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The identification of *Theileria bicornis* in captive rhinoceros in Australia

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ABSTRACT

Poaching of both black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros in Africa has increased significantly in recent years. In an effort to ensure the survival of these critically endangered species, breeding programs were established in the 1990s in Australia, where a similar climate and habitat is available. In this study we examined blood samples from two *C. simum*, including a 16 yr old female (Aluka) who died in captivity, and a 17 yr old asymptomatic male (Umfana). Bloods from seven healthy *D. bicornis* housed at the zoo were also collected. All samples were tested for the presence of piroplasms via blood smear and PCR. A generic PCR for the 18S rRNA gene of the Piroplasmida revealed the presence of piroplasm infection in both dead and asymptomatic *C. simum*. Subsequent sequencing of these amplicons revealed the presence of *Theileria bicornis*. Blood smear indicated that this organism was present at low abundance in both affected and asymptomatic individuals and was not linked to the *C. simum* mortality. *T. bicornis* was also detected in the *D. bicornis* population (n = 7) housed at Taronga Western Plains Zoo using PCR and blood film examination; however only animals imported from Africa (n = 1) tested *T. bicornis* positive, while captive-born animals bred within Australia (n = 6) tested negative suggesting that transmission within the herd was unlikely. Phylogenetic analysis of the full length *T. bicornis* 18S rRNA genes classified this organism outside the clade of the transforming and non-transforming *Theileria* with a new haplotype, H4, identified from *D. bicornis*. This study revealed the presence of *Theileria bicornis* in Australian captive populations of both *C. simum* and *D. bicornis* and a new haplotype of the parasite was identified.

1. Introduction

Parasites and their hosts have co-evolved over the centuries. Benign infections enable the parasite to establish a symbiotic relationship with the host and potentially persist for life. However, factors such as translocation, stress, and suppression of host immunity can allow parasites to proliferate and cause clinical disease. A variety of haemoparasites have been identified in the rhinoceros (Penzhorn et al., 1994) in particular protozoan parasites such as, trypanosomes (McCulloch and Achard, 1969; Mihok et al., 1992a,b) and the recently described piroplasms, *Babesia bicornis* and *Theileria bicornis* (Nijhof et al., 2003). Trypanosomes are commonly transmitted by tsetse flies (*Glossina* spp.) and also cause nagana in livestock (Mihok et al., 1992a), while the genera *Babesia* and *Theileria* are haemoparasites transmitted by arthropod vectors, usually ticks. *Babesia* species such as *B. bigemina* (Figueroa et al., 1992), *B. gibsoni* (Conrad et al., 1991), *B. divergens* (Telford et al., 1993), and *B. bovis* (Adjou Moumouni et al., 2015) has been identified in many wildlife and domestic animals (Penzhorn, 2006; Uilenberg, 2006) and it causes Babesiosis (tick fever) in cattle (Figueroa et al., 1992; Telford et al., 1993; Adjou Moumouni et al., 2015) and haemolytic anaemia in dogs (Conrad et al., 1991). The symptoms of babesiosis include high fever, anorexia, haemolysis, and even death in severe clinical cases (Conrad et al., 1991; Colly and Nesbit, 1992). *B. bicornis* was shown to be fatal in small isolated populations of rhinoceros in Tanzania and South Africa (Nijhof et al., 2003; Otiende et al., 2015).

Theileria species could be classified into two groups based on the pathogenesis and lifecycle (Mans et al., 2015). The transforming *Theileria* species such as *T. annulata* and *T. parva* causes tropical theileriosis (TT) and East Coast fever (ECF) respectively and the diseases have been proven to be fatal in bovines (Uilenberg, 1981; Latif et al., 2002; Bishop et al., 2004). The non-transforming *Theileria* species such as *T. orientalis* causes oriental theileriosis in cattle (Sugimoto and Fujisaki, 2002; Izzo et al., 2010; Sivakumar et al., 2014); symptoms include muscle

