Transboundary and Emerging Diseases

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SHORT COMMUNICATION

Three Novel Haplotypes of *Theileria bicornis* in Black and White Rhinoceros in Kenya

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Summary

Piroplasms, especially those in the genera *Babesia* and *Theileria*, have been found to naturally infect rhinoceros. Due to natural or human-induced stress factors such as capture and translocations, animals often develop fatal clinical piroplasmosis, which causes death if not treated. This study examines the genetic diversity and occurrence of novel *Theileria* species infecting both black and white rhinoceros in Kenya. Samples collected opportunistically during routine translocations and clinical interventions from 15 rhinoceros were analysed by polymerase chain reaction (PCR) using a nested amplification of the small subunit ribosomal RNA (18S rRNA) gene fragments of *Babesia* and *Theileria*. Our study revealed for the first time in Kenya the presence of *Theileria bicornis* in white (*Ceratotherium simum simum*) and black (*Diceros bicornis michaeli*) rhinoceros and the existence of three new haplotypes: haplotypes H1 and H3 were present in white rhinoceros, while H2 was present in black rhinoceros. No specific haplotype was correlated to any specific geographical location. The Bayesian inference 50% consensus phylogram recovered the three haplotypes monophyleticly, and *Theileria bicornis* had very high support (BPP: 0.98). Furthermore, the genetic *p*-uncorrected distances and substitutions between *T. bicornis* and the three haplotypes were the same in all three haplotypes, indicating a very close genetic affinity. This is the first report of the occurrence of *Theileria* species in white and black rhinoceros from Kenya. The three new haplotypes reported here for the first time have important ecological and conservational implications, especially for population management and translocation programs and as a means of avoiding the transport of infected animals into non-affected areas.

Introduction

The eastern black rhinoceros (*Diceros bicornis michaeli*) is indigenous to East Africa, but is currently extant only in Kenya and Tanzania. Black rhinoceros were so abundant in Kenya in the early twentieth century (Brett, 1990) that they were treated as agricultural pests and hampered human settlement in eastern Kenya (Hunter, 1952). There were around 20 000 individuals in the 1970s (Emslie and Knight, 2012) when the species was still widely distributed throughout Kenya. The population then declined catastrophically over the following 20 years as a result of authorized hunting, poaching and human settlements and bottomed out at fewer than 400 animals by 1990 (Okita-Ouma et al., 2007). This decline created a situation of small isolated, demographically unviable populations scattered across fragmented regions of Kenya, many of which now face local extinction. An ambitious translocation programme for isolated rhinoceros populations begun in 1993 (KWS, unpublished data) and focuses on moving

rhinoceros into high-security breeding sanctuaries, thereby enhancing their security and breeding prospects (Merz, 1994). New sanctuaries have been gradually established by translocating offspring from these breeding nuclei and by 2008 the black rhinoceros population in Kenya had risen to over 630 animals in 16 subpopulations (Emslie et al., 2009).

In Kenya today, in addition to the eastern black rhinoceros, there are also populations of both the southern white (*Ceratotherium simum simum*) and northern white (*Ceratotherium simum cottoni*) rhinoceros. The southern white, indigenous to southern Africa, was introduced into Kenya and Uganda in the early 1980s (KWS, unpublished data); on the other hand, the northern white is extinct from its natural range in central African countries (Rookmaaker and Pierre-Olivier, 2012), although four of these rhinoceros were introduced into the Ol Pejeta Conservancy in Kenya in 2009.

Although small in size, the population of the Eastern black rhinoceros in Kenya represents about 90% of this subspecies' global population (Emslie, 2011) and has an annual population growth of about 4%. In Kenya, the translocation of individual rhinoceros from various subpopulations is frequently undertaken to establish, re-establish or augment local populations and is an important conservation tool used to manage the species *in situ* (Woodford and Rossiter, 1993). Translocation has associated disadvantages such as the risk of introducing destructive pathogens into naive wildlife populations and the exposure of translocated animals to pathogens in the new release site (Woodford and Rossiter, 1993; Chipman et al., 2008). The process of capture and translocation is an inherently stressful event for game animals and often compromises animals' immune defences (Woodford and Rossiter, 1993). In natural systems, parasites co-evolve with their hosts and develop equilibrium or endemic stability in which infected hosts do not develop the disease (Penzhorn et al., 1994; Penzhorn, 2006). However, when the host–parasite equilibrium is altered by various stressor conditions, latent infections can develop into disease (Penzhorn et al., 1994; Cindy, 2002; Penzhorn, 2006; Mutinda et al., 2012).

