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Detection of *Babesia* and *Theileria* parasites in white rhinoceroses *(Ceratotherium simum)* in the Kruger National Park, and their relation to anaemia

by

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ABBREVIATIONS

PCR	Polymerase Chain Reaction
RLB	Reverse Line Blot
Bp	Base pairs
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
rRNA	ribosomal Ribonucleic acid
RLB-F	**RLB f**orward primer
RLB-R	**RLB r**everse Primer
dNTP	Deoxynucleotide triphosphate
ECL	Enhanced Chemiluminescence
IFA	Indirect Fluorescent Antibody test
ELISA	Enzyme linked Immunosorbent Assay
RBCs	Red Blood Corpuscles
MCV	Mean Corpuscular Volume
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
PLTs	Platelets
SDS	Sodium dodecyl sulfate
EDAC	1-ethyl 3-(3-dimethylaminopropyl) carbodiimide HCl
EDTA	Ethylene diamine tetra-acetic acid
SANParks	South African National Parks
KNP	Kruger National Park

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Degree:	MSc (Veterinary Tropical Diseases)

ABSTRACT

As part of the larger survey to map the geographical distribution of *Babesia* and *Theileria* parasites in the Southern African rhinoceros population, white rhinoceroses were sampled during routine immobilizations in the Kruger National Park. Polymerase Chain Reaction (PCR) and Reverse Line Blot (RLB) hybridization assays were used to screen for the presence of haemoprotozoa and complete blood counts were used to assess associated changes in clinical parameters. Of the 195 rhinoceroses sampled, 36.4% tested positive for the presence of *Theileria bicornis*, with no significant change in the haematological parameters measured. None of the rhinoceroses sampled tested positive for *Babesia bicornis*, the parasite linked to mortalities in black rhinoceroses.

1. INTRODUCTION

Since their discovery in cattle in the late 19th century by Roumanian microbiologist, Victor Babes (Hunfeld, Hildebrandt, Gray, 2008; Krause, 2000), haemoprotozoal parasites have been detected in a number of wild and domestic hosts, amounting to more than a hundred species to date (Krause, 2000). Both *Babesia* and *Theileria* organisms belong to the Order Piroplasmida in the phylum Apicomplexa (Kreier & Baker, 1987) and both are obligate intracellular parasites that utilize arthropod vectors for transmission to their vertebrate hosts. The main difference for their classification into separate genera involves the first host cell that the organism infects: in *Babesia* spp these are erythrocytes and in *Theileria* spp these are lymphocytes or macrophages (Uilenberg, 2006).

Various *Babesia* and *Theileria* spp have been described from wild animals. In most cases these were incidental findings, but haemoprotozoa have been implicated in losses amongst wild animals, generally related to stress (Penzhorn 2006). The clinical spectrum of piroplasm infection ranges from asymptomatic infection to death. Parasite replication often produces only mild anaemia with few associated symptoms, but it may also cause severe anaemia with numerous clinical manifestations, including high fever, hypotension, pulmonary oedema, disseminated intravascular coagulation, haemoglobinuria, and multiple organ failure (Hunfeld, Hildebrandt & Gray, 2008).

Studying haemoprotozoa in wild animals is difficult. Recently developed molecular techniques, e.g. Polymerase Chain Reaction (PCR) and the Reverse Line Blot (RLB) hybridization assay (Gubbels, De Vos, Van der Weide, Viseras, Schouls, De Vries & Jongejan, 1999), allow for accurate surveys and typing of haemoprotozoa.

Routine immobilization of white rhinoceroses (*Ceratotherium simum*) in Kruger National Park (KNP) for management purposes offered the opportunity of determining the prevalence of piroplasm infection in this population, as well as establishing whether there is a link between anaemia and the presence of piroplasms. The objectives of this study were to detect the presence of *Babesia* and *Theileria* parasites in blood of white rhinoceroses in the KNP using the RLB hybridization assay and to evaluate whether these *Babesia* and *Theileria* parasites, when present, are pathogenic to their host (as manifest by anaemia developing).

2. LITERATURE REVIEW

Due to failure to adapt to human predation and competition for space, white rhinoceros numbers, at the turn of the last century, dwindled to an estimated 20 that survived in the Hluhluwe-iMfolozi Park, KwaZulu-Natal, South Africa (Pienaar, 1994). Through the identification of the fragile nature of this population and concerted efforts to protect them from hunting, the population was brought back from near extinction to their present number of about 11 320 (Emslie, 2004).

The last naturally occurring white rhinoceroses in the Kruger National Park (KNP) region were seen in 1896 (Pienaar, 1970). Reintroductions of white rhinoceroses to the KNP began in 1961 (Pienaar, 1970). Since then their numbers have steadily risen to over 6 000 in the KNP, currently the largest population stronghold for the southern white rhinoceros (*Ceratotherium simum simum*). Efforts to try and restock private reserves within their former range and to strengthen and augment present populations have resulted in a rise in rhinoceros immobilizations and translocations. With very little known about their susceptibility to infection and the role they play in the epidemiology of disease, these translocations can still pose risks (Fischer-Tenhagen, Hamblin, Quandt & Frölich, 2000). Stress associated with capture, confinement in bomas, extreme environmental conditions, pregnancy and poor nutrition can all increase the possibility of opportunistic infections and recrudescence of latent infections (Kock, Jongejan, Kock, Kock & Morkel, 1992; Mihok, Kock & Masake, 1995).

There is evidence that endemic stability is the general pattern of *Babesia* infections in wildlife (Penzhorn, 2006). Animals born in an endemic area are exposed to these tick-borne parasites at a young age, build up immunity and a stable situation ensues. Rhinoceroses born in captivity or non-endemic areas are fully susceptible to these parasites and may develop clinical disease and even die.

Although *Babesia* and *Theileria* parasites are usually quite host specific, similar *Babesia* and *Theileria* parasites have been reported in both white and black (*Diceros bicornis*) rhinoceroses (Bigalke, Keep, Keep & Schoeman, 1970; Nijhof, Penzhorn, Lynen, Mollel, Morkel, Bekker & Jongejan, 2003; Miller & Boever, 1982). Bigalke *et al.* (1970) reported on the presence of a large and a small *Babesia* (or possibly a *Theileria*) species in white rhinoceroses surveyed *ad hoc* in Zululand during routine translocation procedures. Of the 106 animals they examined, 32.1% were positive for the small *Babesia* spp., with only 1.9% positive for the large *Babesia* spp. Bigalke *et al*. (1970) also reported that Neitz (personal communication) had previously observed small piroplasms in both black and white rhinoceroses, while Brocklesby (1967) reported on both small and large *Babesia* spp. in black rhinoceroses in Kenya. McCulloch and Achard (1969) reported on black rhinoceros translocation deaths in Tanzania during 1964 and 1965, with a mortality rate of 3.4% that was

attributable to babesiosis. The publication by Bigalke *et al.* (1970) was the first report to highlight a small and a large *Babesia* in the white rhinoceros; however, the authors could not demonstrate any clinical illness due to either parasite, as all animals translocated were apparently clinically healthy.

