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The occurrence of piroplasms in various South African black rhinoceros (*Diceros bicornis*) populations

by

David Edwin Zimmermann

Submitted in partial fulfillment of the requirements for the degree

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Dedication

To my lovely wife Marilyn, for enduring my absence during field trips, my ‘absence’ while working on my dissertation and for her continued love and support.

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Department: Veterinary Tropical Diseases
Degree: MSc (Veterinary Tropical diseases)

Abstract

Between November 2002 and October 2006, blood samples were collected from 46 black rhinoceroses (*Diceros bicornis*) originating from various national parks and game reserves within South Africa. The samples were divided into two groups, based on the black rhinoceros subspecies from which they originated: twenty-eight (n = 28) of the samples originated from subspecies *D. b. bicornis*, and eighteen (n = 18) from subspecies *D. b. minor*. DNA was extracted; the V4 hypervariable region of the 18S rRNA gene amplified and subjected to the Reverse Line Blot (RLB) hybridization assay. The RLB results demonstrated the presence of either *Theileria bicornis* or *Babesia bicornis* in 9 of the 46 samples examined. A further three PCR products failed to hybridize with any of the *Babesia* or *Theileria* species-specific probes, and only hybridized with the *Babesia*/*Theileria* genus-specific probe, suggesting the presence of a novel species or

variant of a species. Samples collected from black rhinoceroses originating from the more arid areas of South Africa, Tswalu Game Reserve and the old Vaalbos National Park, were found to be apparently free of *T. bicornis* and *B. bicornis* piroplasms. Based on these findings, it was concluded that *B. bicornis* and *T. bicornis* are relatively widespread in black rhinoceros populations in South Africa and pose a potential risk to the success of metapopulation management programs. Of the two black rhinoceros subspecies that occur in South Africa, *D. b. bicornis* is at greater risk due to their apparently *Babesia/Theileria*-naïve status in certain areas, when compared to the subspecies *D. b. minor*. Conservation managers need to carefully evaluate methods and procedures during the translocation of black rhinoceroses, especially when relocating from geographically and climatically diverse ecosystems and more so when dealing with the subspecies *D. b. bicornis*.

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1. Introduction

Black rhinoceroses (*Diceros bicornis*) are classified as critically endangered by the World Conservation Union (IUCN) Red List of Threatened Species and are therefore considered to be facing an extremely high risk of extinction in the wild (Friedmann & Daly 2004). This is mainly due to the illegal demand for rhinoceros horn but in addition diseases, such as babesiosis, have also been identified as poorly understood, ecological threats to black rhinoceros, which could cause local declines in black rhinoceros populations (Friedmann & Daly 2004).

The objectives of this project were to get a better understanding of the epidemiology of piroplasms, especially *Babesia bicornis* and *Theileria bicornis*, in black rhinoceros populations within South Africa by:

1. Determining the prevalence of *B. bicornis* and *T. bicornis* in blood samples collected from various black rhinoceros populations and,
2. Identifying potential vectors of the pathogens by determining the occurrence of tick species on black rhinoceroses.

The long-term managemental aim of this project was to get a better understanding of the potential disease risks involved during the translocation of black rhinoceroses and to help mitigate and anticipate the risk of piroplasm-associated disease during stressful and unfavourable conditions such as translocations and droughts.

2. Literature Review

South African National Parks (SANParks) endorses the basic conservation objectives of the IUCN (Eidsvik 1990) and black rhinoceros conservation has become a sub-goal within this framework.

The idea of metapopulation management was first advocated by Levins (1969), who described a metapopulation as "a population of populations". A metapopulation is linked with the processes of population turnover, extinction and colonization. Although in the same vein as island-biogeographic theory, there is no 'mainland' from which islands are colonized but all the populations are considered as islands interacting with each other, all with a probability of extinction and recolonization (Gilpin & Hanski 1991). The idea of a metapopulation approach to managing biological resources is viewed as a means of tackling the conservation of fragmented populations such as the black rhinoceros. Many isolated black rhinoceros populations are of such a size that they theoretically offer little long-term sustainability. By transferring individuals between such populations, the smaller populations maintain an active role in the single larger metapopulation. Managing black rhinoceros in such a way has associated risks, however, as about 20-30% of translocated animals die during the capture and post-release phase (Adcock 1995). It is therefore imperative that these risks should be reduced as far as possible. Diseases, such as babesiosis, have been identified as poorly understood, ecological threats that could cause local declines in black rhinoceros populations (Friedmann & Daly 2004).

Both *Babesia* and *Theileria* are members of the suborder Piroplasmorina. Babesiosis is caused by infection with species of tick-borne, intra-erythrocytic and generally host-specific protozoan parasites of the genus *Babesia* (de Vos, Potgieter, de Waal & van Heerden 1994). While *Babesia* are primarily parasites of erythrocytes, *Theileria* utilize, successively, leukocytes and erythrocytes for completion of their life cycle in mammalian host.

The presence of piroplasms has long been recognized in both black and white (*Ceratotherium simum*) rhinoceroses. Brocklesby and Vidler (1965) mentioned a *Babesia* species present in very low numbers in a black rhinoceros captured during a severe drought in Tsavo National Park, Kenya. Brocklesby (1967) recalled seeing both large and small piroplasms in blood smears from sick rhinoceroses in Kenya, but was not convinced that the organisms were the cause of illness, maintaining that "piroplasms are often seen in blood smears of wild animals, but unless they are present in large numbers it is dangerous to assume that they are pathogenic".

Two different species of piroplasms have been reported in white rhinoceroses in Zululand, South Africa (Bigalke, Keep, Keep & Schoeman 1970). The parasites were observed in blood smears taken during routine immobilizations. Morphologically they were of two distinct types – a large *Babesia* sp. which was very rare (seen in two out of 106 animals) and a more common smaller piroplasm (seen in 34 out of 106 animals) that was thought to be a *Theileria* sp. due to the presence of rod-shaped forms.

Wildlife has long been known to act as carriers of *Babesia* species, which under stressful conditions, such as translocations or drought, can become pathogenic to the host (Penzhorn 2006). Mortality due to babesiosis may be triggered by stress factors. McCulloch and Achard (1969) reported on mortalities associated with capture and translocation of black rhinoceroses in and around the Grumeti and Lamai Game Control Areas in Tanzania. Two deaths out of 77 rhinoceroses were attributed to babesiosis (3.4%) – large forms of *Babesia* were seen on blood smears.