A group of blood-borne protozoans of the order Piroplasmida and generically referred to as piroplasms have been linked to rhinoceros morbidity and mortality in Tanzania, South Africa and Kenya (Nijhof et al., 2003; Penzhorn, 2006; Obanda et al., 2011). This suggests that infectious diseases (and not only poaching) are an emerging threat to the conservation of rhinoceros (Ramsey and Zainuddin, 1993; Penzhorn et al., 1994). The connection between translocation and the onset of diseases such as piroplasmosis is of interest because translocation is a tool that is frequently employed in the management of this rhinoceros metapopulation *in situ* (Emslie et al., 2009). Piroplasm parasites associated with mortalities in black rhinoceros in South Africa and Tanzania were recognized as the novel species *Theileria bicornis* and *Babesia bicornis* (Nijhof et al., 2003). It is unclear whether or not these lethal species occur and circulate in the Kenyan rhinoceros metapopulation. Moreover, it is not known whether other known or unknown species of piroplasms may latently infect rhinoceros in their various Kenyan subpopulations.

With the advance of current molecular and genetic techniques, more novel species of *Theileria* and *Babesia* are being discovered and their phylogenetic relationships are becoming increasingly well understood. Analyses of 18S rRNA gene fragments have been successfully applied in the identification and classification of several previously unknown *Theileria* and *Babesia* species (Quick et al., 1993, 1993; Persing et al., 1995; Schnittger et al., 2000; Bhoora et al., 2009; Chaisi et al., 2013). Furthermore, the phylogenetic classification of cattle-infecting piroplasms via the analysis, and comparison of 18S rRNA gene fragments has been shown to match traditional taxonomy and provides additional information on their evolutionary relationships (Quick et al., 1993). In the present study, we investigated the genetic diversity and occurrence of piroplasm species infecting both black and white rhinoceros populations in Kenya using a nested amplification of the 18S rRNA genes of *Babesia* and *Theileria*.

Materials and Methods

Study population

In this study, blood samples from 15 rhinoceros (*Diceros bicornis michaeli* $n = 12$ and *Ceratotherium simum simum* $n = 3$) were collected from various national parks and rhinoceros sanctuaries in Kenya (Table 1). Samples were taken from animals from different subpopulations located in different geographical and ecological habitats.

Sampling was carried out opportunistically during scheduled immobilizations for population and health management or during the ear-notching activity that is routinely performed for identification purposes. Rhinoceros were immobilized using 4 μg/kg (body weight) of etorphine hydrochloride (Novartis, Johannesburg, South Africa) combined with 5000 IU hyaluronidase (Kyron Laboratories, Benrose, South Africa) and 80 μg/kg (body weight) total dose of xylazine hydrochloride (Kyron Laboratories). The animals were darted, and upon recumbency blood was drawn from the radial vein of the foreleg and preserved in ethylenediamine tetra-acetic acid tubes. The sex and age of the animals were identified using morphological criteria (Mutinda et al., 2012). Samples were conserved in frozen liquid nitrogen and transported to the laboratory. Animals were revived by the injection of 18 μg/kg (body weight) diprenorphine (M5050®; Kyron Laboratories) and 6 μg/kg (body weight) atipamezole hydrochloride (ANTISEDAN®;

Table 1. *Theileria bicornis* haplotypes identified in the sampled white (*Ceratotherium simum simum*) and black (*Dicerous biconis michaeli*) rhinoceros populations

Species	Age	Sex	Geographical location	*Theileria bicornis* haplotype
D. bicornis michaeli	Subadult	Female	Lake Nakuru National Park	HI
C. simum simum	Adult	Female	Lake Nakuru National Park	H2
C. simum simum	Adult	Male	Meru National Park	H2
C. simum simum	Subadult	Male	Meru National Park	H2
D. bicornis michaeli	Juvenile	Male	Ngulia	H3
D. bicornis michaeli	Adult	Female	Mugie	H3
D. bicornis michaeli	Adult	Male	Mugie	H3
D. bicornis michaeli	Subadult	Male	Meru National Park	H3
D. bicornis michaeli	Subadult	Male	Nairobi National Park	H3
D. bicornis michaeli	Juvenile	Female	Nairobi National Park	H3
D. bicornis michaeli	Juvenile	Female	Nairobi National Park	H3
D. bicornis michaeli	Subadult	Male	Lake Nakuru National Park	H3
D. bicornis michaeli	Subadult	Female	Solio	H3
D. bicornis michaeli	Subadult	Male	Mugie	H3
D. bicornis michaeli	Adult	Male	Mugie	H3