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weakness, ataxia and even death due to the haemolytic properties of the parasite (Izzo et al., 2010). *T. orientalis* has caused disease outbreaks in cattle in Australia and New Zealand in recent years due to introduction of a pathogenic genotype of the parasite (Kamau et al., 2011; McFadden et al., 2011; Eamens et al., 2013a,b,c). *T. bicornis* was first identified in the black rhinoceros *D. bicornis*, in Africa (Nijhof et al., 2003). Since then, there have been reports of *T. bicornis* in wild rhinoceros (Otiende et al., 2015, 2016), cattle (Muhanguzi et al., 2010) and other wildlife (Oosthuizen et al., 2009), indicating that the parasite has a broad host range. Epidemiological studies in Kenya showed that *T. bicornis* is more prevalent in *C. simum* (66%) as compared to *D. bicornis* (43%), while factors such as age, sex, location, and population mix did not have any significant impact on prevalence for either species (Otiende et al., 2015). The pathogenic potential of *T. bicornis* is currently unclear. This parasite is generally considered to be benign, with evidence of endemic stability in some rhinoceros populations (Otiende et al., 2015). However, there have been relatively few studies on *T. bicornis* and many *Theileria* species are known to be capable of inducing disease in the host under stress caused by translocation, pregnancy, lactation and general immunosuppression (Sugimoto and Fujisaki, 2002; Eamens et al., 2013b; Hammer et al., 2016). Furthermore, *T. bicornis* is thought to be related to *T. equi* which causes equine piroplasmosis, a disease known to be induced by stress (Nijhof et al., 2003; Laus et al., 2015; Otiende et al., 2016). In this study, we identified a new haplotype of *T. bicornis* in Australian captive rhinoceros housed at Taronga Western Plains Zoo.

2. Materials and methods

2.1. Animals

Nine rhinoceros, *C. simum* (n = 2), and *D. bicornis minor* (n = 7) between the ages of one and 24 were included in this study and consisted of both wild-caught animals from Africa and captive bred animals (Table 1).

2.2. Blood smear examination

Blood smears were stained with Giemsa or Diff-Quik and viewed under an Olympus BX50 microscope at 100 × magnification and microscopic image was captured with an Olympus DP70 camera.

2.3. Piroplasmida PCRs

DNA extractions were conducted using the DNeasy blood and tissue DNA extraction kit according to manufacturer's protocol (Qiagen). *T. orientalis* qPCR was conducted according to a previously described multiplex hydrolysis probe qPCR assay (Bogema et al., 2015). A generic PCR assay targeting the 18S rRNA gene of the Piroplasmida was also conducted using previously described primers Piroplasmid-F: 5′-CCAG CAGCCGCGGTAATT-3′ and Piroplasmid-R: 5′-CTTTCGCAGTAGTTYG-TCTTTAACAAATCT-3' (Tabar et al., 2008; Baneth et al., 2013). Amplification was carried out with the BIOTAQ™ DNA Polymerase kit (Bioline) to make up a 25 µL PCR cocktail containing: 1 X BIOTAQ buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each primer, 1 unit of BIOTAQ DNA polymerase and molecular grade water. PCR was carried out with the following thermal cycling parameters: Initial denaturation 94 °C for 3 min followed by 35 cycles at 94 °C for 30 s, 64 °C for 45 s, 72 °C for 30 s with a final extension of 72 °C for 7 min. Full length *Theileria bicornis* 18S rRNA gene was amplified with primers 18SAN, 18SBN (Nijhof et al., 2003) and another pair of internal primers designed for this study, 18S-F1: 5′-GATCCTGCCAGTAGTCATATG-3′ and 18S-R1: 5′-TACTCCCCCCAGAACCCA-3'. PCR products were viewed on a 1.5% agarose 0.5 × TBE gel stained with GelRed (Biotium, USA), purified with QIAquick PCR purification kit (Qiagen) and subjected to Sanger sequencing with the primers described above.

