

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

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### ABSTRACT

As part of a larger survey to map the geographical distribution of *Babesia* and *Theileria* parasites in the southern African rhinoceros population, white rhinoceroses were sampled during routine immobilisations in the Kruger National Park. Polymerase chain reaction (PCR) and reverse line blot (RLB) hybridisation assays were used to screen for the presence of piroplasms and complete blood counts were used to assess associated changes in clinical parameters. Of the 195 rhinoceroses sampled, 71 (36.4 %) tested positive for the presence of *Theileria bicornis*, with no significant change in the haematological parameters measured, while 18 (9.2 %) tested positive for *Theileria equi*. None of the rhinoceroses sampled tested positive for *Babesia bicornis*, a parasite associated with mortalities in black rhinoceroses.

**Keywords:** anaemia, *Babesia bicornis*, *Theileria bicornis*, *Theileria equi*, white rhinoceros.

Govender D, Oosthuisen M C, Penzhorn B L **Piroplasm parasites of white rhinoceroses (*Ceratotherium simum*) in the Kruger National Park, and their relation to anaemia.** *Journal of the South African Veterinary Association* (2011) 82(1): 36–40 (En.). Scientific Services, South African National Parks, Private Bag X402, Skukuza, Mpumalanga, 1350 South Africa

### INTRODUCTION

There have been many records of both *Babesia* and *Theileria* spp in wild animals<sup>22,27</sup>. In most cases these were incidental findings, but piroplasmosis has been implicated in losses amongst wildlife, generally related to stress<sup>22</sup>. 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<sup>8</sup>.

There is a paucity of information on the occurrence of piroplasms in African rhinoceroses in general, but particularly in white rhinoceroses (*Ceratotherium simum*), where a single published paper could be traced<sup>1</sup>. This paper reported the results of an *ad hoc* survey of 106 white rhinoceroses in Zululand (northern KwaZulu-Natal, South Africa) during 1967 to 1969, where basic microscopy revealed a 32.1 % prevalence of a *Theileria*-like piroplasm and a 1.9 % preva-

lence of a large *Babesia* sp<sup>1</sup>. The latter is of concern as the recently described *Babesia bicornis* has been associated with fatalities in black rhinoceroses (*Diceros bicornis*) in East and South Africa<sup>2,19</sup>. The recently described *Theileria bicornis*, on the other hand, appears to be apathogenic in black rhinoceroses<sup>19</sup>.

The southern white rhinoceros (*C. s. simum*) population in South Africa increased from near extinction in 1900 to more than 11 320 animals at the beginning of the 21st century<sup>4,23,24</sup>, with over 8000 individuals occurring in the Kruger National Park (KNP) alone. Efforts to restock private reserves within their former range and to strengthen and augment present populations have resulted in a rise in rhinoceros immobilisations and translocations. This may pose a risk, however, as little is known about susceptibility of rhinoceroses to various infections and the role that translocations may play in the epidemiology of disease<sup>5</sup>. 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<sup>11,15,16,17</sup>.

Changes to the haematological picture in babesiosis can be quite varied, with anaemia, leukopenia or leukocytosis and/or thrombocytopenia all being recorded<sup>29</sup>. 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<sup>3,18,21</sup> and the presence of a haemolytic factor in serum, which has been described in *Babesia gibsoni* infection<sup>20</sup>.

Routine immobilisation of white rhinoceroses in KNP for management purposes offered the opportunity to determine the prevalence of *Babesia* and *Theileria* spp. in their blood, using the reverse line blot (RLB) hybridisation assay, and to evaluate whether these piroplasms, when present, are pathogenic to their host (as manifested by development of anaemia).

### MATERIALS AND METHODS

#### Study area

The study was conducted on animals selected in the southern Morula Section of the KNP, South Africa (24°0'41"S 31°29'7"E) bordered by the Sabie and Crocodile Rivers. This area represents the highest white rhinoceros density in KNP (Fig. 1.)

#### Sample collection

White rhinoceroses ( $n = 195$ ) were selected randomly during routine immobilisation between January 2003 and September 2006. These included both males and females of all age classes, a stratified sample representing the age and sex class ratios found in the KNP at the time of the project. Blood was collected from the auricular vein, using 6 ml Vacutainer® (Franklin Lakes, USA) tubes with EDTA anticoagulant, once the animal was immobilised and in lateral recumbency. Blood tubes were then labelled with the animal-specific details as well as date and GPS location. Specimens were placed in a cooler box and transported to the laboratory.