Kyron Laboratories) into an ear vein. The white rhinos were also injected with 25 μg/kg (body weight) naltrexone hydrochloride (Naltrexone; Kyron Laboratories) intramuscularly to prevent re-narcotization. All rhinos were back on their feet in approximately 3 min.

DNA isolation and PCR amplification

Genomic DNA was extracted from blood using a genomic DNA extraction kit (DNeasy blood and Tissue Kit; Qiagen, Southern Cross Biotechnologies, South Africa) following the manufacturer's protocol. A nested PCR amplification specific for the 18S rRNA gene of *Babesia* and *Theileria* was performed (Maamun et al., 2011). A primary amplification was carried out in a 50 μl reaction volume containing 3 μl of the genomic DNA, 45 μl of Platinum blue supermix (Applied Biosystems, Johannesburg, South Africa) and 0.25 μM each of the forward and reverse primers. The forward primer was ILO-9029, (5′-CGGTAATTCCAGCTC CAATAGCGT-3′) and the reverse primer, ILO-9030 (5′-18 TTTCTCTCAAAGGTGCTGAAGGAGT-3′). The amplification (Thermocycler, Veriti; Applied Biosystems, Johannesburg, South Africa) was preceded by a 30-s polymerase activation step at 95°C followed by 30 cycles each of 1 min of denaturing at 94°C, 1 min of annealing at 53°C for 30 s and extension for 1 min at 72°C. Amplification was terminated by a final extension step of 72°C for 9 min. The secondary amplification was performed in a 50-μl reaction volume containing 2 μl of the primary amplification product, 45 μl of platinum blue supermix and 0.3 μM each of the forward and reverse primers. The forward primer was MWG4/70, (5′-AGCTCGTAGTTGAATTTCTGCTGC-3′) and the reverse primer, ILO-7782 (5′-AACTGACGACCTC CAATCTCTAGTC-3′). The secondary PCR (Thermocycler, Veriti; ABI) was begun with an initial denaturation at 95°C for 30 s, followed by 30 cycles of 1 min each at 94°C, annealing at 55°C for 30 s and extension at 72°C for 1 min. The PCR was completed with a final extension step of 72°C for 9 min.

Polymerase chain reaction products showing successful amplification from agarose gel analysis were directly sequenced for both strands. PCR products were purified for direct sequencing by enzymatic treatment using exonuclease I and shrimp alkaline phosphatase (PCR Product Presequencing Kit; Amersham Biosciences, Buckinghamshire, UK). All DNA sequencing was carried out by direct cycle sequencing on both strands of purified PCR DNA products from PCR amplification using MWG4/70 and ILO-7782 primers (0.1 μM from each). Sequencing reactions were carried out with the ABI PRISM DigDye Terminator v3.1 cycle sequencing kit and analysed on an ABI310 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Molecular analyses

The complementary reads were used to resolve rare ambiguous base-calls in Sequencher v.4.9. Sequences were aligned in Seaview v.4.2.12 (Gouy et al., 2010) under ClustalW (Larkin et al., 2007) default settings. Incomplete terminal sequences were removed from the alignment. Nucleotide substitutions and *p*-uncorrected distances were performed in MEGA v5 (Tamura et al., 2011), and phylogenetic analyses were performed with Mr. Bayes v.3.1.2 (Huelsenbeck and Ronquist, 2001). Sequence BLAST searches were conducted in GenBank to identify homologous sequences (Altschul et al., 1990). Sequences with

98% or greater similarity to our target sequences and others belonging to the same genus (as suggested from the BLAST searches) of African origin were also included in the alignment. The designated out-group was *T. gondii* following Nijhof et al. (2003).