2.4. Molecular phylogeny

T. bicornis 18S rRNA sequences were aligned using Geneious version (7.1.9) (Kearse et al., 2012) and subjected to a nucleotide BLAST comparison with the Genbank database. Phylogenetic analysis was conducted using DNAdist within the PHYLIP package (Felsenstein, 2005) and a neighbour-joining tree was generated with 1000 bootstrap replicates to estimate phylogenies (Fig. 2). The analysis included 19 *Theileria* spp., six *Babesia* spp., one *Cytauxzoon* sp. and *Toxoplasma gondii* as the outgroup.

3. Results

3.1. Blood smear examinations

Piroplasms were observed on blood smears from Aluka, but in Umfana, there were observations of red cell inclusions reflected by dot forms, signet ring forms and also rod forms on Giemsa-stained smears, but not Diff-Quik-stained smears. Given that Umfana was an asymptomatic animal, the clinical significance of these inclusions was doubtful.

Serum biochemistry results from the *D. bicornis* cohort were generally unremarkable, with minor elevations in alkaline phosphatase and aspartate aminotransferase (AST) in some animals. Siabuwa additionally displayed elevated creatine kinase (CK; 1154 U/L, reference range 142–742 U/L) and blood urea nitrogen (BUN; 9.0 mmol/L, reference range 2.5–8.1 mmol/L). Siabuwa was positive for piroplasms on blood smear (Fig. 1), while all other *D. bicornis* tested negative. Piroplasm morphology resembled and was in the size range (1.5 µm) of *Theileria* spp (Izzo et al., 2010). rather than *Babesia* spp. and was generally of the comma-shaped form (Fig. 1), although ring forms were also occasionally observed.

3.2. PCR amplification and sequencing

All animals tested negative for *Theileria orientalis*. Despite a negative blood smear, Aluka tested positive for Piroplasmida 18S rRNA using a generic piroplasmid PCR. Umfana, the asymptomatic male *C. simum* which returned a positive blood smear, also tested positive for

Table 1
Summary of the nine animals used in this study.

Rhinoceros name	Year of birth	Origin (year of introduction to Australia)	Species	Gender	Date sampled
Aluka	1996	Ex-wild, Africa (2002)	*Ceratotherium simum*	Female	18/3/2012
Umfana	1995	Ex-wild, Africa (2002)	*Ceratotherium simum*	Male	20/3/2012
Bakhita	2002	Captive-born, Australia	*Diceros bicornis minor*	Female	16/5/2016
Chikundo	2000	Captive-born, Australia	*Diceros bicornis minor*	Male	16/5/2016
Dafari	2015	Captive-born, Australia	*Diceros bicornis minor*	Male	16/5/2016
Kufara	2010	Captive-born, Australia	*Diceros bicornis minor*	Female	16/5/2016
Kwanzaa	1992	Captive-born, Australia	*Diceros bicornis minor*	Male	16/5/2016
Mpenzi	2005	Captive-born, Australia	*Diceros bicornis minor*	Male	16/5/2016
Siabuwa	1992	Ex-wild, Africa (1993)	*Diceros bicornis minor*	Male	17/5/2016