#### Sample processing

At the laboratory, the blood tubes were placed on a roller for c. 5 min or until the contents were properly mixed. Five  $\mu$ l of the sample was run on an automated blood counter machine (ABC Vet) to

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Received: January 2011. Accepted: March 2011.

attain complete blood counts. The remainder of the blood sample was pipetted into 3.7 ml NUNC tubes and frozen as EDTA-buffered whole blood in a -10 °C freezer. Samples were then batched and taken to the Molecular Biology Laboratory, Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria.

#### DNA extraction

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.

#### PCR

One set of primers was used to amplify a 460- to 520-bp fragment of the 18S SSU rRNA spanning the V4 hypervariable region<sup>7,14</sup>. The forward primer, RLB-F [5'-GAC ACA GGG AGG TAG TGA CAA G-3'] and the biotin-labelled reverse primer, RLB-R [5'-Biotin-CTA AGA ATT TCA CCT CTA ACA GT-3'], hybridised with regions conserved for *Theileria* and *Babesia* spp. Methods described were similar to those followed by Gubbels *et al.*<sup>7</sup>, with minor changes. 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 2nd cycle by 2 °C 5 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 min at 72 °C, and left at 4 °C.

#### RLB hybridisation

Two *Theileria/Babesia*-specific membranes were used to allow for the maximum number of samples to be run concurrently. Membrane 1 was prepared according to the method described by Gubbels *et al.*<sup>7,25</sup>. Membrane 2 was a ready prepared Isogen Life Science membrane (Maarssen, the Netherlands). Both membranes utilised the same probe sequences (Table 1) and probe concentrations. The RLB hybridisation method was adapted from Gubbels *et al.*<sup>7</sup>, the only difference being that 10 µl of PCR product was used instead of 40 µl.

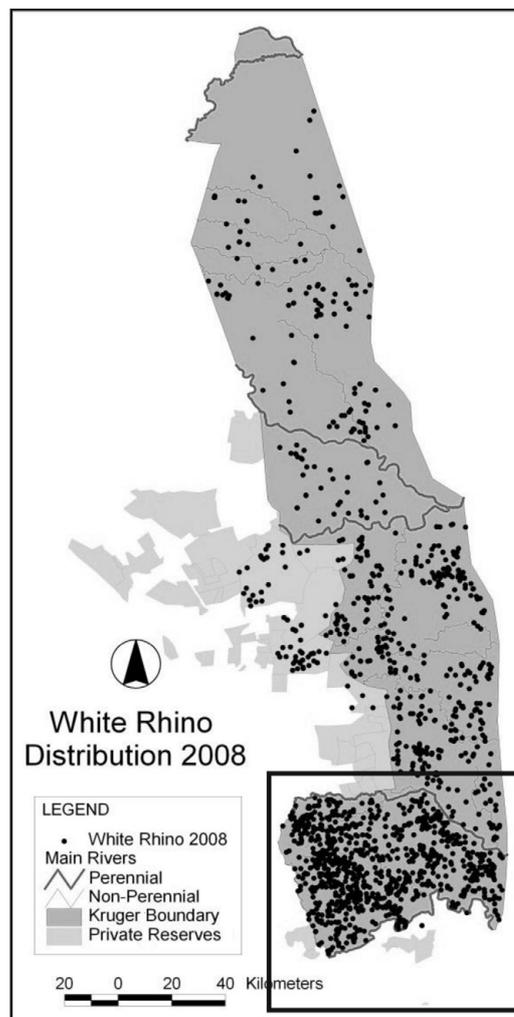


Fig. 1: Map of the Kruger National Park showing white rhinoceros densities, with the study area (defined by the box) having the highest white rhinoceros densities in the Park (GIS Laboratory, SANParks, Skukuza).

Table 1: List of genus- and species-specific RLB hybridisation probes and their corresponding probe sequence. Symbols used to indicate degenerate positions: R = A/G; W = A/T; Y = C/T.

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

### Data analysis

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

## RESULTS

### Frequencies of infection

Using the RLB hybridisation assay, none of the 195 samples tested positive for the presence of *B. bicornis*, while a prevalence of 36.4 % was found for *T. bicornis* (Table 2). There were no significant differences between the various age/sex classes when the 5 groups were compared (Table 2); when compared with the rest of the population, however, the subadult females showed a significantly higher prevalence ( $\chi^2 = 5.68$ ;  $P = 0.01$ ).

There was an unexpected 9.2 % prevalence of *Theileria equi* in the study population. There appeared to be a significant correlation ( $\chi^2 = 19.26$ ;  $P < 0.0001$ ) between membrane used and detection of these parasites, with the Isogen Life Science membrane detecting higher rates of infection compared with the 'in-house' prepared membrane. All animals positive for *T. equi* were also positive for *T. bicornis*; the signal for *T. equi*, although strong, was significantly weaker than the *T. bicornis* signal or the *T. equi*-positive control.

