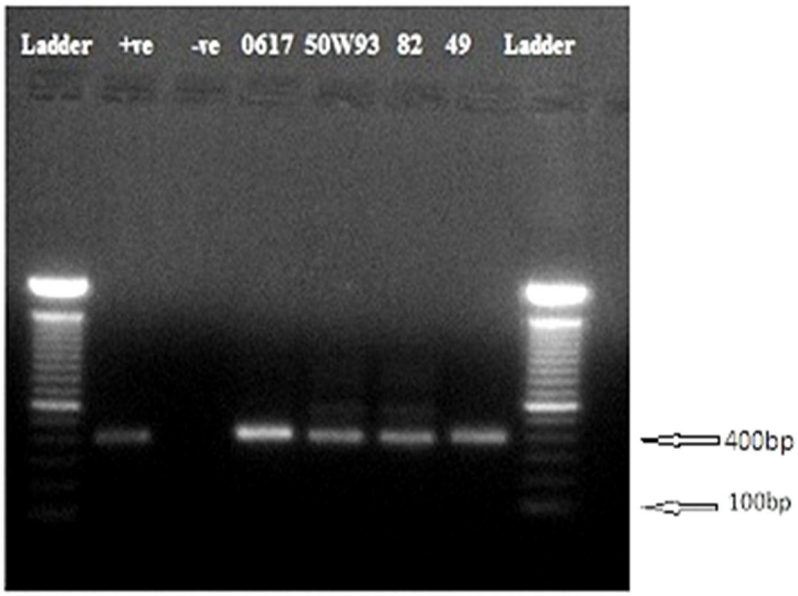

Figure 2:*Gel electrophoresis PCR amplification of a partial sequence of 18S rRNA of Babesia spp./Theileria spp. gene, product size approximately 400 bp. Lanes (From Left): (1) Molecular weight marker = 100bp; (2) Positive control; (3) Negative control; (4-7) Products of the amplified PCR product obtained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

3.2 Infection Rates in Black and White Rhinoceroses

Overall infection rate in the white rhinoceroses was significantly higher (65.6%) than that of the black rhinoceroses (42.7%, χ^2=0.028, Figure 3). Ngulia and Solio Rhino Sanctuaries did not have white rhinoceros representatives because they are purely black rhinoceros conservation areas. Piroplasm infection rates in the two rhinoceros species from the six conservation areas sampled is summarized in Table 2. 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.

Table 2: Piroplasm infection rates in D. bicornis and C. simum from the six 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

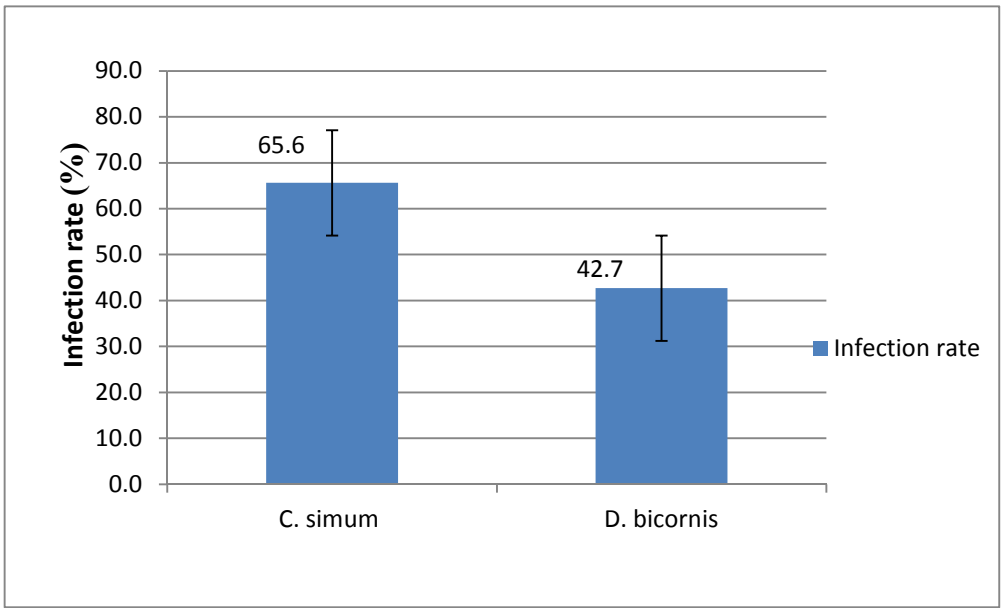

Figure 3: Overall piroplasm infection rates in C. simum and D. bicornis from six different rhinoceros conservation areas in Kenya

3.3 Infection Rates in Male and Female Rhicoceroses

Overall infection rate in the males (53.7%) was insignificantly higher compared to that of females (45%, χ^2 =0.353, Figure 4) in both rhinoceros species.Piroplasm infection rates in female and male rhinoceroses of both species from the six sampled conservation areas varied and are summarized in Table 3.