The most appropriate substitution model for the Bayesian inference was determined by the Bayesian information criterion (BIC) in Model test v.0.1.1 (Posada, 2008). Mr. Bayes was used with default parameters and Markov chain settings and with random starting trees. The gamma shape parameter and proportion of invariant sites were estimated from the data. Each run consisted of four chains of 10 000 000 generations, sampled every 10 000 generations for a total of 1000 trees. A plateau was reached after few generations with 25% (250 trees) of the trees resulting from the analyses discarded as 'burn-in'.

Ethics

The Committee of the Department of Veterinary and Capture Services of the Kenya Wildlife Service (KWS) approved the study and the animal capture, translocation and sample collection. KWS guidelines on Wildlife Veterinary Practice-2006 were followed. All KWS veterinarians complied with the Veterinary Surgeons Act, Cap. 366, Laws of Kenya, that regulates veterinary practice in Kenya.

Results and Discussion

Amplification products of 385 bp were generated from the samples taken from Kenyan black and white rhinoceros. The PCR products were sequenced, and BLAST analyses indicated that the sequences were most similar to *T. bicornis* (Nijhof et al., 2003). Three haplotypes were recovered (H1, H2 and H3) from the sampled animals and are shown in Table 1. Their sequences are deposited in GenBank with accession numbers KC771140 (H1), KC771141 (H2) and KC771142 (H3). Haplotypes H1 and H3 were present in white rhinoceros, while H2 was presented in black rhinoceros. No specific haplotype was correlated with any specific geographical location. The best-fitting model for the BML tree was TIM3+I+G ($-\ln L = -1584.9442$, BIC = 3396.1116). The Bayesian inference 50% consensus phylogram recovered all the haplotypes monophyletic with *T. bicornis* with very high support (BPP: 0.98) (Fig. 1). Furthermore, the genetic *p*-uncorrected distances and substitutions between *T. bicornis* and the three haplotypes were the same in all three haplotypes, indicating very close genetic affinity (Table 2).

Members of the Order Piroplasmidae include an assemblage of intra-erythrocytic protozoans that are vectored by diverse ticks and cause disease in humans, livestock and wildlife. Infections with piroplasms range from severe acute to mild subclinical forms. Piroplasms, especially *T. parva*, cause major economic losses in the livestock industry. Although wild animals are just as susceptible to piroplasms, most parasites occur latently due to co-evolution with their hosts. However, under stressful conditions, latent infections can flare up and cause clinical piroplasmosis. Capture and translocation is a management strategy that is widely used to manage populations *in situ*, and, in many cases, the stress of the translocation can be linked to the onset of

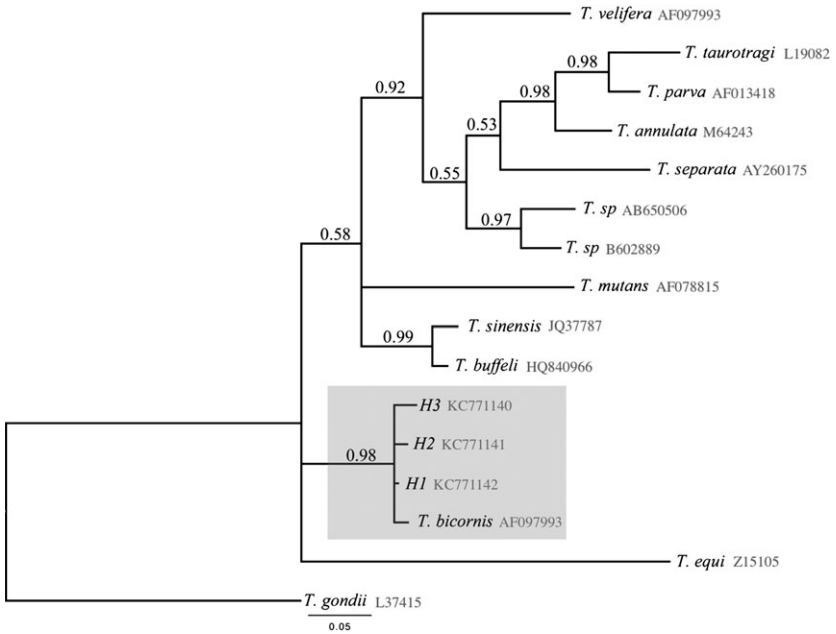

Fig. 1. Bayesian inference 50% consensus phylogram of *Theileria* species (18S rRNA partial sequence). Values by nodes are the posterior probabilities recovered from the Bayesian analysis. All three recovered haplotypes (H1, 2, 3) and *T. bicornis* are shown in the shaded area. GenBank accession numbers are in grey colour.

