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Original article

Advancing disease surveillance in rhinoceroses: A multiplex real-time PCR assay for detecting *Theileria bicornis* and *Babesia bicornis*

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ABSTRACT

Black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses in Southern Africa face multiple threats, including poaching, habitat loss, and translocation stress. Infections with *Theileria bicornis* (in both rhino species) and *Babesia bicornis* (confirmed only in black rhinos) add further health risks, with stressors such as translocation potentially increasing susceptibility. Effective management requires sensitive molecular diagnostic assays for accurate detection and surveillance. To address this, we developed a multiplex qPCR assay (MqTbBb) using species-specific TaqMan™ minor groove binder (MGB) probes for the simultaneous detection of *T. bicornis* and *B. bicornis*. The assay targets 18S rRNA gene regions, amplifying an 87 bp fragment for *T. bicornis* and a 51 bp fragment for *B. bicornis*, with efficiencies of 100 % and 98 %, respectively. Probit analysis determined a 95 % Limit of detection of 1.00×10^{-6} % and 6.27×10^{-6} % equivalent parasitized erythrocytes for *T. bicornis* and *B. bicornis*, respectively. No cross-reactivity was observed with other related protozoa tested. A total of 223 field samples from rhinos (101 black and 122 white) in Mpumalanga province were screened using both the MqTbBb and Reverse Line Blot (RLB) hybridization assays. The MqTbBb detected *T. bicornis* in 57 % of black and 99 % of white rhinos, with co-infections in 40 % of black rhinos. RLB detected *T. bicornis* in 96 % of black and 95 % of white rhinos, with a *Babesia* catch-all probe signal in 75 % and 32 %, respectively. *B. bicornis* was not detected by RLB and was never detected as a single infection by qPCR. These findings highlight high *T. bicornis* prevalence and rare *B. bicornis* infections (co-infections). The MqTbBb assay strengthens detection, surveillance, and conservation efforts.

1. Introduction

The International Union for the Conservation of Nature (IUCN) has classified the black rhinoceros (*Diceros bicornis*) as Critically Endangered and the white rhinoceros (*Ceratotherium simum*) as Near Threatened (Emslie, 2020a;b). In Southern Africa, where the Southern white rhino (*C. simum*) and two subspecies of black rhino (*D.* bicornis *bicornis* and *D. bicornis minor*) are native, conservation is crucial for maintaining regional biodiversity (Knight et al., 2015; Department of Forestry, 2024). Over the past two decades, conservation efforts have led to an increase in black rhino populations in South Africa; however, poaching, habitat loss, and translocation-related stress remain major threats. Many rhinos are now managed in fenced sanctuaries, conservation areas, or intensively protected zones (Wielgus et al., 2023). Translocation, while vital for maintaining genetic diversity and managing metapopulations, can induce chronic stress leading to immunosuppression and increased susceptibility to infection (Mellya et al., 2023).

Among disease threats to rhinos, piroplasms such as *Babesia* and *Theileria* species are of particular concern. These bloodborne protozoan parasites are transmitted by ixodid ticks and infect a wide range of wild and domestic mammals worldwide (Mugera and Wandera, 1967; McCullogh and Achard, 1969; Otiende et al., 2015). *Theileria bicornis* has been reported in both white and black rhinos, whereas *Babesia bicornis* has, to date, only been confirmed in black rhinos (Nijhof et al., 2003; Otiende et al., 2016; Zimmermann et al., 2021). While many infections are benign, stress from events like translocation or overpopulation can suppress the immune system, potentially triggering parasite proliferation and occasionally leading to fatalities (Nijhof et al., 2003; Otiende et al., 2016).

Babesia species were first reported in black rhinos in Kenya in 1967

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(Brocklesby, 1967). Between 1967 and 1969, blood smears from immobilized white rhinos during a translocation event in KwaZulu-Natal (KZN), South Africa, revealed a smaller *Theileria* species in 34 animals and a larger *Babesia* species in two female calves (Bigalke et al., 1970). In 2003, a molecular investigation into the fatalities of four black rhinos, two from Tanzania's Ngorongoro Conservation Area, one from Addo Elephant National Park in South Africa, and one from Hluhluwe-iMfolozi Park (KZN), led to the official description of these parasites as *T. bicornis* and *B. bicornis* (Nijhof et al., 2003). *Babesia bicornis* was linked to the deaths of three rhinos and was also found in five healthy black rhinos from the Great Fish River Reserve complex in South Africa (Nijhof et al., 2003). Subsequent molecular studies indicate that non-pathogenic *T. bicornis* is prevalent in white and black rhinos in South Africa and Kenya (Govender et al., 2011; Otiende et al., 2015; Zimmermann et al., 2021).