Nijhof, Penzhorn, Lynen, Mollel, Morkel, Bekker & Jongejan (2003) reported on a further four black rhinoceros that had died as a result of babesiosis: in 1995 a young male rhinoceros (Benji) at Addo Elephant National Park, South Africa; in 1996 a 30-year-old pregnant animal at Hluhluwe-iMfolozi Park, South Africa; and in 2001 two females in Ngorongoro Conservation Area in Tanzania. In all four cases the animals died soon after capture or during periods of environmental stress. Blood and tissue samples from each of these animals were subjected to polymerase chain reaction (PCR) and the Reverse Line

Blot (RLB) hybridization assay and two new species, named *Babesia bicornis* and *Theileria bicornis*, were identified. A number of black rhinoceros deaths at Addo Elephant National Park after a very dry period during the 1990s were also thought to be primarily due to babesiosis. After the death of the two female rhinoceroses in the Ngorongoro Conservation Area, prophylactic treatment of the other rhinoceroses was undertaken by means of darting, using diminazine aceturate, to apparent good effect (Fyumagwa, Mkumbo & Morkel 2004).

Blood samples collected from black rhinoceroses from Etosha National Park and Damaraland, Namibia, were subjected to PCR using *Theileria* and *Babesia* genus-specific primers and the RLB hybridization assay, with negative results (Penzhorn, Oosthuizen, Bosman, Kilian & Horak 2008). Black rhinoceroses translocated from these areas to *B. bicornis*-endemic areas, could potentially be at risk of infection, and clinical signs may ensue. In that study, all the rhinoceroses were found to be infested with *Hyalomma truncatum* ticks, while *Hyalomma marginatum rufipes* was less abundant. A single *Rhipicephalus longipes* was recovered from one rhinoceros in Damaraland.

The vectors of *B. bicornis* and *T. bicornis* are still unknown, although *Amblyomma rhinocerotis* and *Dermacentor rhinocerinus*, both ixodid ticks, are known to feed specifically on black and white rhinoceroses (Knapp, Krecek, Horak & Penzhorn 1997). The majority of tick species found on rhinoceroses are not host-specific but are found on a variety of other vertebrates as well (Penzhorn, Krecek, Horak, Verster, Walker, Boomker, Knapp & Quandt 1994). Forty tick species have been recovered from black and white rhinoceroses. Three species, *Amblyomma rhinocerotis*, *Dermacentor rhinocerotis* and *Amblyomma personatum*, are primary rhinoceros parasites (Penzhorn *et al*. 1994). Common sites of tick attachment are skin folds in the perineal region, in and around the ears and around the eyes.

Detecting piroplasms in wild animals can be difficult. Traditionally, piroplasms were identified by microscopic examination of Giemsa-stained blood smears or serodiagnostic techniques, such as the immunofluorescent antibody (IFA) test (Gubbels, de Vos, van der

Weide, Viseras, Schouls, de Vries & Jongejan 1999). Advances in molecular biology have led to the development of more sensitive and specific diagnostic tests based on the detection and discrimination of parasite nucleic acid (DNA or RNA) sequences, and have decreased the subjectivity that usually occurs in interpreting results. The RLB hybridization assay, which was developed for the simultaneous detection and identification of tick-borne parasites infecting cattle and small ruminants (Gubbels *et al*., 1999; Schnittger, Yin, Gubbels, Beyer, Niemann, Jongejan & Ahmed 2003**)**, has successfully been used to discover previously undescribed *Theileria* and *Babesia* species infecting African wildlife species (Nijhof *et al*., 2003; Nijhof, Pillay, Steyl, Prozesky, Stoltsz, Lawrence, Penzhorn & Jongejan 2005; Oosthuizen, Zweygarth, Collins, Troskie & Penzhorn 2008).

The aim of this study was to get a better understanding of the epidemiology of piroplasms, especially *Babesia bicornis* and *Theileria bicornis*, in black rhinoceros populations within South Africa, with the long-term managemental aim of getting a better understanding of the potential disease risks involved during the translocation of black rhinoceroses and to help mitigate and anticipate the risk of piroplasm-associated disease during stressful and unfavourable conditions such as translocations and droughts.

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3. Materials and Methods

3. Materials and Methods

Study animals

Blood specimens were collected from black rhinoceroses from various localities in South Africa over a period of five years, from 2002 to 2006. The rhinoceroses comprised two ecotypes or subspecies: the more common southern-central black rhinoceros, *Diceros bicornis minor*, and the arid or south-western black rhinoceros, *Diceros bicornis bicornis*. In total forty-six (n = 46) blood samples were examined – 18 southern-central black rhinoceroses from Kruger National Park (Mpumalanga Province), Marakele National Park (Limpopo Province), Pilanesberg National Park (North West Province) and Double Drift Game Reserve (Great Fish River Reserve Complex, Eastern Cape) and 28 south-western black rhinoceroses from Vaalbos National Park (Northern Cape), Addo Elephant National Park (Eastern Cape), Mountain Zebra National Park (Eastern Cape) and Tswalu Game Reserve (Northern Cape) (Figure 1).