Table 2. Nucleotide substitutions (above diagonal) and *p*-uncorrected distances (below diagonal) for each pairwise comparison between *Theileria* species

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

All three recovered haplotypes (H1, 2, 3) and *T. bicornis* are shown in the shaded area. Abbreviations on the top of the table correspond to the three first letters of the specific name. For GenBank accession numbers, see Fig. 1.

clinical piroplasmosis. Various *Babesia* and *Theileria* spp. have been found incidentally to occur in wildlife. Nijhof et al. (2003) showed that *Babesia bicornis* could be linked to fatal disease, although these authors presented no evidence to suggest that *T. bicornis* is pathogenic for the black rhinoceros.

Findings from this study confirm for the first time the presence of new *T. bicornis* haplotypes circulating among both white and black rhinoceros in Kenya; however, these haplotypes were not shared between these two rhinoceros species. *Theileria bicornis* was first isolated from black rhinoceros but since then has been detected in nyala, white rhinoceros and cattle (Muhanguzi et al., 2010; Pfitzer et al., 2011), thereby suggesting that it has a wide host range. Although this parasite has never been associated with fatal clinical infection in black rhinoceros in South Africa, it is closely related to *T. equi* (Nijhof et al., 2003), which is pathogenic in equids and causes stress-induced piroplasmosis in, for instance, recently captured Grevy's zebra and plains zebra in Kenya and Uganda (Dennig, 1966).

Our results indicate that the new *T. bicornis* haplotypes are widespread among the major rhinoceros subpopulations in Kenya. Such a wide distribution may be advantageous as many individuals are not immunologically naive to this pathogen, and hence, there is less risk of clinical disease in cases where individuals are released into endemic areas.

Despite the positive impact of rhinoceros population management in Kenya, translocations have key shortcomings associated with the risk of introducing destructive pathogens into naive wildlife population and the exposure of translocated animals to pathogens in the new release sites (Woodford and Rossiter, 1993; Chipman et al., 2008). The findings of our study suggest that these translocations could have exposed the naive rhinoceros population to endemic areas where there are other piroplasm hosts such as buffalo (*Syncerus caffer*), hartebeest (*Alcelaphus buselaphus*), eland (*Taurotragus oryx*) (Burridge, 1975) and bushbuck *Tragelaphus scriptus*, among others (Benson et al., 2006). This type of management may therefore increase the possibility of successfully transmitting piroplasms from the alternative host to naive rhinoceros and is likely to account for the maintenance of these three new haplotypes. These parasites have co-evolved with their hosts and have developed an equilibrium or endemic stability in which infected hosts do not develop the disease (Penzhorn et al., 1994; Penzhorn, 2006). However, when the host–parasite equilibrium is altered by various stressor conditions, latent infections may develop into disease (Penzhorn et al., 1994; Cindy, 2002; Penzhorn, 2006).

Nevertheless, the transmission dynamics and the specific vectors of *T. bicornis* are still unknown, although ixotid ticks of the species *Amblyomma rhinoceroscerotis* and *Dermacentor rhinoceros,* known to specifically feed on both

black and white rhinoceros, are suspected to play a role in parasite transmission between individuals (Horak and Penzhorn, 1997). Tick distribution is dynamic and it is possible that the tick species that are vectors for *T. bicornis* are widely spread in all the parks sampled and thus help to ensure transmission.

Conclusions

This is the first record of *T. bicornis* and its three new haplotypes in both black and white rhinoceros in Kenya. The parasite is well established in this country's rhinoceros population because several major subpopulations were found to be positive, thereby suggesting that the majority of the rhinoceros are immunologically challenged and exist in a state of endemic stability with this parasite. This may explain the lack of clinical theileriosis, even during capture. However, as this parasite could be multihost, it is likely to be maintained in a habitat even in the absence of rhinoceros. Further epidemiological studies that include the identification of other hosts within rhinoceros sanctuaries still need to be carried out. Although we did not detect *B. bicornis,* further investigations in other subpopulations are required in the future.

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Declaration of Interest

The authors report no conflict of interests. The authors alone are responsible for the research and the writing of this paper.

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