Genetic characterization of the *T. bicornis* 18S rRNA gene has revealed four haplotypes (H1–H4) present in both white and black rhinos (Otiende et al., 2016; Yam et al., 2018; Zimmermann et al., 2021). Although *T. bicornis* is generally considered non-pathogenic, its potential role in rhino morbidity and mortality, following translocation, cannot be excluded given the lack of definitive evidence. The identification of distinct *T. bicornis* haplotypes in Kenyan rhinoceros populations highlights genetic diversity and suggests caution when relocating naïve animals (Otiende et al., 2016). *Theileria bicornis* has also been reported in other species, including nyalas (*Tragelaphus angasii*), impala (*Aepyceros melampus*), sable antelope (*Hippotragus niger*), and eland (*Tragelaphus oryx*) (Pfitzer et al., 2011; King'ori et al., 2019; Clift et al., 2020), though these findings may reflect diagnostic limitations rather than true host plasticity.

Molecular assays, including RLB hybridization, nested and conventional PCR, cloning and sequencing, have improved detection of hemoparasites in rhinos, providing insights into prevalence and distribution (Nijhof et al., 2003; Govender et al., 2011; Otiende et al., 2015; Zimmermann et al., 2021). Between 2003 and 2006, a study of 195 white rhinoceroses in Kruger National Park (KNP) reported T. bicornis in 36.4 % and *T. equi* in 9.2 % of individuals, with no *B. bicornis* detected (Govender et al., 2011). In black rhinoceros populations, molecular screening found *T. bicornis* in 42.1 %, B. bicornis in 15.8 %, and *T. equi* in 5.3 % of individuals (Zimmermann et al., 2021). While these methods have improved parasite detection and characterization, limitations remain for routine diagnostics.

Pre-translocation screening is crucial for rhino conservation, yet existing molecular diagnostic methods, such as conventional PCR and RLB hybridization, are often limited by their inability to quantify parasite loads and susceptibility to contamination (Buling et al., 2007). To address these limitations, this study developed a multiplex qPCR assay (MqTbBb) using species-specific TaqMan™ minor groove binder (MGB) probes to simultaneously detect and quantify *T. bicornis* and *B. bicornis* infections in both white and black rhinos. Quantitative PCR has been successfully applied to other hemoparasites (Buling et al., 2007; Kim et al., 2008; Sibeko et al., 2008; Bhoora et al., 2010; Troskie et al., 2019; Bhoora et al., 2020), and its application to rhino parasites is expected to enable rapid, accurate detection, facilitating epidemiological studies and generating essential baseline diagnostic data to better inform conservation management strategies.

2. Methods

2.1. Ethical approval, sample collection and DNA isolation

Ethical approval was obtained from the University of Pretoria Animal Ethics Committee (AEC) and Research Ethics Committee (REC) [REC030–24]. In addition, permission to perform research in terms of Section 20 of the Animal Diseases Act, 1984 (Act No 35 of 1984) was obtained from the Department of Agriculture, Land Reform and Rural Development (DALRRD) [Ref no 12/11/1/1/6 (5014 MG) and 12/11/1/1/6 (6691 KL)].

EDTA blood samples from 223 white and black rhinoceroses were obtained from the KNP South African National Parks (SANParks) Biobank (BMTA 012/23, $n = 196$) and the Care for Wild (CfW) Rhino Sanctuary ($n = 27$) both located in Mpumalanga Province, South Africa. The Biobank samples were collected between 2020 and 2024 from various sections of the KNP, including Skukuza, Malelane, Stolznek, Tshokwane, Houtboschrand, N'wanetsi, Satara, Lower Sabie, Pretoriuskop, Crocodile Bridge, Letaba and Kingfisherspruit. These included an equal number of black ($n = 98$) and white ($n = 98$) rhinoceroses, comprising adults, sub-adults, and juveniles of both sexes. Sampling was primarily conducted during dehorning procedures, animal translocations and the clinical management of gunshot wounds. Additional samples from CfW were obtained from 24 white and three black rhinos during a routine dehorning procedure during the winter of 2024. These animals were temporarily housed in bomas at the time of sampling.