In the case report by Nijhof *et al.* (2003), a novel *Babesia* species, designated *Babesia bicornis*, was identified in three black rhinoceroses that died in wildlife areas in Tanzania and South Africa. Screening of black rhinoceroses in South Africa revealed a second parasite, designated *Theileria bicornis*. That report made it clear that *B. bicornis* can cause fatal disease in black rhinoceroses, although there is no evidence to prove that *T. bicornis* is pathogenic to black rhinoceroses. Also, although dual infections do occur, the authors could merely speculate about the possible effects of such infections.

The vector of these parasites is still unknown, though the ixodid ticks *Amblyomma rhinocerotis* and *Dermacentor rhinocerinus* are known to feed specifically on black and white rhinoceroses (Knapp, Krecek, Horak & Penzhorn, 1997). In two white rhinoceroses sampled in the KNP, 6 tick species were found, with *Amblyomma hebraeum* and *Dermacentor rhinocerinus* making up the bulk of the sample, while *Hyalomma truncatum, Rhipicephalus simus*, *R. appendiculatus* and *R. zambeziensis* were found in smaller numbers (Knapp *et al.,* 1997).

When studying haemoprotozoa, identification of the individual species has been traditionally based on the host specificity and piroplasm morphology (de Gopegui, Peñalba, Goicoa, Espada, Fidalgo, Espino, 2007). Though microscopy still remains a cheap and easily achievable modality for diagnosis, it lacks both sensitivity and species specificity, especially when dealing with morphologically indistinguishable organisms. In an attempt to overcome this, serological tests were developed that measure antibody response. Some of these tests include the indirect fluorescent antibody test (IFA) and the enzyme-linked immunosorbent assay (ELISA). These were more specific, but could not differentiate between current and previous parasitic infections or identify carrier animals that have low antibody titres. Therefore, molecular/DNA-based diagnostics were developed over the past 20 years. With our current knowledge and the availability of the Polymerase Chain Reaction (PCR) and the Reverse Line Blot (RLB) hybridization assays, it is now possible to determine whether white rhinoceroses carry both *B. bicornis* and *T. bicornis* and if so, whether anaemia can be related to infection. The RLB assay is a versatile diagnostic tool to sensitively and simultaneously detect and differentiate haemoparasites in blood, tissue or within the tick vector itself.

Changes to the haematological picture in babesiosis can be quite varied, with anaemia, leukopenia or leukocytosis and/or thrombocytopenia all being recorded with infection (Zobba, Ardu, Niccolini, Chessa, Manna, Cocco & Pinna Parpaglia, 2008). The haemolytic anaemia

associated with piroplasm infection is thought to be induced by trophozoite intra-erythrocyte binary fission, secondary immune-mediated haemolytic anaemia, oxidative damage to erythrocytes (Murase, Ueda, Yamato, Tajima & Maede, 1996; Orinda, Commins, Waltisbuhl, Goodger & Wright, 1994), and the presence of a haemolytic factor in serum, which has been described in *Babesia gibsoni* infection (Onishi, Ueda, Horie, Kajikawa & Ohishi, 1990; de Gopegui *et al*., 2007).

3. MATERIAL AND METHODS

3.1. Study Area:

The Kruger National Park, with an area of 19 485 km^2, is South Africa's largest wildlife refuge. Located in the north eastern section of the country, it borders Zimbabwe to the north and Mozambique to the east. The study was conducted on animals selected in the southern Morula Section of the park, bordered by the Sabie and Crocodile Rivers. This area represents the highest white rhinoceros densities in KNP (Figure 1). As a result approximately 100 animals are captured each year for sale.

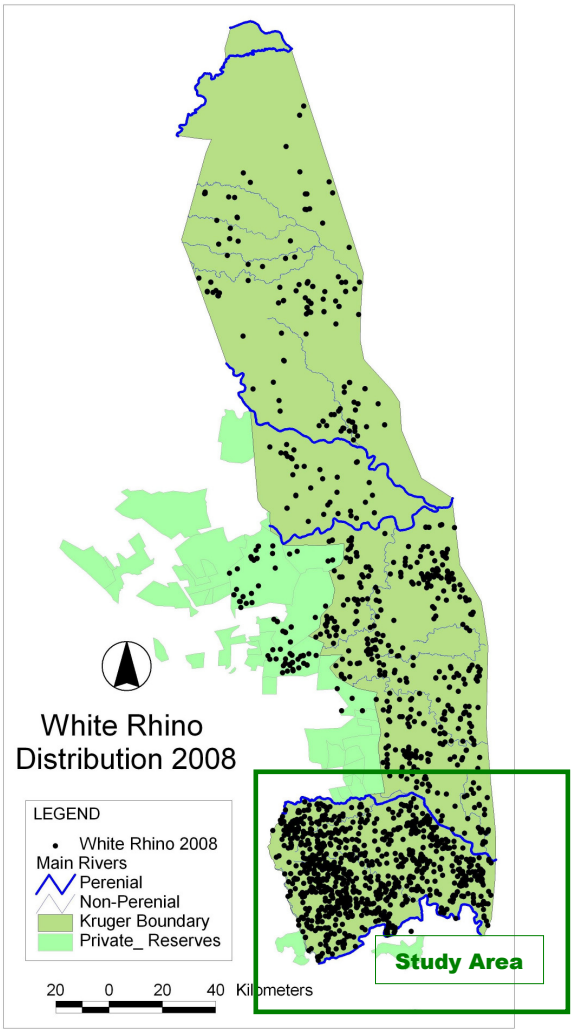

FIGURE 1. Map of the Kruger National Park showing white rhinoceros densities, with the study area (defined in the box), having the highest white rhinoceros densities in the Park (GIS Laboratory, SANParks, Skukuza).

3.2. Animals:

White rhinoceroses (n = 195) were selected randomly during routine immobilization for sales to various private owners. These included both males and females of all age classes. The animals were sampled between January 2003 and September 2006. The 195 samples represent a stratified sample through the population representing the age and sex class ratios found in the KNP at the time of the project.

3.3. Sampling:

Rhinoceroses were immobilized following South African National Parks (SANParks) standard operating procedure for game immobilization, transport and holding of wild animals (revised March, 2006), using a combination of etorphine, azaperone and hyaluronidase (Addendum 1).