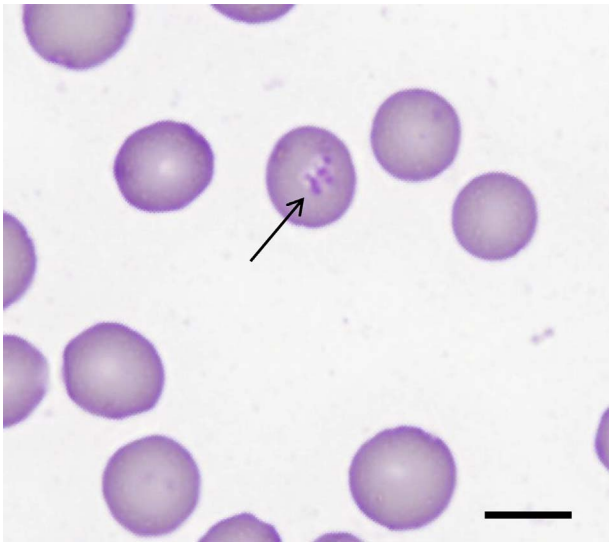

Fig. 1. Photomicrograph of Diff-Quik stained blood smear from black rhinoceros, Siabuwa infected with *T. bicornis*. The morphology of *T. bicornis* is not well-described in the literature. We observed comma-shaped piroplasms approximately 1.5 μm in length closely resembling *T. orientalis*. Ring forms were also occasionally observed (not shown). Bar = 5 μm.

piroplasmids. Of the seven *D. bicornis* tested, only one (Siabuwa) returned a positive PCR result for Piroplasmida, which was also consistent with the blood smear results. Sequencing of the 18S rRNA amplicons revealed the presence of *T. bicornis* in all three samples. Additional PCR amplification of the 18S rRNA gene resulted in 1729 bp, 1691 bp and 1616 bp of sequence from the samples of white rhinoceros Umfana (MF536661), Aluka (MF536660) and black rhinoceros Siabuwa (MF536659) respectively.

3.3. Molecular phylogeny

Phylogenetic analysis of 1474 bp of the 18S rRNA gene of the *C. simum* and *D. bicornis* piroplasmid strains demonstrates the close relationship to *T. bicornis* (Fig. 2). Furthermore, the *T. bicornis* cluster falls outside the non-transforming and transforming *Theileria* groups along with *Cytauxzoon felis* and *Theileria equi* (Fig. 2), which is consistent with prior studies (Nijhof et al., 2003; Schreeg et al., 2016). Alignment of the 18S rRNA genes also revealed the presence of two haplotypes of *T. bicornis* H2, which was previously described (Otiende et al., 2016) and alignment of the 396 bp *T. bicornis* haplotypes revealed a new *T. bicornis* haplotype, H4, identified in this study (see Figs. 2 and 3). The *T. bicornis* haplotype in the two infected white rhinoceros, Aluka and Umfana was 100% homologous to haplotype H2; however, a new haplotype, H4, was identified in *D. bicornis*. H4 (accession number MF567493) in the infected black rhinoceros (Siabuwa) is 99% homologous to the H2 (accession number KC771141).

4. Discussion

In this study, we examined blood samples from captive *C. simum* and *D. bicornis* housed at Taronga Western Plains Zoo, Australia for piroplasmid parasites. Haemoparasites such as trypanosomes (McCulloch and Achard, 1969; Mihok et al., 1992a,b) and *Babesia bicornis* (Nijhof et al., 2003) have been associated with rhinoceros mortalities in Africa and were considered in the differential diagnosis of a 16 yr old female *C. simum*; however, neither of these parasites was detected. *Theileria bicornis* was detected in blood samples from both dead (Aluka) and asymptomatic (Umfana) *C. simum* via PCR; however, piroplasms were only observed in blood films from Umfana, suggesting that the *T. bicornis* infection in Aluka was of a low intensity. The pathogenic potential of *T. bicornis* is not fully understood as this organism has been poorly studied; however asymptomatic infections are common in both black and white rhinoceroses in Africa (Otiende et al., 2015). Only a single case of *T. bicornis* infection has been recorded in association with rhinoceros deaths although it presented as a coinfection with *B. bicornis* (Nijhof et al., 2003; Otiende et al., 2015). Thus, the presence of *T. bicornis* was considered an incidental finding unlikely to be involved in the *C. simum* mortality.

PCR screening of the captive population of *D. bicornis* for piroplasmids revealed only a single *T. bicornis*-positive animal (Siabuwa), which was also confirmed by blood smear. This animal had elevated AST and CK levels that were potentially linked to tissue damage, but blood parameters were otherwise normal. The blood profile of Siabuwa was similar to a piroplasm prevalence study in white rhinoceroses by Govender et al. (2011) where blood parameters of the animals had no significant changes as well. Siabuwa was translocated from Africa as were the two *T. bicornis*-positive *C. simum*, while the remaining 6 captive-bred *D. bicornis* were *T. bicornis* negative. This suggests that *T. bicornis* was introduced to Australia with wild-caught rhinoceros and that transmission amongst the Australian captive population did not occur.