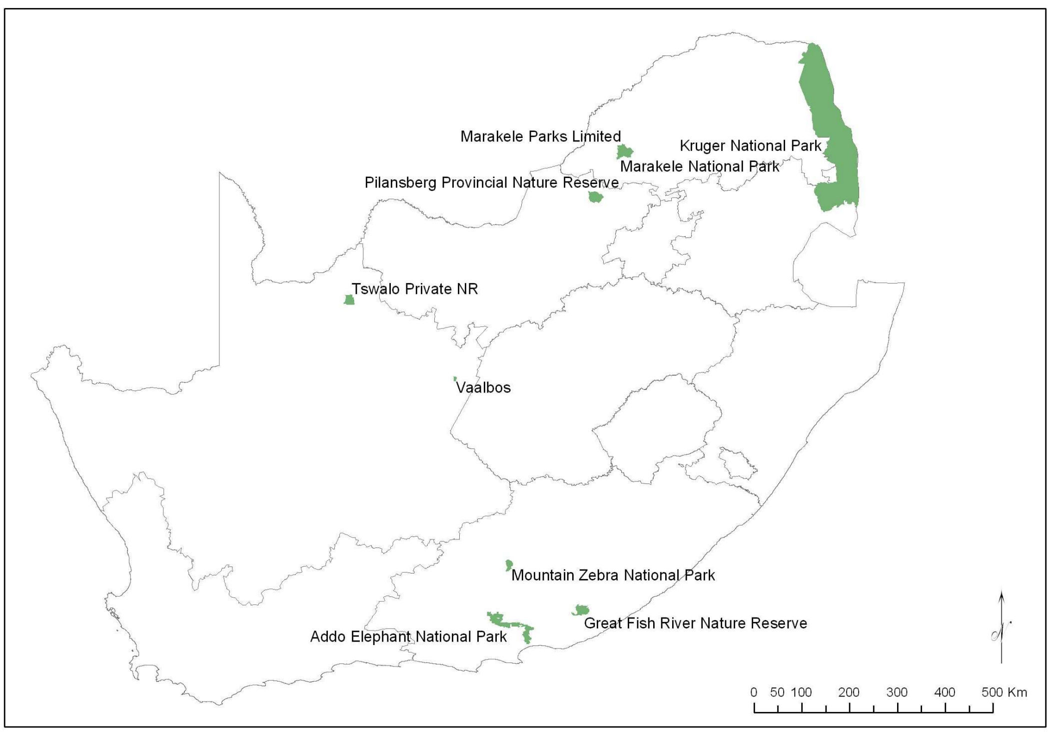

FIGURE 1. Map showing the various localities of the National Parks and Game Reserves, in South Africa, from which the blood specimens were collected from black rhinoceroses.

Black rhinoceroses were selectively immobilized for metapopulation management, according to the SANParks's standard operating procedure for immobilization, transport and holding of wild animals. A combination of etorphine hydrochloride (M99, Novartis), azaperone (Stresnil, Janssen-Cilag) and hyaluronidase (Hyalase, Kyron Laboratories) was used to immobilize the animals.

Sample collection and handling

Samples were collected routinely from immobilized black rhinoceroses. Blood samples were collected by venipuncture from the auricular vein into ethylenediamine tetraacetic acid (EDTA) tubes, and labeled with the animals' specific details and date of capture. All visible ticks were collected from seven rhinoceroses and preserved in separate vials containing 70% ethanol for identification. The blood samples were stored on ice in the field and kept refrigerated on transportation to the laboratory. Once at the laboratory, the EDTA samples were placed on a roller for approximately five minutes after which they were pipetted out into a 3.7 ml NUNC tube and stored frozen as EDTA-buffered whole blood in a -10°C freezer.

DNA extraction

DNA was extracted from 200 µl of EDTA-anticoagulated blood using a DNA extraction kit (DNeasy Tissue kit, QIAGEN, Southern Cross Biotechnologies, SA), following the manufacturer's protocol. In short, stored blood was thawed and mixed at room temperature. Twenty µl proteinase K (20 mg/ml) and 180 µl of AL lysis buffer was added to 200 µl of blood, and mixed thoroughly. It was incubated at 56ºC for 10 minutes whereafter 200 µl 100% ethanol was added before transferring the mixture into the QIAamp mini spin column. It was centrifuged at full speed for 1 minute and the flow through discarded. The column was placed back in a 2 ml collection tube and 500 µl AW1 wash buffer was added before centrifuging for 1 minute at 8000 rpm. After discarding the flow through, the washing step was repeated by adding 500 µl AW2 wash buffer. The column was placed in a new 2 ml collection tube and centrifuged for 1 minute at full speed to remove the residual AW buffer. The column was transferred to a 1.5 ml tube and 100 µl elution buffer AE added directly onto the DNeasy membrane. The column was incubated at room temperature for 1 minute and centrifuged for 1 minute at 8000 rpm to elute the DNA. Additional centrifugation was then carried out at full speed for 1 minute to optimize DNA recovery. DNA samples stored at -20 ºC or 4°C for further analysis.

Polymerase chain reaction (PCR)

PCR was conducted as described by Nijhof *et al.* (2003). The V4 hypervariable area of the 18S rRNA gene was amplified using the *Theileria* and *Babesia* genus-specific forward primer RLB F2 [5'-GAC ACA GGG AGG TAG TGA CAA G-3'] and the reverse-biotin-labeled primer RLB R2 [5'-Biotin-CTA AGA ATT TCA CCT CTG ACA GT-3']. The primers used were obtained from Inqaba Biotec (South Africa). The PCR reaction mixture consisted of 12.5 µl of Platinum Quantitative PCR Supermix-UDG; 20 pmol (0.25 µl) of both the forward and reverse primers; 9.5 µl of molecular grade water; and 2.5 µl of the extracted DNA template. The total volume of 25 µl was mixed in each PCR tube by pipetting up and down. The tubes were put directly into a preheated and programmed thermocycler (programmed for *Babesia/Theileria* touchdown PCR). The PCR program was 3 min at 37^{o}C; 10 min at 94^{o}C; and 10 cycles of 94^{o}C for 20 sec (denaturation step), 67^{o}C for 30 sec (annealing step), and 72^{o}C for 30 sec (extension step), with lowering of the temperature of annealing step by 2^{o}C after every second cycle (touchdown). This was followed by 40 cycles of 94^{o}C for 20 sec, 57^{o}C for 30 sec, and 72^{o}C for 30 sec. The PCR was completed with a final extension cycle of 7 minutes at 72^{o}C, and left at 4^{o}C (See Table 1.)

TABLE 1. Thermocycler programme

Number of cycles	Time	Temperature	Purpose
1 cycle	3 min	37 °C	Activate UDG
1 cycle	10 min	94 °C	Inactivate UDG & activate *Taq* polymerase
2 cycles	20 sec	94 °C	Denaturing of template DNA
	30 sec	67 °C	Annealing of primers
	30 sec	72 °C	Extending of PCR products by *Taq* polymerase
2 cycles	20 sec	94 °C	Denaturing of template DNA
	30 sec	65 °C	Annealing of primers
	30 sec	72 °C	Extending of PCR products by *Taq* polymerase
2 cycles	20 sec	94 °C	Denaturing of template DNA
	30 sec	63 °C	Annealing of primers
	30 sec	72 °C	Extending of PCR products by *Taq* polymerase
2 cycles	20 sec	94 °C	Denaturing of template DNA
	30 sec	61 °C	Annealing of primers
	30 sec	72 °C	Extending of PCR products by *Taq* polymerase
2 cycles	20 sec	94 °C	Denaturing of template DNA
	30 sec	59 °C	Annealing of primers
	30 sec	72 °C	Extending of PCR products by *Taq* polymerase
40 cycles	20 sec	94 °C	Denaturing of template DNA
	30 sec	57 °C	Annealing of primers
	30 sec	72 °C	Extending of PCR products by *Taq* polymerase
1 cycle	7 min	72 °C	Final extension
		4 °C	Hold

Reverse Line Blot (RLB) hybridization assay

The PCR products were analyzed using the RLB hybridization technique, first described by Gubbels *et al.* (1999).