Blood was collected from the auricular vein, using 6 ml Vacutainer® (Franklin Lakes, USA) tubes with EDTA anticoagulant, once the animal was immobilized and in lateral recumbency. Blood tubes were then labeled with the animal specific details as well as date and GPS location where it was caught. The specimen was then placed in a cooler box, and kept cool until it reached the laboratory.

3.4. Sample Handling:

Once at the laboratory, the EDTA samples were placed on a roller for approximately 5 min or until they were properly mixed. Five microliters of the sample was then run on an automated blood counter machine (*ABC Vet*) in order to attain complete blood counts. These results were filed and analysed at a later stage.

The remainder of the EDTA blood sample was pipetted into 3.7 ml NUNC tubes and frozen as EDTA-buffered whole blood in a -10^{o}C freezer. Once all the samples were available, they were taken to the Molecular Biology Laboratory, Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, where they were subjected to the Reverse Line Blot (RLB) hybridization assay for the simultaneous detection of *Theileria* and/or *Babesia* parasites.

3.5. DNA extraction from blood using the QIAamp® DNA Extraction Kit

DNA was extracted from 200 µl of EDTA-buffered whole blood using the QIAamp® DNA Extraction Kit (Qiagen, Southern Cross Biotechnology, South Africa) following the manufacturer's instructions. In short, 20 µl Proteinase K and 200 µl lysis buffer AL was added to 200 µl whole blood, mixed thoroughly and incubated at 56°C for 10 min. Two hundred µl of 100% ethanol was added, thoroughly mixed and then transferred to a QIAamp Spin Column. It was centrifuged at 6000 x *g* (8000 rpm) for 1 min after which the flow through was discarded. Five hundred µl wash buffer AW1 was added to the spin column and centrifuged at 6000 x *g* (8000 rpm) for 1 min. After discarding the flow through, the washing step was

repeated by adding 500 µl wash buffer AW2 and centrifuging at full speed (20,000 x *g*; 14,000 rpm) for 3 min. The spin column was then placed in a clean 1.5 ml micro centrifuge tube, 100 µl elution buffer AE was added directly onto the spin column membrane and incubated at room temperature (15–25°C) for 1 min. The DNA was eluted by centrifuging at 6000 x *g* (8000 rpm) for 1 min. The DNA was stored at –20°C until further analysis.

3.6. PCR

One set of primers was used to amplify a 460- to 520-bp fragment of the18S SSU rRNA spanning the V4 hypervariable region (Gubbels, de Vos, van der Weide, Viseras, Schouls, de Vries & Jongejan, 1999: Matjila, Penzhorn, Bekker, Nijhof & Jongejan, 2004) . The forward primer, RLB-F [5'-GAC ACA GGG AGG TAG TGA CAA G-3'] and the biotin-labeled reverse primer, RLB-R [5'-Biotin-CTA AGA ATT TCA CCT CTA ACA GT-3'], hybridized with regions conserved for *Theileria* and *Babesia*. Primers were obtained from Isogen (Maarssen, The Netherlands). Reaction conditions in a 25 µl volume were as follows: 12.5 µl of Platinum Quantitative PCR Supermix-UDG (Invitrogen), 20 pmol (0.25 µl) of both the forward and reverse primers (Inqaba Biotec, South Africa), 2.5 µl of the extracted DNA template and 9.5 µl of molecular grade water. A touch-down PCR programme was followed, starting with 3 min at 37°C; 10 min at 94°C; and 10 cycles of 94°C for 20 s, 67°C for 30 s, 72°C for 30 s with decreasing of the annealing temperature after every second cycle by 2°C for five times. These cycles continued until the annealing temperature reached 57°C. Finally, 40 cycles of 94°C for 20 s; 57°C for 30 s and 72°C for 30 s were performed. The PCR was completed with a final extension cycle of 7 minutes at 72°C, and left at 4°C.

3.6. RLB hybridization.

Preparation of membrane: A Biodyne C blotting membrane (Pall Biosupport, Ann Arbor, Mich.) was activated by a 10-min incubation in 10 ml of 16% (wt/vol) 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDAC) (Sigma, St. Louis, Mo.) at room temperature. The membrane was washed for 2 min with distilled water and placed in an MN45 miniblotter (Immunetics, Cambridge, Mass.). Specific oligonucleotides (Table 1) containing a N-terminal *N*-(trifluoracetamidohexyl-cyanoethyl,*N*,*N*-diisopropyl phosphoramidite [TFA])-C6 amino linker (Isogen), were diluted to a 200- to 1,600 pmol/150 ml concentration in 500 mM $NaHCO_3$ (pH 8.4) and covalently linked to the membrane by filling the miniblotter slots with the oligonucleotide dilutions; and incubating for 1 min 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 10 min at room temperature. The membrane was washed with shaking in 125 ml of 2x SSPE (360 mM NaCl, 20 mM NaH_2PO_4, 2 mM EDTA [pH 8.4]) containing 0.5% sodium dodecyl sulphate (SDS) for 5 min at 60°C.

TABLE 1. List of genus and species-specific RLB probes and their corresponding probe sequence.

Probe Identification	**Probe Sequence * (from 5' – 3')**
Theileria/*Babesia* catch-all	TAA TGG TTA ATA GGA RCG GTT G
Babesia felis	TTA TGC GTT TTC CGA CTG GC
Babesia divergens	ACT RAT ATC GAG ATT GCA C
Babesia microti	GRC TTG GCA TWC TCT GGA
Babesia bigemina	CGT TTT TTC CCT TTT GTT GG
Babesia bovis	CAG GTT TCG CCT GTA TAA TTG AG
Babesia rossi	CGG TTT GTT GCC TTT GTG
Babesia canis canis	TGC GTT GAC GGT TTG AC
Babesia canis vogeli	AGC GTG TTC GAG TTT GCC
Babesia major	TCC GAC TTT GGT TGG TGT
Babesia bicornis	**TTG GTA AAT CGC CTT GGT C**
Babesia caballi	GTT GCG TTK TTC TTG CTT TT
Theileria sp (kudu)	CTG CAT TGT TTC TTT CCT TTG
Theileria sp (sable)	GCT GCA TTG CCT TTT CTC C
Theileria bicornis	**TTG GTA AAT CGC CTT GGT C**
Theileria annulata	CCT CTG GGG TCT GTG CA
Theileria buffeli	GGC TTA TTT CGG WTT GAT TTT
Theileria sp (buffalo)	CAG ACG GAG TTT ACT TTG T
Theileria mutans	CTT GCG TCT CCG AAT GTT
Theileria parva	GGA CGG AGT TCG CTT TG
Theileria taurotragi	TCT TGG CAC GTG GCT TTT
Theileria velifera	CCT ATT CTC CTT TAC GAG T
Theileria equi	**TTC GTT GAC TGC GYT TGG**
Theileria lestoquardi	CTT GTG TCC CTC CGG G