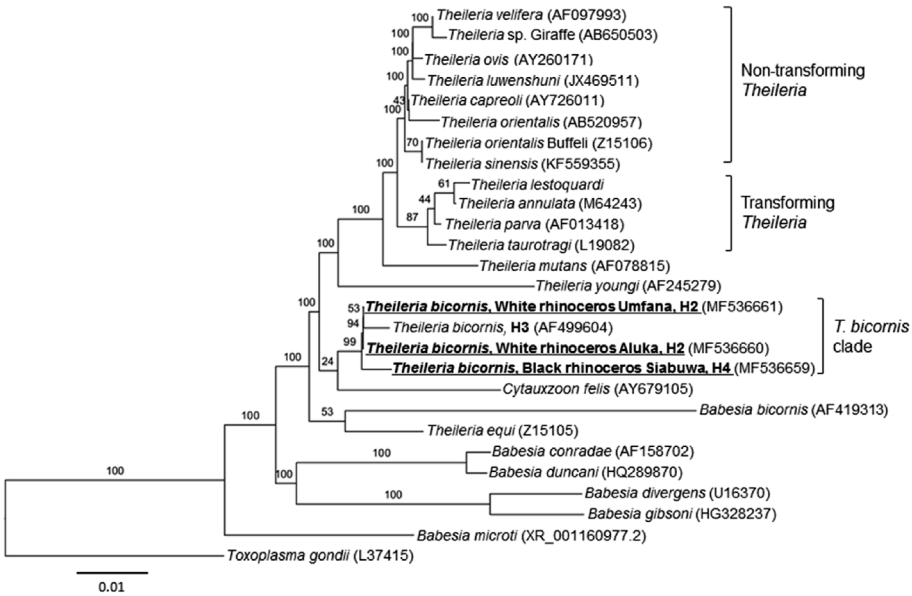

Fig. 2. Molecular phylogenetic analysis of the piroplasm 18S rRNA gene, including the three rhinoceros samples (Aluka, Umfana and Siabuwa) used in this study. The 18S rRNA sequences were extracted from Genbank during BLAST analysis. The rooted phylogenetic tree was constructed using the neighbour-joining method with *T. gondii* forming the outgroup. Bootstrap percentages are represented on each node based on 1000 replicates. Phylogenetic analyses were conducted using the PHYLIP packages (Felsenstein, 2005). The haplotypes of the three *T. bicornis* sequences from this study are indicated at the end of the sequence label.

Fig. 3. Truncated alignment of the 18S rRNA *T. bicornis* haplotypes including the new haplotype H4 identified in this study. Geneious version (7.1.9) (Kearse et al., 2012) was used to generate alignments to highlight the differences between the four *T. bicornis* haplotypes. 18S rRNA sequences of the three previously described *T. bicornis* haplotypes H1 to H3 (accession numbers KC771140 to KC771142 respectively) were extracted from Genbank for this analysis. The new *T. bicornis* haplotype H4 was submitted to Genbank and assigned accession number MF567493.

Currently, the vectors identified to be capable of transmitting *T. bicornis* are *Dermacentor rhinocerinus* and *Amblyomma rhinocerotis* (Knapp et al., 1997; Otiende et al., 2016). These ticks are present in Africa, have not been identified in Australia and are characterized by long, sturdy mouthparts capable of penetrating the thick rhinoceros hide (Horak et al., 2017). Whether endemic Australian tick species are competent vectors of *T. bicornis* and other rhinoceros blood parasites is unclear, but they may lack the necessary mouthparts to achieve transmission. Transplacental transmission has been demonstrated for several piroplasmid species (Phipps and Otter, 2004; Fukumoto et al., 2005; Mierzejewska et al., 2014; Zakian et al., 2014; Sudan et al., 2015; Swilks et al., 2017) but tends to occur only at low frequencies and there was no evidence from this study that *T. bicornis* was transmitted via this route within the captive population.