Preparation of membrane: A Biodyne C blotting membrane (Pall Biosupport, Ann Arbor, Mich.) was activated by a 10-minute incubation in 10 ml freshly prepared 16% (wt/vol) 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDAC) (Sigma, St Louis, Mo.) at room temperature. The membrane was washed for 2 minutes with demineralized water and placed in a MN45 miniblotter (Immunetics, Cambridge, Mass.) Specific oligonucleotides (Table 2) [containing a N-terminal N-(trifluoracetamidehexyl-cyanoethyl,N,N-diisopropyl phosphoramidite [TFA]) – C6 amino linker (Isogen)] were diluted to 150 µl and were subsequently covalently linked to the membrane with the amino linker by filling the miniblotter slots with the oligonucleotide dilutions; they were then incubated for 2-3 minutes at room temperature. The oligonucleotide solutions were aspirated, and the membrane was inactivated by incubation in 100 ml of a 100 mM NaOH solution for 8 minutes at room temperature. The membrane was washed in 100 ml 2X SSPE/0.1% sodium dodecyl sulfate (SDS) for 5 minutes at 60^{o}C. A *Theileria* and *Babesia* genus-specific probe, as well as the *B. bicornis* and *T. bicornis* species-specific probes, were included on the membrane.

TABLE 2. List of genus and species-specific probes used during the Reverse Line Blot (RLB) hybridization assay.

Probe Number	Probe Identification	Probe Sequence * (from 5' – 3')
1	*Theileria/Babesia* catch-all	TAA TGG TTA ATA GGA RCR GTT G
2	*B. felis*	TTA TGC GTT TTC CGA CTG GC
3	*B. divergens*	ACT RAT ATC GAG ATT GCA C
4	*B. microti*	GRC TTG GCA TCW TCT GGA
5	*B. bigemina*	CGT TTT TTC CCT TTT GTT GG
6	*B. bovis*	CAG GTT TCG CCT GTA TAA TTG AG
7	*B. rossi*	CGG TTT GTT GCC TTT GTG
8	*B. canis canis*	TGC GTT GAC GGT TTG AC
9	*B. canis vogeli*	AGC GTG TTC GAG TTT GCC
10	*B. major*	TCC GAC TTT GGT TGG TGT
11	*B. bicornis*	TTG GTA AAT CGC CTT GGT C
12	*B. caballi*	GTT GCG TTK TTC TTG CTT TT
13	*Theileria* sp. (kudu)	CTG CAT TGT TTC TTT CCT TTG
14	*Theileria* sp. (sable)	GCT GCA TTG CCT TTT CTC C
15	*T. bicornis*	GCG TTG TGG CTT TTT TCT G
16	*T. annulata*	CCT CTG GGG TCT GTG CA
17	*T. buffeli*	GGC TTA TTT CGG WTT GAT TTT
18	*Theileria* sp. (buffalo)	CAG ACG GAG TTT ACT TTG T
19	*T. mutans*	CTT GCG TCT CCG AAT GTT
20	*T. parva*	GGA CGG AGT TCG CTT TG
21	*T. taurotragi*	TCT TGG CAC GTG GCT TTT
22	*T. velifera*	CCT ATT CTC CTT TAC GAG T
23	*T. equi*	TTC GTT GAC TGC GYT TGG
24	*T. lestoquardi*	CTT GTG TCC CTC CGG G

* Symbols used to indicate degenerate positions: R = A/G W = A/T Y = C/T

RLB hybridization: Prior to use the membrane was washed for 5 minutes at room temperature with approximately 10 ml 2X SSPE/0.1%SDS and placed in the miniblotter with the slots perpendicular to the previously applied specific oligonucleotides. Diluted PCR products (10 ul PCR product diluted with 150 ul 2X SSPE/0.1% SDS) was heated to 96^{o}C in a thermalcycler and then immediately cooled on ice. The denatured PCR samples were then applied into the slots and incubated for 60 minutes at 42^{o}C. The samples were removed by aspiration and the membrane removed from the blotter and washed twice in preheated 2X SSPE/0.5%SDS for 10 minutes at 50^{o}C in a water bath under gentle shaking. Subsequently the membrane was incubated with 10 ml 2X SSPE/0.5% SDS + 2.5 ul streptavidin-POD (peroxidase labeled) conjugate (1.25 U) for 30 minutes at 42^{o}C. The membrane was washed twice in 125 ml preheated 2X SSPE/0.5% SDS for 10 minutes at 42°C in a water bath under gentle shaking. The membrane was then rinsed twice with 125 ml 2X SSPE for 5 minutes at room temperature under gentle shaking. The membrane was transferred to an overhead sheet and incubated for 1 minute in 10 ml of ECL (5 ml ECL1 + 5 ml ECL2) detection fluid (Amersham, Little Chalfont, Buckinghamshire, United Kingdom) at room temperature. The membrane was placed between 2 (clean) overhead sheets, placed in an exposure cassette and exposed to X-ray film for 5-20 minutes. The ECL reaction reached a peak after 20 minutes. The X-ray film was then developed for detection of hybridized PCR products which were visualized by chemiluminescence.

After use, all PCR products were stripped from the membrane by two washes in 1% SDS for 30 minutes each time at 100^{o}C. The membrane was rinsed in 20 mM EDTA (pH 8.0) for 15 minutes at room temperature under gentle shaking and stored in fresh EDTA solution at 4^{o}C in a sealed container for reuse.

For each DNA extract, the PCR and RLB were repeated twice to increase the sensitivity; the results of the RLB were combined and recorded.

Tick identification

Ticks were identified to species level using a stereomicroscope and a key (Walker, Bouattour, Camicas, Estrada-Pena, Horak, Latif, Pegram & Preston, 2003). Specimens identified were compared to species descriptions in Hoogstraal (1956) and Walker *et al*. (2003).

4. Results

PCR and RLB results:

Between November 2002 and October 2006, blood samples were collected from 46 black rhinoceroses originating from various national parks and game reserves within South Africa. The samples were divided into two groups based on the black rhinoceros subspecies from which they originated: twenty-eight (n = 28) of the samples originated from subspecies *D. b. bicornis,* and eighteen (n = 18) from subspecies *D. b. minor*. DNA was extracted from all blood samples collected, the V4 hypervariable region of the 18S rRNA gene were amplified using *Theileria* and *Babesia* genus specific primers and subjected to the RLB hybridization assay. In total 12 of the 46 samples subjected to PCR amplification and RLB hybridization tested positive for the presence of piroplasms – six from the *D. b. bicornis* group and six from the *D. b. minor* group. The combined RLB results are summarized in tables 3 and 4, respectively. The RLB results demonstrated the presence of either *Theileria bicornis* or *Babesia bicornis* in 9 of the 46 samples examined. A further three PCR products failed to hybridize with any of the *Babesia* or *Theileria* species-specific probes, and only hybridized with the *Babesia*/*Theileria* genus-specific probe, suggesting the presence of a novel species or variant of a species. Samples collected from black rhinoceroses originating from the more arid areas of South Africa, Tswalu Game Reserve and the old Vaalbos National Park, were found to be apparently free of *T. bicornis* and *B. bicornis* piroplasms.