### Seasonal differences in prevalence

Monthly and seasonal occurrences of *T. bicornis* were compared (Fig. 2 and Table 3). Although the prevalence of infection was higher during the wet season, this did not differ significantly from the prevalence during the dry season.

### Blood profiles

Haematology values 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, red blood corpuscles (RBCs), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) or platelets (PLTs) (Table 4). There was a slight elevation in white blood corpuscles count (WBC) (leucocytosis) in infected animals, but the difference was not significant ( $P = 0.068$ ; Mann-Whitney non-parametric test).

## DISCUSSION

The prevalence of *T. bicornis* in the KNP white rhinoceros population is similar to

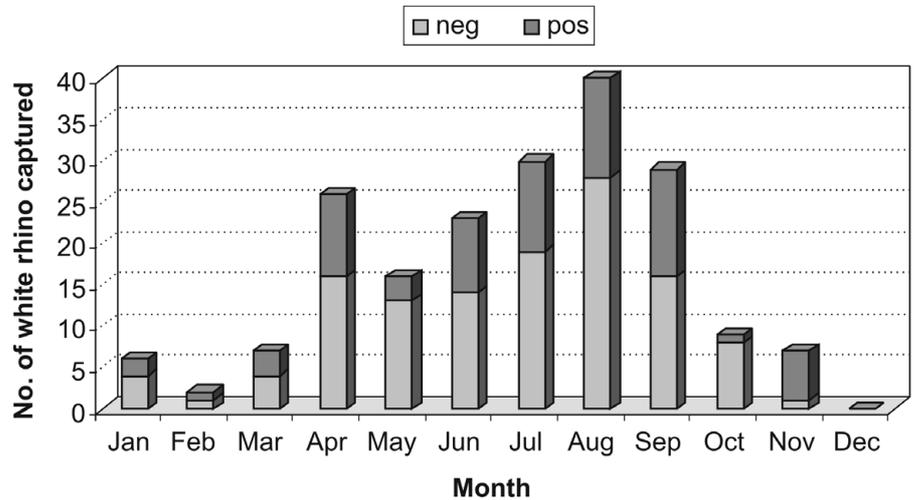


Fig. 2: Monthly variation in prevalence of *Theileria bicornis* infection in white rhinoceroses in the Kruger National Park.

Table 2: Prevalence of infection of *Theileria bicornis* and *Theileria equi* in white rhinoceroses of various age and sex classes in the Kruger National Park.

	Number	<i>T. bicornis</i> -positive	<i>T. equi</i> -positive
Adult bull <sup>a</sup>	55	18 (32.7 %)	6 (10.9 %)
Adult cow <sup>b</sup>	21	6 (28.6 %)	0
Subadult bull <sup>c</sup>	34	10 (29.4 %)	3 (8.8 %)
Subadult cow <sup>d</sup>	67	32 (47.8 %)	7 (10.5 %)
Calf <sup>e</sup>	18	5 (27.8 %)	2 (11.1 %)
Total	195	71 (36.4 %)	18 (9.2 %)

<sup>a</sup>Male, breeding animal 8 years or older.

<sup>b</sup>Female, breeding animal 6 years or older.

<sup>c</sup>Male animal, 4–8 years old, that has not yet acquired a territory.

<sup>d</sup>Young female animal that has not yet calved.

<sup>e</sup>Both male and female animals still with the dam and usually less than 2.5 years old.

that reported in Zululand (northern part of KwaZulu-Natal)<sup>1</sup>. The RLB hybridisation assay was not able to detect *B. bicornis* in any of the samples. These could be either true negative results or due to a very low *B. bicornis* parasitaemia which was below the detection limit of the test. Blood smears of a debilitated black rhinoceros in KNP showed erythrocytes parasitised by large *Babesias* (R Bengis, Skukuza State Veterinary Services, pers. comm., 2008). The 2 rhinoceros species in the KNP are hosts to the same tick species<sup>10</sup>, suggesting that *B. bicornis* could be expected to occur in both rhinoceros species.

Given the density of white rhinoceroses in the KNP, a concerted effort was made to select only healthy, nutritionally unstressed animals for translocation. This may have

biased our results and account for the absence of *B. bicornis* in the study population.

The complete blood-count parameters that were measured did not show significant changes, although there was an indication of an increase in white blood cells in infected rhinoceroses. Anaemia, although sometimes seen as a classical early detection sign for babesiosis, was not detected in animals infected with *T. bicornis*.

The subadult female proportion of the population showed a significantly higher rate of infection with *T. bicornis* compared with the rest of the population. Subadult females are most likely the segment of the population undergoing the most dramatic changes: they are becoming reproductively active, participate in courtship and mating and are often early

Table 3: Dry and wet season variation in prevalence of *Theileria bicornis* in white rhinoceroses in the Kruger National Park.