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

RLB hybridization: Before use, the membrane was washed for 5 min at 42°C with 125 ml of 2x SSPE/0.1% SDS and placed in the miniblotter with the slots perpendicular to the previously applied specific oligonucleotides (Gubbels *et al.*, 1999; Schnittger, Yin, Qi, Gubbels, Beyer, Niemann, Jongejan & Ahmed, 2004). A volume of 10 µl of PCR product was diluted in 150 µl of 2x SSPE/0.1% SDS, heated for 10 min at 100°C, and cooled on ice immediately. Denatured PCR samples were applied into the slots and incubated for 60 min at 42°C. PCR products were aspirated, and the blot was washed twice in 125 ml of 2x SSPE/0.5% SDS for 10 min at 50°C with shaking. Subsequently the membrane was

incubated in 10 ml of 1:4,000-diluted peroxidase-labeled streptavidin (Boehringer, Mannheim, Germany) in 2x SSPE/0.5% SDS for 30 min at 42°C. The membrane was washed twice in 125 ml of 2x SSPE/0.5% SDS for 10 min at 42°C with shaking. After two rinses in 125 ml of 2x SSPE for 5 min each time at room temperature, an incubation for 1 min in 10 ml of ECL detection fluid (Amersham, Little Chalfont, Buckinghamshire, United Kingdom) followed before exposure to an ECL hyperfilm (Amersham) for 5 to 20 min. After development of the film, spots occurred at the sites where genus and species-specific oligonucleotide and PCR product hybridized and the identity of the micro-organism(s) in the sample was established.

After use, all PCR products were stripped from the membrane by two washes in 1% SDS for 30 min each time at 80°C. The membrane was rinsed in 20 mM EDTA (pH 8.0) and stored in fresh EDTA solution at 4°C for reuse.

Two membranes were used in the current study to allow for maximum number of samples to be run concurrently. Membrane 1 was prepared according to the methodology described above and Membrane 2 was a ready prepared Isogen membrane (Maarssen, The Netherlands). Both membranes utilized the same probe sequences and probe concentrations.

3.7. Statistical analysis

The Statistical Analysis System (SAS®) was used to analyze the data. The Chi-square test and the Mann-Whitney non-parametric test were used for the statistical comparison between haemoprotozoal prevalence, age and sex class, season, as well as for the haematology results. The significance level was set at $P<0.05$.

4. RESULTS

1. PCR and RLB Results

1.1. Frequencies of infection

Out of the 195 samples tested, no animals tested positive for the presence of *Babesia bicornis* using the RLB hybridization assay. A prevalence of 36.41% for *T. bicornis* was seen in the population (Table 2).

TABLE 2. Frequencies of infection of *Theileria bicornis* and *Theileria equi* in white rhinoceroses and between different age and sex classes of white rhinoceroses in the Kruger National Park.

	No. of Individuals	***T. bicornis* positive (%)**	***T. equi* positive (%)**
Total Sampled	195	36.41 (71)	9.23 (18)
Adult Bull[a]	55	32.73 (18)	10.91 (6)
Adult Cow[b]	21	28.57 (6)	0
Sub-adult Bull[c]	34	29.41 (10)	8.82 (3)
Sub-adult Cow[d]	67	47.76 (32)	10.45 (7)
Calf[e]	18	27.78 (5)	11.11 (2)

[a] = male, breeding animal 8 years or older

[b] = female, breeding animal 6 years or older

[c] = refers to a male animal between the ages of 4 to 8 years that has not yet acquired a territory

[d] = refers to a young female animal that has not yet calved

[e] = refers to both male and female animals still with the dam and usually less than 2.5 years

There was no significant difference between the various age/sex classes when the five groups were compared (Table 2). However, the sub-adult females, when compared against the rest of the population, were significantly more affected (5.68, p= 0.01; chi-square test).

There was also an unexpected 9.23% prevalence of *Theileria equi* in the study population. However, there appeared to be a significant correlation (19.26, P< 0.0001; chi-square test) between membrane used and detection of these parasites with the Isogen Membrane detecting higher rates of infection as compared to the "in-house" prepared membrane. It was shown that all animals positive for *T. equi* were also positive for *T. bicornis* and that the signal, though strong, was significantly weaker than the *T. bicornis* signal or the *T. equi*-positive control.

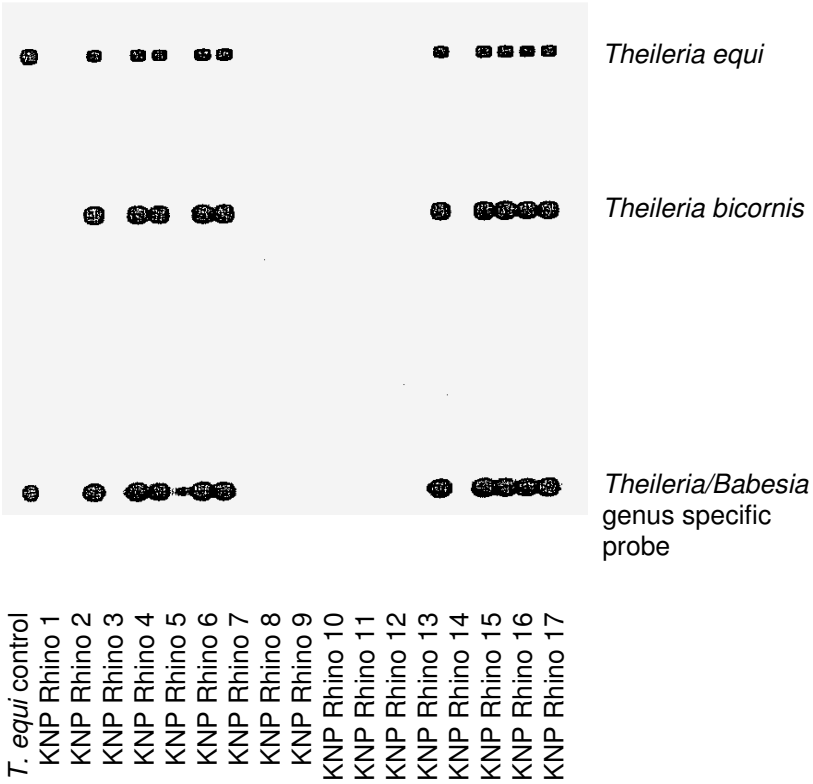

FIGURE 2. RLB with PCR products derived from DNA extracted from white rhinoceros blood samples collected in the KNP. On this membrane a *Theileria/Babesia* genus-specific probe and species-specific probes were used. Several rhinoceroses tested positive with the *Theileria/Babesia* genus specific probe, as well as both the *T. bicornis* and *T. equi* probe.