Table 3: 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

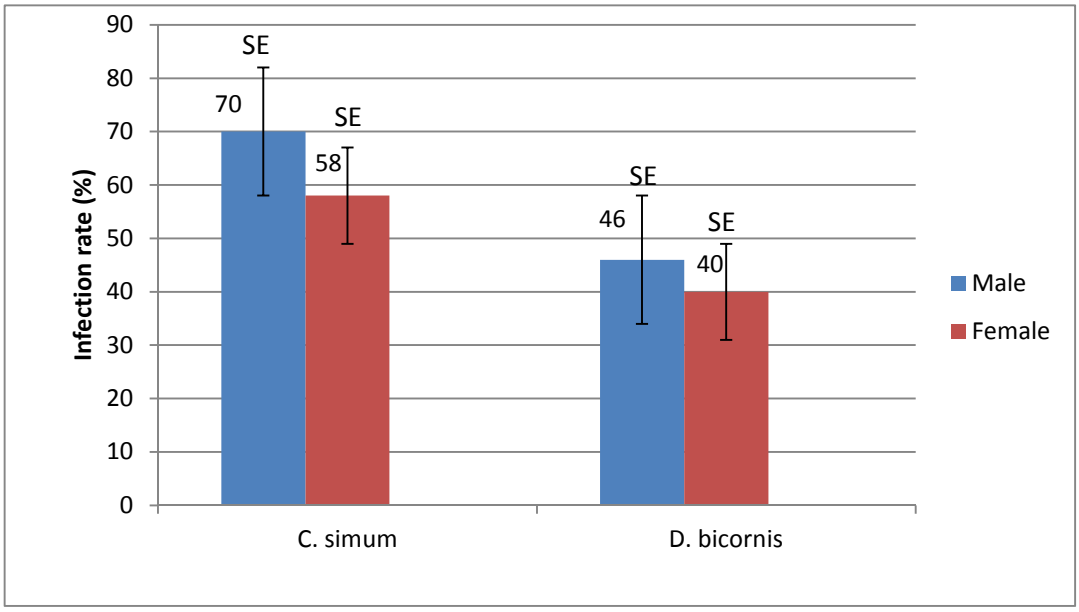

Figure 4: Overall piroplasm infection rate in males and females in both rhinoceros species from different conservation areas in Kenya

3.4 Infection Rates in the Juvenile, Adult and Sub-adult Rhinoceroses

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 differences were not statistically significant(χ^2 = 0.465, Figure 5).Piroplasm infection rates in adults, sub-adults, and juveniles of both rhinoceros species from the six sampled rhinoceros conservation areas are summarized in Table 4.

Table 4: 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

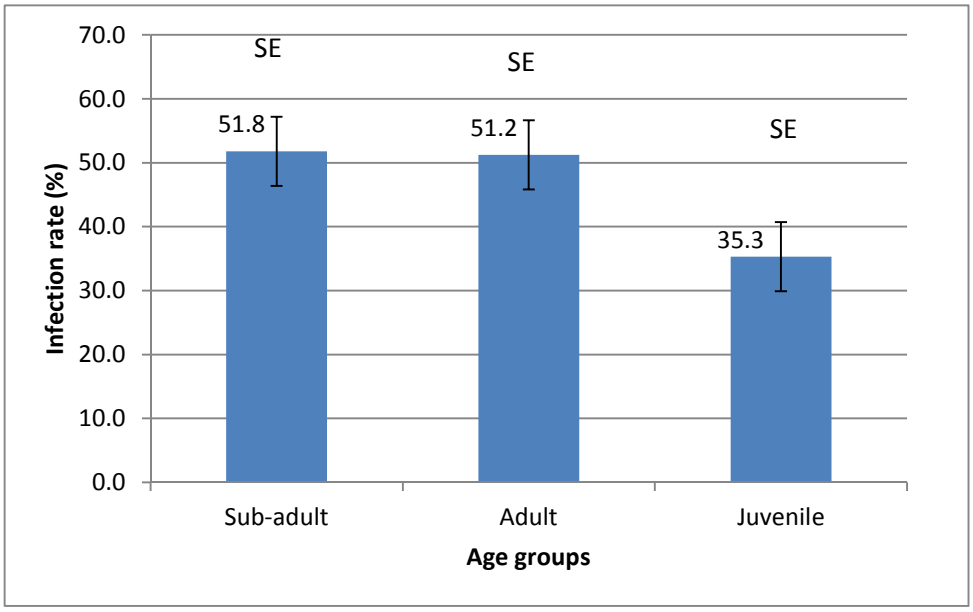

Figure 5: Overall piroplasm infection rates in the juveniles, sub-adults and adults in both rhinoceros species from six rhinoceros conservation areas in Kenya

3.5 Infection Rates in the Different Rhinoceros Conservation Areas Sampled

Infection rates varied among the six sampled sub population with Meru National Park having the highest infection rate (66.7%), while Solio Rhino Sanctuary had the least (12.5%) thoughthe differences were not significant($\chi^2 = 0.140$).