Four populations of *D. b. bicornis* were examined (Table 3); RLB results can be summarized as follows:

- Addo Elephant National Park – *B. bicornis* was detected in 2 of the 12 blood samples examined and *T. bicornis/ T. equi* was detected in one of the samples.
- Vaalbos National Park – a positive *Theileria/Babesia* catch-all signal, without a species-specific signal, was detected in 2 of the 11 blood samples examined, thus suggesting the presence of a possible novel species or variant of a species.
- Mountain Zebra National Park (MZNP) – *B. bicornis* was detected in 1 of the 2 samples examined. This bull had recently been moved to MZNP from Addo

Elephant National Park and could thus be grouped as part of the Addo Elephant National Park population.

- Tswalu Game Reserve – with all three blood samples examined, RLB results indicated that no PCR products could be amplified and therefore no hybridization with any of the probes present on the blot could be seen.

Four *D. b. minor* populations were examined (Table 4); RLB results can be summarized as follows:

- Marakele National Park – *T. bicornis* was detected in 3 of the 11 blood samples examined and *B. bicornis* in one of the samples. One of the samples that tested positive for *T. bicornis* (Alicia) also had a faint positive signal for *T. equi* on one of the blots.
- Kruger National Park – *T. bicornis* was detected in 1 of the 3 blood samples examined.
- Pilanesberg National Park – a positive *Theileria/Babesia* genus-specific signal, without a species-specific signal, was detected in 1 of the 3 blood samples examined, thus suggesting the presence of a possible novel species or variant of a species.
- Great Fish River Reserve Complex (GFRRC) – one animal was examined with negative results.

Figure 2 shows the results of the RLB which tested positive for *T. bicornis*, *B. bicornis* or a *Theileria/Babesia* genus-specific signal, as a percentage of the total blood samples collected from the various localities.

TABLE 3. Combined results of blood samples from 28 desert black rhinoceroses (*Diceros bicornis bicornis*), examined for piroplasms (*Babesia* and *Theileria* spp.), using the Reverse Line Blot (RLB) hybridization assay.

Sample Number	Rhinoceros identification	Locality when sample collected*	Origin of Rhino*	Date collected	Combined RLB result
1	S/adult male	Vaalbos NP	Vaalbos NP	19/02/2005	Negative**
2	S/adult female	Vaalbos NP	Vaalbos NP	14/05/2005	Negative
3	S/adult male	Vaalbos NP	Vaalbos NP	30/05/2005	*Theileria*/*Babesia* genus-specific probe positive
4	Leanne	Vaalbos NP	Vaalbos NP	13/09/2006	*Theileria*/*Babesia* genus-specific probe positive
5	Tiffany	Vaalbos NP	Vaalbos NP	13/09/2006	Negative
6	Ubejane	Vaalbos NP	Vaalbos NP	14/09/2006	Negative
7	Tshkudu	Vaalbos NP	Vaalbos NP	14/09/2006	Negative
8	Rathie	Vaalbos NP	Vaalbos NP	14/06/2006	Negative
9	Nkombe	Vaalbos NP	Namibia	15/09/2006	Negative
10	Jabula	Vaalbos NP	Vaalbos NP	19/09/2006	Negative
11	Bwana	Vaalbos NP	Tswalu GR	06/10/2006	Negative
12	Adult male 1	Tswalu GR	Tswalu GR	25/05/2005	Negative
13	S/adult male 2	Tswalu GR	Tswalu GR	25/05/2005	Negative
14	S/adult male 3	Tswalu GR	Tswalu GR	25/05/2005	Negative
15	Muleka	MZNP	Addo Elephant NP	25/11/2002	*Theileria*/*Babesia* genus-specific probe positive *B. bicornis*
16	Alfie	MZNP	Addo Elephant NP	25/11/2002	Negative
17	Buffelskuil male	Addo Elephant NP	Addo Elephant NP	11/02/2003	Negative
18	03/173	Addo Elephant NP	Addo Elephant NP	08/04/2003	*Theileria*/*Babesia* genus-specific probe positive *B. bicornis*

19	03/176	Addo Elephant NP	Addo Elephant NP	12/04/2003	Negative
20	03/177	Addo Elephant NP	Addo Elephant NP	13/04/2003	*Theileria*/*Babesia* genus-specific probe positive *T. bicornis* *T. equi* (faint)
21	Kleinalec	Addo Elephant NP	Addo Elephant NP	08/10/2003	*Theileria*/*Babesia* genus-specific probe positive *B. bicornis*
22	Darling	Addo Elephant NP	Addo Elephant NP	08/10/2003	Negative
23	Nabab	Addo Elephant NP	Addo Elephant NP	09/10/2003	Negative
24	HM	Addo Elephant NP	Namibia	17/05/2004	Negative
25	Kenges calf	Addo Elephant NP	Addo Elephant NP	18/05/2004	Negative
26	Tswaros calf	Addo Elephant NP	Addo Elephant NP	18/05/2004	Negative
27	Chipenbere	Addo Elephant NP	Addo Elephant NP	24/05/2005	Negative
28	Gamka	Addo Elephant NP	Addo Elephant NP	05/04/2006	Negative

[*] **:** Vaalbos NP = Vaalbos National Park; Tswalu GR = Tswalu Game Reserve; MZNP = Mountain Zebra National Park; Addo Elephant NP = Addo Elephant National Park.

[**] **:** Negative results were either true negatives or below detection limits of the test.

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TABLE 4. Combined results of blood samples from 18 southern black rhinoceroses (*Diceros bicornis minor*), examined for piroplasms (*Babesia* and *Theileria* spp.), using the Reverse Line Blot (RLB) hybridization assay.