1.2. Seasonal differences in infection frequencies

There was no significant difference when infection rates were compared between seasons (Figure 3 and Table 3). Of the 164 animals tested during the dry season (April to September), 58 (35.37%) tested positive, as opposed to 13 out of 31 (41.94%) that were tested in the wet season (Table 3).

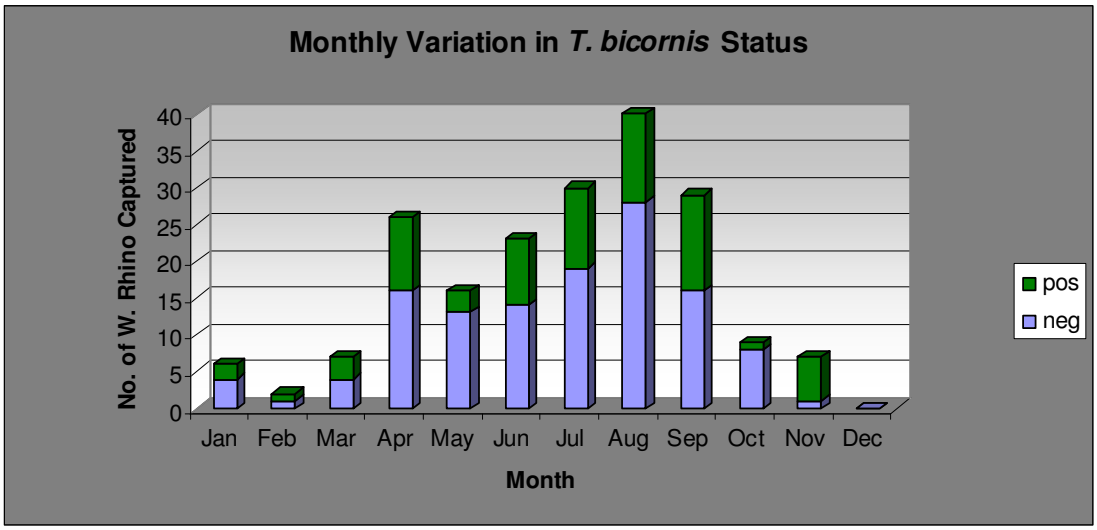

FIGURE 3. Monthly variation in *T. bicornis* status in white rhinoceroses in KNP

TABLE 3. Dry and wet season variation of *T. bicornis* status in white rhinoceroses in KNP

	Tot. Animals Captured	**Negative %**	**Positive %**	**P- Value [c]**
Dry season[a]	164	64.63%	35.37%	0.467
Wet Season[b]	31	58. 06%	41.94%	

a = April to September, b= October to March, c= chi-square test

2. Blood Profiles

Results obtained are shown in Table 4. There was no significant change to the blood profile with *T. bicornis* infection, *i.e.* no significant change in haematocrit, RBCs, MCV, MCH, MCHC or PLTs (Table 4). There was, however, a slight elevation in WBC count (leucocytosis) in those animals infected (P=0.068; Mann-Whitney non-parametric test).

TABLE 4. Haematology values for white rhinoceroses sampled.

Parameter	Positive *T. bicornis*				Negative *T. bicornis*				P-Value[a]
	Mean	Std Dev	Min	Max	Mean	Std Dev	Min	Max	
WBC[b] X 10^3/ul	24.50	8.052	10.10	39.5	19.97	6.75	8.2	36.9	0.067
RBC[c] X 10^6/ul	6.90	1.77	3.44	11.56	7.32	2.77	2.94	17.6	0.490
HGB[d] g/dl	14.71	4.01	7.70	25.00	14.79	3.56	6.40	24.70	0.740
Hct[e] %	40.23	7.46	23.10	48.70	42.86	8.84	19.60	56.30	0.274
MCV[f] FL	62.62	3.78	56.00	69.00	62.90	4.56	55.00	71.00	0.803
MCH[g] pg/cell	21.28	1.51	19.80	25.00	20.86	1.47	18.40	23.30	0.607
MCHC[h] g/dl	34.02	1.71	32.70	39.00	33.22	1.23	29.10	34.90	0.478
Platelets	561.54	163.03	299.00	885.00	541.45	168.74	185.00	797.00	1.000

[a]= Mann-Whitney non-parametric test for statistical significance

[b]= White Blood Corpuscles; [c]= Red Blood Corpuscles; [d]= Haemoglobin; [e]= Haematocrit; [f]= Mean Corpuscular Volume; [g]= Mean Corpuscular Haemoglobin; [h]= Mean Corpuscular Haemoglobin Concentration

5. DISCUSSION

As part of the larger survey to map the geographical distribution of *Babesia* and *Theileria* parasites in the Southern African rhinoceros population, 195 white rhinoceroses were sampled during routine immobilizations in the KNP. The RLB hybridization results indicated a 36.41% prevalence of *T. bicornis*: however, *B. bicornis* was not demonstrated in this study.

In white rhinoceroses surveyed *ad hoc* in Zululand by basic microscopy, Bigalke *et al*. (1970) reported a 32.1% prevalence of a small *Babesia* sp. (probably *Theileria* sp.), which is consistent with our findings in the KNP. Bigalke *et al*. (1970) also reported 1.9% prevalence of a large *Babesia* sp. The latter parasite concerns us most as it has been linked to fatalities in black rhinoceroses in East Africa and South Africa (Nijhof *et al*., 2003). One possible explanation for lack of its detection might be that the parasite is not present in our study population or that there was PCR failure, whereby the parasite DNA was not amplified. Blood smears of a debilitated black rhinoceros in KNP, however, showed erythrocytes parasitized with large *Babesias* (Dr Roy Bengis, pers. comm.). Tick surveys conducted on rhinoceroses in the KNP (Knapp *et al.* 1997) have also demonstrated shared tick parasites in both species of rhinoceroses, making geographic absence of parasite rather improbable.

A clue might come in when trying to understand the immune response that the parasite elicits. Piroplasm-infected erythrocytes reveal membrane alterations (Sun, Tenenbaum, Greenspan, Teichberg, Wang, Degnan & Kaplan, 1983) resulting in enhanced erythrocyte removal upon spleen passage (Commins, Goodger, Waltisbuhl & Wright, 1988). Initial work with *Babesia argentina* (also known as *Babesia bovis*) showed that the erythrocyte surface antigenicity varied over time (Curnow, 1968). Subsequent work showed that multiple membrane antigenic changes could take place, beginning with a single parasite clone, indicating that *Babesia* spp. might persist by evading the host's immune response through parasite-directed alterations in the erythrocyte membrane (Krause, Daily, Telford, Vannier, Lantos & Spielman, 2007). These variant antigens are encoded by a multi-gene family. When different parasites were compared, greater diversity of these genes resulted in greater capability of evading the host's immune response (Krause *et al*., 2007). This might be why what seems a more pathogenic haemoparasite, like *B. bicornis*, is not able to survive an intact immune system, due to possibly fewer parasite-directed alterations in the erythrocyte membrane, resulting in early recognition and elimination of the parasite. *Theileria bicornis*, in comparison, manages to evade the host's immune system leading to piroplasms appearing in the peripheral blood circulation.