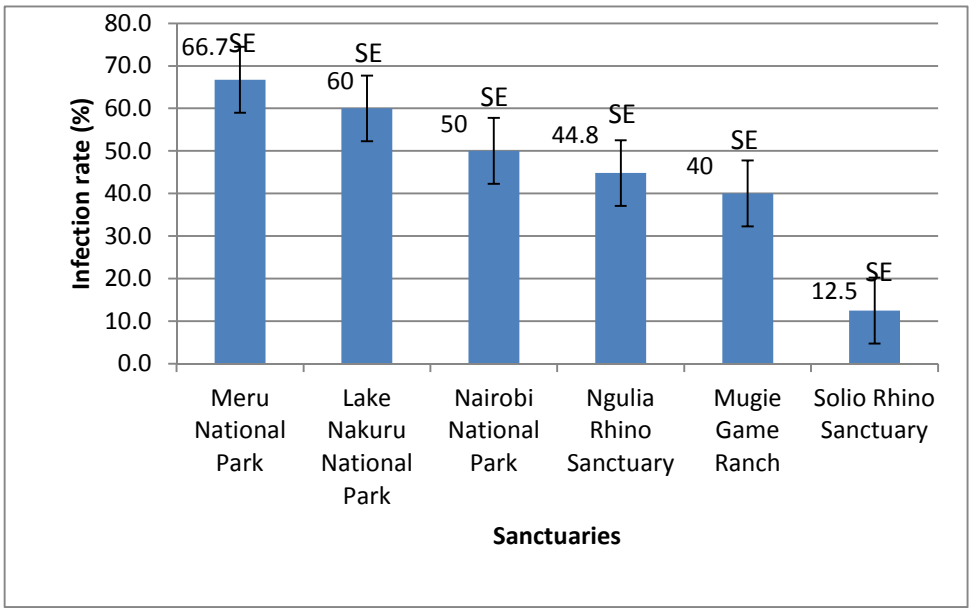

Figure 6: Rhinoceros infection rates in the six conservation areas in Kenya

4.0 Discussion

In this study piroplasms were isolated in all the rhino sub-populations sampled and infected individuals comprised both black and white rhinoceros of both sexes and age groups. Piroplasm occurrence in these sub-populations suggests that both species in Kenya are susceptible. Further, it suggests that Kenyan rhinoceroses are sub-clinically infected with piroplasms, which makes them reservoirs for host.Similar findings were found in Tanzania and South Africa's black and white rhinoceroses (Nijhof *et al*., 2003; Zimmermann, 2009; Govender *et al*., 2011).

The infection rate in white rhinoceroses was significantly higher than that of the black rhinoceroses (figure 3), 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 *et al*., 2005). In addition territorial males have been shown to be exposed and susceptible to parasitic infections (Ezenwa, 2003; Cross *et al*., 2009), because territorial defence 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 *et al*., 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 (Figure 4).

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 (Figure 5). Rhinoceros sub-adults are usually subjected to variable stressful conditions, such as reproductive maturity, courtship, territorial defense (Brett, 1988; Govender *et al*, 2011) or conquest, mating, and pregnancy, which may lead to immune suppression and trigger infection (Glaser *et al*., 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 supports 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 *et al*, 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 piroplasms in all the sampled rhinoceros meta-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 ($\chi^2 = 0.140$). 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 thisstudy does not support the above statement since although 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, trabsmission 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 piroplasms, 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 *et al*., 1967; Nijhof *et al*., 2003; Nijhof *et al*., 2005). This is because it is able to physiologically suppress host immune competence (Agarwal *et al*., 2001; Meuhlenbein, 2006), allowing proliferation of parasites in the host to a threshold that leads to clinical state which may become fatal (Nijhof *et al*., 2003).

It is probable that the high rates 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.

5.0 Conclusions

The findings of this study confirmed the occurrence of piroplasmsin both the Kenyan white (*Ceratotheruim simum simum*) and black (*Diceros bicornis michaeli*) rhinoceros of both sexes and all age groups in all the rhino conservation areas sampled. The sampled animals didn't show any clinical signs of piroplasmosis and therefore the rhino's co-existence with the parasites seems to represent an instance of endemically stable situation. The occurrence of piroplasmsin all the sub populations of rhinoceros in Kenya suggests that both species are susceptible. In addition, it suggests that all sub-populations are immunologically challenged against piroplasms. Further, it suggests that Kenyan rhinos are sub-clinically infected with proplasms, which makes them maintenance hosts. Lastly it suggests that all locations of rhinos harbor competent tick vectors of piroplasms.

6.0 Recommendations

Following the findings of this study I recommend that an epidemiological study to be carried out using larger and random sample size from each rhinoceros conservation area to characterize thepiroplasm species infecting Kenyan Rhinoceroses.Further study to be carried out on piroplasm tick vectors, and piroplasm-vector- host interaction so as to understand the transmission dynamics, and possible disease-transmission in the human-wildlife-livestock ecosystem.

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