Sample Number	Rhinoceros identification	Locality when sample collected*	Origin of the Rhino*	Date collected	Pooled RLB results
1	Akura	Marakele NP	Frankfurt Zoo	28/11/2002	*T. bicornis*
2	Tim	Marakele NP	Frankfurt Zoo	28/11/2002	Negative**
3	Kates calf	Marakele NP	Marakele NP	15/04/2003	*Theileria*/*Babesia* genus-specific probe positive *B. bicornis*
4	Alicia	Marakele NP	Marakele NP	15/09/2003	*Theileria*/*Babesia* genus-specific probe positive *T. bicornis* *T equi*
5	S/adult male 1	Marakele NP	Marakele NP	16/09/2003	Negative
6	Adult male 2	Marakele NP	Marakele NP	16/09/2003	*T. bicornis*
7	Adult male 3	Marakele NP	Marakele NP	18/09/2003	Negative
8	Male calf	Marakele NP	Marakele NP	07/07/2004	Negative
9	Adult female	Marakele NP	Marakele NP	07/07/2004	Negative
10	S/adult female	Marakele NP	Marakele NP	07/07/2004	Negative
11	S/adult male	Marakele NP	Marakele NP	06/05/2006	Negative
12	06/90	Kruger NP	Kruger NP	24/04/2006	Negative
13	06/96	Kruger NP	Kruger NP	24/04/2006	*T. bicornis*
14	Danny	Kruger NP	Kruger NP	??	Negative
15	Adult female 1	Pilanesberg NP	Pilanesberg NP	26/11/2002	Negative
16	Adult male 2	Pilanesberg NP	Pilanesberg NP	26/11/2002	Negative
17	Adult female 3	Pilanesberg NP	Pilanesberg NP	26/11/2002	*Theileria*/*Babesia* genus-specific probe positive

18	5yr male	GFRRC	GFRRC	12/05/2006	Negative

*****: Marakele NP = Marakele National Park; Kruger NP = Kruger National Park; Pilanesberg NP = Pilanesberg National Park; GFRRC = Great Fish River Reserve Complex.

******: Negative results were either true negatives or below detection limits of the test.

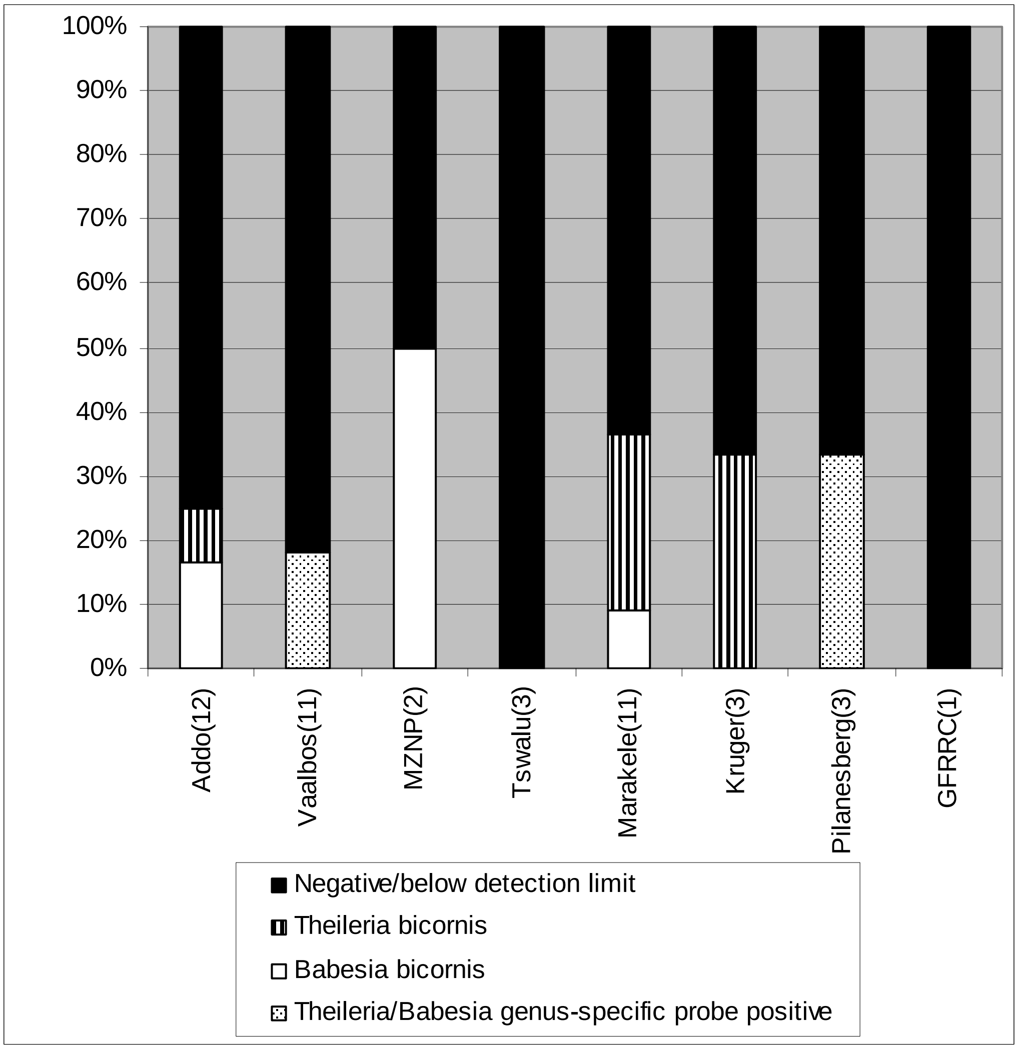

FIGURE 2. Bar-chart showing the percentage of blood samples, from the various localities, which tested positive for the presence of piroplasms.

Tick Identification:

Only seven of the black rhinos, from which blood samples were collected, were examined for ticks. All seven of the rhinoceros originated from Vaalbos National Park in the Northern Cape. Two species of ticks, *Hyalomma truncatum* and *Hyalomma marginatum rufipes*, were identified on the rhinoceroses (Table 5).

TABLE 5. Ticks collected and identified on black rhinoceroses originating from Vaalbos National Park, Northern Cape, South Africa.