After experimental infection of a domestic cat with *Babesia leo*, no parasites were observed on blood smears for the first 42 days (Lopez-Rebollar, Penzhorn, de Waal & Lewis, 1999). At that time the cat was splenectomised and soon afterwards parasites started appearing in the

peripheral blood smears, eventually peaking at an infection rate of 45%. Immune compromise from capture stress, handling or boma confinement, may act in the same way as a splenectomy would: the animal may no longer be able to phagocytose parasitised erythrocytes, resulting in their rise and multiplication in peripheral circulation, eventually causing clinical disease and/or death.

Another factor that may play a role in the absence of *B. bicornis* in the study population is that given the density of white rhinoceroses in the KNP, a concerted effort is made to select only healthy, nutritionally unstressed animals for translocations, and this may have biased our results.

The complete blood count parameters that were measured did not seem to show significant changes, though there may be some indication of a rise in white blood cells in those rhinoceroses infected. Anaemia, though sometimes seen as a classical early detection sign for babesiosis, was not detected in those animals infected with *T. bicornis*.

The sub-adult female proportion of the population showed a significantly higher rate of infection with *T. bicornis* when compared with the rest of the population (5.680, P= 0.0172; chi-square test). Sub-adult females are, most likely, the proportion of the population that is undergoing the most dramatic changes: they are starting to become reproductively active, participate in courtship and mating, and are often early pregnant when captured. This may result in high stress levels and a hormonal milieu that may induce immune suppression, making these animals less able to fight off these haemoparasites (Zhang, Zhang, Miao, Hanley, Stuart, Sun, Chen & Yin, 2008; Serafeim & Gordon, 2001; Glaser & Kiecolt-Glaser, 2005).

A 9% prevalence of *T. equi* was seen in the study population. It was shown (Fig. 2) that only animals positive for *T. bicornis* were positive for *T. equi* and that the signal, though strong, was significantly weaker than the *T. bicornis* signal or the *T. equi*-positive control. Possible reasons for this would be cross reaction with the *T. bicornis* probe or contamination with other target DNA. The full-length 18S RNA gene sequence from *T. bicornis*, when determined and analysed phylogenetically, showed the parasite clustering with *T. equi* on the parsimony tree (Nijhof *et al*., 2003; Katzer, McKellar, Kirvar, Shiels, 1998), making *T. bicornis* a close relative of *T. equi*. The *T. equi* probe also shares the same first and last 4 base nucleotides with that of the *T. bicornis* probe, which under normal circumstances would not be enough for a cross reaction. If the temperature of the hybridization was not optimal, however, some cross-reaction may have been possible.

The apparent lack of seasonal variation in *T. bicornis* infection, though one might expect it due to the seasonality of their tick vectors, might be explained in that these parasites are

known to cause chronic asymptomatic infection that can persist for months or years (Krause, 2000). This may result from failure of the adaptive immune response to clear the pathogen, as was noted in *B. bovis* infected cattle, where the untimely release of anti-inflammatory cytokine IL-10 dampened the effect of interferon gamma, an activator of nitric oxide production by macrophages (Goff, Johnson, Parish, Barrington, Tuo & Valdez, 2001). Studies of *Babesia* suggest that the parasite-induced erythrocyte membrane antigen variability and cytoadherence result in long-term asymptomatic infection of the vertebrate host, increasing the probability of transmission to arthropod vectors and to new hosts, thereby assuring pathogen survival (Krause *et al*., 2007).

6. CONCLUSION

Although this study confirms a relatively high prevalence of *T. bicornis* in the white rhinoceros population in the KNP, it also revealed that the parasite did not seem to cause any ill health to the animals concerned. It seems to represent another instance of an endemically stable situation, where co-evolution of parasite and host has been pushed to the degree where long-term asymptomatic infection ensures parasite survival and persistence in the environment.

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Addendum 1

STANDARD OPERATING PROCEDURES FOR THE CAPTURE, TRANSPORTATION AND MAINTENANCE IN HOLDING FACILITIES OF WILDLIFE **2. Species specific considerations** **2.1 Chemical capture** 2.1.2 White rhino **VETERINARY WILDLIFE SERVICES** **SOUTH AFRICAN NATIONAL PARKS**	Document No.	5
	Effective date	2nd Sept 2005
	Revision history	
	Revision No.	New
	Page No	30 to 37
	Approval	

1. **Purpose**
   This procedure establishes guidelines for the capture using chemical capture agents, transport or maintenance in holding facilities of one or several white rhino to meet an objective of SANParks. The process begins with the preparations and planning of the actions to be taken by the Veterinary and Operations staff of Veterinary Wildlife Services and ends when the task has been completed successfully and the results recorded.

2. **Revision history**

Date	Rev. No	Change	Reference section
2/9/2005	1.0	New procedure drafted	Not applicable

3. **Persons affected**
   - Veterinary Wildlife Services, SANParks.
   - SANParks staff involved in the capture, transport or holding of animals.
   - Researchers conducting research in National Parks.
   - Private veterinarians involved in the capture, transport or holding of animals on behalf of SANParks.
   - Private game capture companies or individuals involved in the capture, transport or holding of animals on behalf of SANParks.

4. **Policies**
   Refer to:
   - SABS 10331: 2000 South African Standard, Code of Practice, Translocation of certain species of wild herbivores, The South African Bureau of Standards
   - SABS 1884 – 1: 2004 South African National Standard Holding pens for temporary housing of animals Part 1: Holding pens for wild herbivores at auctions and in quarantine facilities. Standards South Africa (a division of SABS)
   - SABS 1884 – 1: 2004 South African National Standard Holding pens for temporary housing of animals Part 2: Vehicles for transportation of wild herbivores by road to holding pens and other facilities. Standards South Africa (a division of SABS) (Currently being developed)

   The policy of Veterinary Wildlife Services is to ensure:

   - That the safety of people involved in the capture, transport or holding of animals is not compromised at any time.
   - That operations are planned and the techniques selected to ensure the safety and welfare of the animals.