	Number	Positive (%)	Negative (%)	<i>P</i> -value <sup>c</sup>
Dry season <sup>a</sup>	164	58 (35.4)	106 (64.6)	0.467
Wet season <sup>b</sup>	31	13 (41.9)	18 (58.1)	

<sup>a</sup>April to September; <sup>b</sup>October to March; <sup>c</sup>Chi-square test.

Table 4: Haematology values for white rhinoceroses positive and negative for *Theileria bicornis*, respectively.

Parameter	<i>T. bicornis</i> -positive				<i>T. bicornis</i> -negative				P-value <sup>a</sup>
	Mean	SD	Min	Max	Mean	SD	Min	Max	
WBC <sup>b</sup> (10 <sup>3</sup> /uL)	24.50	8.052	10.10	39.50	19.97	6.75	8.20	36.90	0.067
RBC <sup>c</sup> (10 <sup>6</sup> /μL)	6.90	1.77	3.44	11.56	7.32	2.77	2.94	17.60	0.490
HGB <sup>d</sup> (g/dL)	14.71	4.01	7.70	25.00	14.79	3.56	6.40	24.70	0.740
Hct <sup>e</sup> (%)	40.23	7.46	23.10	48.70	42.86	8.84	19.60	56.30	0.274
MCV <sup>f</sup> (fl)	62.62	3.78	56.00	69.00	62.90	4.56	55.00	71.00	0.803
MCH <sup>g</sup> (pg/cell)	21.28	1.51	19.80	25.00	20.86	1.47	18.40	23.30	0.607
MCHC <sup>h</sup> (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

<sup>a</sup>Mann-Whitney non-parametric test for statistical significance.

<sup>b</sup>White blood corpuscles.

<sup>c</sup>Red blood corpuscles.

<sup>d</sup>Haemoglobin.

<sup>e</sup>Haematocrit.

<sup>f</sup>Mean corpuscular volume.

<sup>g</sup>Mean corpuscular haemoglobin.

<sup>h</sup>Mean corpuscular haemoglobin concentration.

pregnant when captured. This may result in high stress levels and a hormonal milieu that may induce immune suppression, making these animals more susceptible to haemoparasites<sup>6,26,28</sup>.

A 9.2% prevalence of *T. equi* was seen in the study population. Only animals positive for *T. bicornis* were positive for *T. equi* and the RLB signal, although strong, was significantly weaker than the *T. bicornis* signal or the *T. equi*-positive control. This could suggest possible cross reaction of the *T. equi* probe with the *T. bicornis* amplicons. When investigating the *T. equi* RLB probe region within the V4 hyper-variable area, it was shown that *T. equi* probe differed by 8 base nucleotides which, under normal circumstances, should be sufficient to prevent hybridisation of the probe and the PCR amplicons. If the temperature of the hybridisation was not optimal, however, some cross-reaction may have been possible. It should also be kept in mind that it has previously been shown with 18S rRNA gene sequencing and phylogenetic analysis that *T. bicornis* clustered closely with *T. equi*<sup>9,19</sup>. Sequencing and cloning of the parasite 18S rRNA gene of the samples in question will have to be done to clarify the matter.

The apparent lack of seasonal variation in *T. bicornis* infection, although it might be expected due to the seasonality of their tick vectors, might be explained by the fact that these parasites are known to cause chronic asymptomatic infection that can persist for months or years<sup>12</sup>. 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<sup>13</sup>.

## CONCLUSION

Although none of the rhinoceroses sampled tested positive for *B. bicornis*, a parasite associated with mortalities in black rhinoceroses, this study confirmed a relatively high prevalence of *T. bicornis* in the white rhinoceros population in the KNP. It also revealed that *T. bicornis* did not seem to be detrimental to the infected animals. This could represent an endemically stable situation, where coevolution of parasite and host has been pushed to the degree where long-term asymptomatic infection ensures parasite survival and persistence in the environment.

## ACKNOWLEDGEMENTS

We thank the National Research Foundation (though grant GUN 65022 to B L Penzhorn) and the Wildlife Group of the South African Veterinary Association for funding this research; SANParks for laboratory support and access to biological samples; Miss Jenny Joubert and Mr Khosi Maseko for sample collection and storage; Mrs Rina Owen for assisting with the statistical analysis; Dr Raksha Bhoora and Dr Kgomotso Sibeko for assisting with the analysis of samples. This project (V045/07) was approved by the Research Committee of the Faculty of Veterinary Science and the Animal Use and Care Committee of the University of Pretoria.

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