Date	Locality	Rhinoceros Identification	Tick Species	Male	Female
13/09/2006	Vaalbos NP	S/Adult female (Tiffany)	*Hyalomma truncatum*	2	4
			Hyalomma marginatum rufipes	7	0
13/09/2006	Vaalbos NP	S/Adult female (Leanne)	*Hyalomma truncatum*	0	0
			Hyalomma marginatum rufipes	5	2
14/09/2006	Vaalbos NP	Juvenile (Tshkudu)	*Hyalomma truncatum*	5	0
			Hyalomma marginatum rufipes	4	2
14/09/2006	Vaalbos NP	S/Adult female (Rathie)	*Hyalomma truncatum*	6	0
			Hyalomma marginatum rufipes	2	1
14/09/2006	Vaalbos NP	Adult female (Ubejane)	*Hyalomma truncatum*	0	0
			Hyalomma marginatum rufipes	2	2
19/09/2006	Vaalbos NP	Adult female (Umkhombe)	*Hyalomma truncatum*	1	2
			Hyalomma marginatum rufipes	1	1
19/09/2006	Vaalbos NP	Young Male (Jabula)	*Hyalomma truncatum*	2	5
			Hyalomma marginatum rufipes	1	0

5. Discussion

Metapopulation Management:

Black rhinoceroses were historically found throughout sub-Saharan Africa, with the exception of the Congo Basin (Kingdon, 1979). Poaching, in combination with man-made alteration of the landscape leading to the fragmentation and isolation of a once continuous wildlife ecosystem, has resulted in black rhinoceroses existing in only a few isolated pockets within their former range (Friedmann & Daly, 2004). The majority of these populations are in formal conservation areas, although some are also held on private land. The more common ecotype or subspecies, *D. b. minor*, once ranged southwards from central Tanzania to eastern South Africa (Kingdon, 1979). *Diceros b. bicornis* was distributed from southern Angola to the western and southeastern Cape. At the African Rhino Workshop, Cincinnati, October 1986, it was recommended that both *in situ* and captive-management programs should attempt to maintain the genetic integrity of the various ecotypes, i.e. they should not be allowed to interbreed, unless future genetic and other studies indicate that this separation is unjustifiable (Brooks, 1989). In order to keep isolated and small populations genetically viable, metapopulation management is required to avoid further loss of genetic variability as a result of inbreeding.

Metapopulation management also involves the translocation of rhinoceroses from areas of high density to low-density areas or areas that are suitable for the establishment of new populations. This is to attain sufficiently large populations to ensure the survival of the species. It is therefore a dynamic process that integrates the management of various subpopulations, population genetic management and habitat management, with the ultimate aim of reintroducing black rhinoceroses to areas where they have been extirpated. These translocation policies are considered from the perspective of maximizing the growth rate of the populations and establishing new populations (Hearne & Swart 1991). Appropriate translocations can be beneficial but can also lead to problems such as the transfer of pathogens. Ideally the IUCN guidelines (IUCN, 1987) should be followed.

Disease Risk Assessment:

The spread of diseases to new environments, via translocations, may have important effects on wildlife, agriculture or public health, and may affect the success of the translocation effort itself (Leighton, 2002). Any animal translocation contains two main types of risks or health concerns: (a) the introduction of the disease or infectious agent by the translocated animals to the destination ecosystem may result in ecological or economic harm, and/or (b) health hazards present at the destination ecosystem may adversely affect the translocated animals.

Ideally animals should never be translocated without a thorough understanding of the potential disease agents present at both the site of origin and destination. In all too many cases there is much we do not know about the range of diseases affecting or potentially affecting the species of interest, particularly in the wild.

The process of health risk assessment of wild animal translocations should evaluate the probability that the possible health hazard will occur and the magnitude of negative consequences if it does occur. It is the combination of probability of occurrence and magnitude of consequences. Emphasis should be placed on diseases that fulfill the following three criteria (Leighton, 2002):

1. May be carried by animals to be translocated from the source ecosystem to the destination ecosystem.
2. May infect or cause disease in one or more wild or domestic animal species or in humans in the destination ecosystem.
3. May have significant ecological or economic consequences if introduced into the destination ecosystem.

Wild animals are known to be carriers of piroplasms and in the presence of a suitable vector are able to infect or cause disease in other animals in the destination ecosystem. Both *Babesia* and *Theileria* species have been identified in black rhino, namely *B. bicornis* and *T. bicornis*. At this stage there is no evidence to suggest that *T. bicornis* is pathogenic, but *B. bicornis* has proven to result in mortalities in black rhinoceroses

(Nijhof *et al.* 2003). Being a highly endangered species, the death of one black rhinoceros can have serious negative impacts on a metapopulation management program. For this reason it is important for conservation managers to have a better understanding of the parasites and the potential negative ecological consequences if introduced into an ecosystem or if a *Babesia*-naïve animal is introduced into an infected ecosystem at destination.

***Diceros bicornis bicornis* ecotype metapopulation management in South Africa:**
The repatriation of *D. b. bicornis* populations into two arid South African national parks, Augrabies National Park in 1985 and Vaalbos National Park in 1987, was the initial phase of a plan by the SANParks to repatriate this ecotype to its former range in South Africa (Hall-Martin, 1986). At the time there was the possibility of increased poaching pressure on the Namibian populations and as a result there was a need to establish other secure populations outside Namibia. Given the relatively small size of the parks into which the rhinoceroses were to be repatriated, it was decided from the outset that a metapopulation approach to managing the *D. b. bicornis* population would be implemented. This would also help reduce the chance of the entire population succumbing to some stochastic event such as drought, the probability of which was considered rather high given that most of the parks were in arid areas and therefore prone to such events. The initial founding population of 12 animals (plus a further three added later) was brought into South Africa from Etosha National Park, Namibia.

The habitat in the Augrabies and Vaalbos National Parks is very similar to the habitat from which the rhinoceroses originated. For various reasons both these founder populations have since been relocated to new areas within the National Parks system, with subpopulations having since been established in Karoo National Park, Mountain Zebra National Park, Addo Elephant National Park and the newly proclaimed Mokala National Park (Northern Cape). Black rhinoceroses were also privately imported, from Namibia, to Tswalu Game Reserve in the Northern Cape in 1995. By 2006 the *D. b. bicornis* population in South Africa had increased substantially to just over 100 individuals.

The environment within these parks varies from a very dry, semi-desert/karoo habitat in the Northern Cape to moister, thicket and fynbos habitats in the southern areas of the Eastern and Western Cape. The exposure to various diseases and potential vectors, such as ticks, also varies according to the environment and habitat type.

At the time of the initial introductions to South Africa, piroplasms had not been recognized as potential disease hazards for black rhinoceroses and even if they were, PCR techniques were not available to identify subclinical carriers of the parasites. Recent research suggests that black rhinoceroses originating from Namibia are apparently not infected with piroplasms (Penzhorn *et al*., 2008) and therefore did not pose a risk to the destination ecosystems. This would also suggest that any rhinoceroses that have tested positive for piroplasms would have been exposed to a tick vector, and had become infected, since their reintroduction to South Africa from Namibia.