- That the animal survives the immobilization procedure with the minimum effect on its natural behaviour or other activities.
- That the minimum amount of restraint consistent with accomplishing the task is used.
- That no capture, transport, holding or manipulation of any animals for the purposes of research is undertaken without the approval of the Animal Use and Care Committee.

The administration of immobilizing drugs including, but not restricted to, opioids and cyclohexylamines, and the performing of veterinary procedures are the responsibility of the responsible veterinarian for each operation

5. Definitions

Refer to appendix I

6. Responsibilities

The manager of the VWS is responsible for ensuring:

- The compliance to this procedure
- The detailed planning of this procedure with the assistance of other members of VWS or specialists in the handling and care of the animals to be captured and translocated that he/she deems fit to include in the team chosen to undertake the task

The assigned leader of the team that undertakes the planned procedure will ensure:

- That the procedure is completed as planned and that the objectives are met
- That changes are made to the procedure to meet the needs of the situation that may develop when the planned actions are set in motion and
- That such changes are communicated clearly to all the members of capture team to enable them to complete their supportive roles in completing the procedure

Veterinarian:

- Administration of chemical agents, including immobilizing drugs, performing of veterinary procedures, and health and welfare of animals.

Veterinary technologist:

- Assist veterinarian; collection and processing of biological samples; and record keeping.

Veterinary assistants:

- Provide operational support to the veterinarian and veterinary technologist.

Operations Manager:

- Manage and ensure compliance to this procedure in the capture, holding and relocation operations according to SANParks strategy and policy.

Operations coordinator:

- Implement and coordinate operations including vehicles, equipment, facilities and staff.

Capture supervisor:

- Implementation of capture and transport operations.

Capture assistants:

- Provide operational support to operations coordinator and capture supervisor

Boma supervisor:

- Operation of holding facilities, including aspects of animal husbandry and welfare

Boma assistants:

- Provide operational support to boma supervisor

7. Procedures

7.1 Preparation and planning

The preparation for the capture will be undertaken by members of the capture team (SOP 1. General principles; 1.1 Chemical capture)

7.2 Drug administration

7.2.1 Choice of drugs and dosages to be used.

Drugs used in SANParks for the immobilization and transport of white rhino.

	Immobilizing drug mixture		Tranquillization			
			Boma		Tranquillizers administered as required during transportation	
	Etorphine (mg)	Azaperone (mg)	Zuclopenthixol acetate (mg)	Azaperone (mg)	Zuclopenthixol acetate (mg)	Azaperone (mg)
Adult bull	**4-5**	**40**	**50**	**80**	**50**	**80-160**
Adult cow	**4**	**40**	**50**	**80**	**50**	**80-160**
Sub-adult	**3-4**	**40**	**50**	**60-80**	**50**	**60-120**
Juvenile	**2-3**	**20-30**	**25**	**-**	**25**	**40-80**

All doses should be administered intramuscularly.

The dose of the drugs administered should be adjusted to the circumstances in which the capture will take place. Pregnant animals may need higher doses, animals in poor condition less.

7.2.2 Administration of antidotes (IV or IM)

- Nalorphine should be titrated intravenously in incremental doses of 10mg to effect as a partial antagonist (Nalorphine is currently not available and nalbuphine is a potential alternative)
- Diprenorphine will be given at 3 to 5 times the etorphine dose in mg.
- Naltrexone will be given at 25 to 50 times the etorphine dose in mg if the veterinarian decides to reverse the opioid effects completely.
- Adult and sub-adult animals can be given diprenorphine (0.6-1.2mg) to effect plus nalorphine administered intravenously soon after being immobilized. This reduces muscle tremors, decreases heart rate and appears to improve blood oxygenation (although some initial blood gas determinations do not appear to support this). Repeated doses of diprenorphine and nalorphine can be given to effect as required. The antagonists are given, in the dosages described above, to enable the animal to rise to its feet and be loaded into a crate. Nalorphine can be used alone in animals that are not very deeply immobilized.
- Diprenorphine only partially reverses the effects of etorphine in white rhino and is used in animals that will be transported after capture as they remain heavily sedated due to the continuing effects of the opioids for approximately 3 to 6 hours.
- Naltrexone is a pure antagonist and should only be given to animals that are being released into bomas or back into the field following capture.

7.2.3 Alternative immobilizing opioids

- Carfentanil, Fentanyl and thiafentanil have been used successfully in the immobilization of white rhino.

7. 2.4 Tranquillizers

- Azaperone should be added to the dart for its synergistic effects with etorphine. It appears to reduce the opioid induced excitement during the induction phase and assists in reducing the

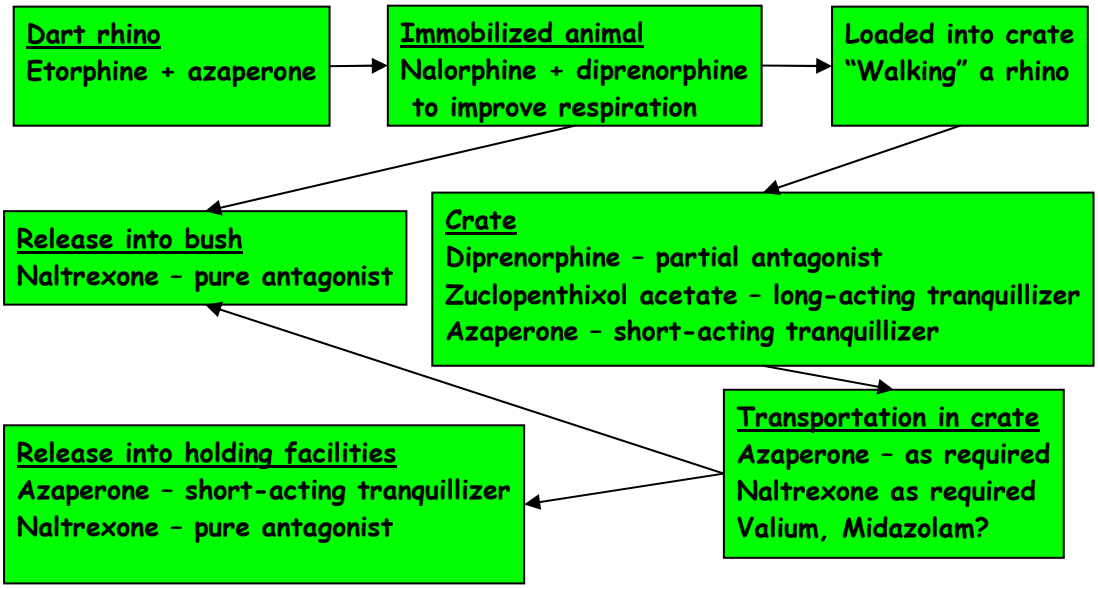
opioid induced increase in blood pressure. Only low doses should be used otherwise the animal may be effected excessively and have difficulty standing on recovery as a result. Administer additional doses of azaperone to animals being transported in crates and immediately prior to release into bomas if greater tranquillization is needed.