Sequence alignments of H2 and H4 revealed a difference of a single nucleotide substitution and a single thymine nucleotide insertion. Current literature of *T. bicornis* haplotype indicates H1 and H3 to only occur in black rhinoceros and H2 in white rhinoceros (Table 1 in Otiende et al., 2016). Whether haplotype H4 only occurs in black rhinoceros or any of these haplotypes contributes to disease remains unknown, but further studies could be done to determine haplotype specificity for a particular host or if the haplotypes play a pathogenic role in infected animals. Phylogenetic analysis placed the *T. bicornis* clade in this study outside the transforming and non-transforming clades of the *Theileria* group (Fig. 2). *T. bicornis* appears to be close relatives of *C. felis*, *T. youngi*, *B. bicornis* and *T. equi* which are consistent to previous studies (Nijhof et al., 2003; Otiende et al., 2016). The complete lifecycle of *T. bicornis* has not been established but it has similar characteristics to the non-transforming *Theileria* group suggesting it may be a largely benign parasite (Nijhof et al., 2003). However as both horses and rhinoceros are odd-toed ungulates classified under the order Perissodactyla, it is worth to noting that *T. equi* that causes clinical equine piroplasmosis (Nijhof et al., 2003; Laus et al., 2015; Otiende et al., 2015, 2016), was recently detected in rhinoceros (Govender et al., 2011).

Piroplasmids have coevolved with their hosts (Otiende et al., 2015). Benign infections are common in non-transforming *Theileria* species, for example *T. orientalis* genotype Buffeli in cattle (Kamau et al., 2011) and *T. velifera* (Uilenberg, 1981; Mans et al., 2015). Some of these haemoparasites can persist as lifelong infections in the host, only causing clinical signs when the animals are immunosuppressed or undergo stress from translocation, rearing conditions or pregnancy (Sugimoto and Fujisaki, 2002). Translocation of the rhinoceros in Africa to suitable and safe environments is integral for the conservation of these magnificent animals. However, translocation stress has been reported to decrease PCV levels (Kock et al., 1999) and is also linked to immune suppression in the animals which can lead to morbidity and/or fatality (Glaser and Kiecolt-Glaser, 2005; Martin, 2009; Otiende et al., 2015). Thus screening of animals for haemoparasites prior to translocation would be prudent for future breeding programs.

5. Conclusion

We revealed for the first time in Australia the presence of *T. bicornis* in both white and black rhinoceros. Evidence from this study suggests that the parasite was acquired in Africa and was not transmitted within the captive rhinoceros population within Australia. A new *T. bicornis* haplotype, H4, has been identified. *T. bicornis* infection intensity was low and haematological parameters within infected rhinoceros were unremarkable suggesting that infection with this parasite was likely incidental rather than the cause of the 2012 white rhinoceros mortality event. However, given that translocation-induced stress is a major trigger factor for theileriosis; future screening of translocated rhinoceros would be prudent to ensure successful breeding programs.

Accession numbers

The three 18S rRNA sequences and new *T. bicornis* haplotype H4 sequence of the rhinoceroses have been deposited in Genbank and were assigned accession numbers: MF536659 (Siabuwa), MF536660 (Aluka), MF536661 (Umfana) and MF567493 (*T. bicornis* haplotype H4).

Author's contributions

CJ and DB conceived the study and together with JY designed the study. JY performed the molecular analysis, interpretation of data and drafted the manuscript. SG performed the microscopic analysis and imaging. BB and MC collected the samples and performed the biochemical analysis. CJ assisted with data interpretation and provided critical comments on the manuscript. All authors read and approved the final manuscript.

Declaration of interest

Declarations of interest: none.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijppaw.2017.12.003.

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