In this study, blood samples from black rhinoceros populations in the Northern Cape, namely Vaalbos National Park and Tswalu Game Reserve, tested negative for both *T. bicornis* and *B. bicornis*. This would suggest these populations have still not been exposed to these piroplasms and/or the tick vector does not occur in the area. The tick species, *Hyalomma truncatum* and *Hyalomma marginatum rufipes*, identified on the black rhinoceroses in Nambia (Penzhorn *et al.*, 2008), were also found, in this study, on the rhinoceroses in Vaalbos National Park. This would suggest that these ticks are not vectors of the piroplasms, but one has to be careful of making such assumptions as the ticks may not have been exposed to diseased or carrier animals, and could potentially still be vectors of the piroplasms. Two of the samples originating from Vaalbos National Park gave a positive *Theileria/Babesia* genus-specific signal, without a species-specific signal, suggesting a possible novel species or variant of a species in the area. Both these animals were born at Vaalbos National Park and must have been exposed to the parasite and vector while in that ecosystem. This would also suggest that the two tick species identified on rhinoceros, in the area, could be potential vectors of piroplasms.

The only *D. b. bicornis* blood samples that tested positive for *B. bicornis* or *T. bicornis* originated from either Addo Elephant National Park or Mountain Zebra National Park, both in this study and that conducted by Nijhof *et al*. (2003). The rhinoceros bull, Muleka, who tested positive for *B. bicornis* at Mountain Zebra National Park, had originally been in Addo Elephant National Park. The blood samples were collected soon after his introduction to Mountain Zebra National Park and it is likely that he became infected with *B. bicornis* while in Addo Elephant National Park. This would suggest the presence of both the piroplasms and their vectors in the Addo Elephant National Park environment.

This has a number of important implications for conservation managers doing a disease risk assessment. When moving black rhinoceroses from the more arid Northern Cape or Namibia, to the moister, southern parts of the Eastern Cape, one is potentially moving *Babesia*-naïve animals into an 'infected' area and they could potentially develop clinical disease if exposed to the piroplasms. The probability that they will be exposed to piroplasms after release into the destination environment is very high. In the case of black rhinoceroses, there has been one confirmed mortality due to babesiosis in Addo Elephant National Park. This was a young bull (Benji) and it is speculated that there was a certain degree of stress-related immunosuppression, which may have triggered the mortality by reducing the resistance of the animal to *Babesia* parasites. It has also been speculated that a number of deaths in the late 1990s, after a drought period, may also have been as a result of stress-induced babesiosis – this was never confirmed. It is therefore important to carefully monitor the rhinoceroses post-release, especially when individuals are moved from non-infected areas to known infected areas such as Addo Elephant National Park. One could also look at prophylactically treating animals with an anti-*Babesia* compound such as diminazene aceturate, as recommended by Penzhorn *et al.* (2008).

In most natural ecosystems, a dynamic relationship is maintained between parasitic organisms, their indigenous hosts, and the environment, what is called endemic stability (Penzhorn, 2006). This is more likely the case within the black rhinoceros population of Addo Elephant National Park. If rhinoceroses are translocated from Addo Elephant

National Park (and the southern parts of the Eastern Cape) to the more arid areas of the country, there is a high probability that they may carry piroplasms from the source to the destination environment. At this stage it has not been established whether there is a suitable vector in the arid areas of the country, to transmit the parasites from host to host. The consequences of such translocations could be potentially disastrous for the destination ecosystem, in the event that the disease of concern infects the susceptible hosts. This could be a major setback for the metapopulation management program and it would therefore be important to monitor the whole population post-release of a potentially infected animal.

Diceros bicornis minor **ecotype metapopulation management in South Africa:**
Black rhinoceroses were formerly widespread throughout most of South Africa, but by the 1930s they had been reduced to two relict populations comprising 100 to 150 animals of the southern-central subspecies (*D. b. minor*) in Hluhluwe-iMfolozi Park and Mkuzi Game Reserve in Zululand (Brooks, 1989). From these founder populations, animals were reintroduced to Kruger National Park (1971-82), Pilanesberg National Park (1981-83), Great Fish River Reserve Complex (1986) and subsequently from Kruger National Park to Marakele National Park in the 1990s. At the time, as with the south-western ecotype of black rhinoceros, piroplasms had not been recognized as potential disease hazards for rhinoceroses. A number of these populations have since been found to be positive for *Theileria* and *Babesia* species. Nijhof *et al*. (2003) reported on mortalities of black rhinoceroses associated with tick-borne parasites from KwaZulu-Natal and found the parasite in samples taken from rhinoceroses originating from the Great Fish River Reserve Complex. In this study *T. bicornis* was found in both Kruger National Park and Marakele National Park, and *B. bicornis* was found in one individual originating from Marakele National Park. The presence of the piroplasm suggests that the parasites are, or have become, endemic to these areas. What is of interest is that Akura, one of the rhinoceroses that tested positive for *T. bicornis*, was born and bred in a captive environment (Frankfurt Zoo, Germany) and had been reintroduced to Marakele National Park. The rhinoceros would only have been exposed to the parasite on reintroduction and

this would suggest there is a certain degree of innate resistance to the parasite resulting in endemic stability.

From a disease risk point of view, the probability that black rhinoceroses of the subspecies *D. b. minor* having been exposed to piroplasms is very high. Therefore, the magnitude of risk during translocations between various populations is reduced. There is more of a risk of the translocated animals succumbing to babesiosis due to stress-related immunosuppression.

Two of the samples, Alicia from Marakele National Park and 03/177 from Addo Elephant National Park, tested positive for the presence of both *T. bicornis* and *T. equi*. Phylogenetic analysis revealed (Nijhof *et al*., 2003) that *T. bicornis* clustered with *T. equi*, suggesting a close relationship between the two parasites. From the probe sequences, it is unlikely that they would cross react. One could speculate that there was contamination with *T. equi*, but this is also unlikely since one would expect *T. equi* to be detected in more than two samples. The most likely explanation is that this was a dual infection with both *T. equi* and *T. bicornis*.

6. Conclusion

In conclusion, based on findings to date, *B. bicornis* and *T. bicornis* are relatively widespread in black rhinoceros populations in South Africa and pose a potential risk to the success of metapopulation management programs. Of the two black rhinoceros subspecies that occur in South Africa, *D. b. bicornis* is at greater risk due to their apparently *Babesia/Theileria*-naïve status in certain areas, when compared to the subspecies *D. b. minor*. Conservation managers need to carefully evaluate methods and procedures during the translocation of black rhinoceroses, especially when relocating from geographically and climatically diverse ecosystems and more so when dealing with the *D. b. bicornis* ecotype.

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