- Zuclopenthixol acetate is an effective long-acting tranquillizer for white rhino being transported or held in bomas.

7.2.5 Additional drugs

- Add 2500 to 5000 IU of hyaluronidase to the immobilizing drug mixture to assist drug absorption and thereby reduce the induction time.

7.2.6 Drug combinations used in the immobilization and transport of white rhino



7.2.7 Drug delivery.

- White rhino can be darted on foot although this technique can be very hazardous to the inexperienced as the animals must be darted at relatively close quarters. Rhino are extremely agile and quick on their feet. Vehicles can be used as a darting platform, however, locating and following animals may be difficult especially over rough terrain or through thick bush.
- Darting rhino from a helicopter makes it possible to locate suitable animals quickly. The induction of immobilization can be monitored from the air and the selected individual can be guided to a suitable recovery area. An added advantage is that the helicopter can be used to guide the ground crew via the shortest route to the immobilized animal and to chase away family members or other rhino nearby.
- Darts should be placed in the neck or low on the hindquarters when fired from the ground. Aim for the top of the hindquarters if a helicopter is used. Avoid placing the dart in the shoulders as the skin is thick in this area.
- Long needles (6 – 8 cm) with a barb or collar should be used.
- If mothers and calves are immobilized together the mother will be darted first followed immediately by the calf.

- Induction time should be less than five minutes. If the darted animal is not significantly affected by the drugs within this time it should be given a second full dose.
- Signs of induction include a slowing of pace, elevation of the head and a high stepping gait. The animal will slow to a standstill with its head markedly elevated and then become recumbent.

7.3 Handling of the immobilized animal

- Blindfold the animal as soon as possible.
- Immobilized rhinos can be placed in either sternal or lateral recumbency. The lateral position is generally preferred as it facilitates the circulation of blood to the legs following the physical exertion associated with capture. Rhino that are immobilized for any length of time should be rolled from one side to the other to prevent pressure ischaemia of the limbs.
- Blood oxygenation must be assessed regularly throughout the immobilization period as rhinos soon develop hypoxia. The respiration rate should be at least 6 to 8 breaths per minute. Blood oxygenation will be measured by pulse oximetry or by observing the colour of the arterial blood.
- Heart rates are frequently elevated to 120 to 180 beats/min and this should fall to below a 100 beats/min with the administration of diprenorphine and/or nalorphine. A heart rate of 60 to 80 beats/min. is ideal.
- Body temperature should be monitored and animals that tend to become hyperthermic should be doused liberally with cold water. Hyperthermia can be a serious problem as these bulky animals tend to run long distances after being darted.

7.4 Loading into crates from the field

Add table of drug dosages used for loading-peter and Danny

- Place a crate on the ground in front of the immobilized rhino. Tie a cotton rope around the head behind the second horn. Pass the free end of the rope through the front and out the back of a crate.
- Partially revived the animal using diprenorphine and/or nalorphine, and when it is able to stand pull and guide it into the crate using the rope attached to its head. Use electrical prodders judiciously to encourage the animal to stand and move forward into the crate.
- Give additional doses of diprenorphine to partially revive the animal in the crate. Administer short-acting or long-acting tranquillizers as required.

7.5 Loading into crates from bomas

- Administer 0.8 to 1.0 mg etorphine to an adult rhino in a holding boma.
- When the animal is affected by the drug after 10 to 25 minutes and develops a mild high-stepping action with its head elevated it can be coaxed into the transport crate. This is achieved as the rhino in this state follows a white or light coloured cloth waived in front of it.

7.6 “Walking” a rhino

- An immobilized rhino can be “walked” out of an inaccessible area before it is loaded into a crate.
- Blindfold the anima and fastened a thick rope around the head as is done when these animals are loaded into a crate. Tie a second rope around a hind foot.
- Administer repeated 10 mg doses of nalorphine combined with 0.6 to 1.2 mg diprenorphine intravenously until the rhino can get to its feet when prodded with an electrical prodder. Guide the animal and pull it forward using the head rope. Use the rope on the hind leg to slow the animal

down if necessary. Continue prodding the animal to keep it walking forward.

7.7 Transportation

- Captured rhino can be loaded and transported directly to their destination or they can first undergo an adaptation period of 2 to 4 weeks in bomas before being transported.
- Transport all animals separately in crates. These are either rectangular or rhomboid in shape. Transport the animals facing backwards in rectangular crates and at right angles to the length of the truck in rhomboid crates.
- It is essential that an animal stands normally on all its feet immediately after loading; it is only after this that it can be allowed to lie down. This prevents the animals from lying in an abnormal position and developing neuromuscular problems as a result.
- After the animal is loaded into a crate administer diprenorphine which only partially reverses the effects of the etorphine. This assists in keeping the animal sedated for 3 to 6 hours.
- Inject 50 to 100mg of zuclopenthixol acetate for long-acting tranquillization or repeated doses of azaperone as required during the journey.
- Rhinos that have been tranquillized with excessive doses of drugs may push their horns into a corner of the crate and this increases the risk that the horn will be broken. In these cases small doses of naltrexone (5 mg) can be given intramuscularly to partially reverse the effects of the etorphine. (Modify!)
- Teff or lucerne should be added to the crate as food and as bedding.

7.8 Maintenance in holding facilities

- Bomas are used for an adaptation and training period before transportation of captured animals to another area. Alternatively, they can be used at the destination point to familiarize the rhino with the local conditions before release. Bomas can be used to hold injured animals that need treatment.
- Give both a short together with a long-acting tranquillizer and treat with a “pour-on” acaracide before releasing it into the boma.
- White rhino are social animals and prefer to be in visual or tactile contact with other animals of the same species. Groups of these animals that adapt well to captivity are: a cow with calf at foot; animals that are running together in the wild; and sub- adult animals.
- Bomas should be built with walls strong enough to contain a rhino at full charge and at the same time allow them to see each other and the outside environment.
- Vertical tannalized (CCA) poles sunk 1 m into the ground with two horizontal poles affixed to the outside of the vertical poles forms an ideal wall.
- Use a system of large and small pens to boma train these animals. Large back pens (20m×20m) are used to habituate the animals to captivity after which they are moved to the smaller front pens (12m×6m to accustom them to close confinement.
- Feed the animals twice a day with highly palatable, good quality lucerne and teff. Provide water with an added mineral supplement *ad lib.*
- Monitor food intake and defaecation on a daily basis as some animals may refuse to eat. It is essential that these animals be released back into the free-ranging environment after 10 to 14 days.