Genomic DNA was extracted from 200 µL of each EDTA blood sample using the PureLink® Genomic DNA Kit (Invitrogen, ThermoFisher Scientific, USA), according to the manufacturer's instructions.

2.2. PCR amplification and reverse-line blot (RLB) hybridization

Primers RLB-F2 (5′-GAC ACA GGG AGG TAG TGA CAA G-3′) and RLB-R2 (5′-biotin-CTA AGA ATT TCA CCT CTG ACA GT-3′), targeting *Theileria* and *Babesia* species, were used to amplify the V4 hypervariable region of the 18S rRNA gene of the parasites, as described by Nijhof et al. (2005). Resulting PCR products were analyzed using the RLB hybridization assay as described by Nijhof et al. (2005). The oligonucleotide probes used for detecting *T. bicornis* and *B. bicornis* are detailed in Table 1.

2.3. Design of the TaqMan® MGB™ qPCR assays for detecting T. bicornis and B. bicornis

A total of 35 published 18S rRNA gene sequences of *T. bicornis* (GenBank accession numbers: AF499604; KC771140 – KC771142; MF536659 – MF536661; MF567493; MN595046 – MN595051; MT903279 – MT903297; MT90330 – MT903303) and five of *B. bicornis* (GenBank accession numbers: AF419313; MT903298 – MT903301) were retrieved and aligned using the BioEdit Sequence Alignment Editor (Hall, 1999). Conserved regions were identified, and real-time PCR primers and probes were designed.

Primer Express© Software v3.0.1 (Applied Biosystems, ThermoFisher Scientific, USA) was used to design species-specific forward and reverse primers and TaqMan® minor groove binder (MGB) probes. The specificity of each primer-probe set was evaluated in silico, using BLAST (NCBI). The T. bicornis qPCR assay amplifies an 87 bp fragment, while B. bicornis qPCR assay amplifies a 51 bp fragment, both within the V4 hypervariable region of the 18S rRNA gene (Table 2).

Singleplex assays for each target species were first optimized on a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems, ThermoFisher Scientific, USA), using Micro-Amp optical 96-well reaction plates. Each 10 µl reaction consisted of 1X KAPA Probe Fast Universal Master

Table 1
Oligonucleotide probes used in the RLB hybridization assay.

Species	Probe sequence (5′–3′)	Reference
Theileria/Babesia catch-all	TAATGGTTAATAGGARCRGTTG	Gubbels et al. (1999)
Theileria catch-all	ATTAGAGTGCTCAAAGCAGGC	Nijhof (unpublished)
Babesia catch-all 1	ATTAGAGTGTTTCAAGCAGAC	Nijhof (unpublished)
Theileria bicornis	GCGTTGTGGCTTTTTTCTG	Nijhof et al. (2003)
Babesia bicornis	TTGGTAAATCGCCTTGGT	Nijhof et al. (2003)

*Ambiguity codes: $R = A/G$.

Table 2
Species-specific oligonucleotide primer and probe sequences used in singleplex and multiplex qPCRs for detecting and distinguishing between Theileria bicornis and Babesia bicornis.

Species	Primer/Probe	Sequence (5′-3′)	Size (bp)*
Theileria bicornis	TbicqFwd653	GTTGTGGCTTTTTTCTGGTTGAT	23
	TbicqRev739	CCATGCTAA AGTATTCAAGGCAAA	24
	TbicqPr677	FAM-TGGCTTCGGCCTTT-MGB	14
Babesia bicornis	BbicqFwd572	TCTGCTGCGTCGGTTGGT	18
	BbicqRev622	CCAAAGGCAACCGGAAAAA	19
	BbicqPr602	VIC-TAAATCGCCTTGGTCGTGG-MGB	19

* bp: base pairs.

Mix (KAPA Biosystems, Merck), 0.25 µM of each primer, 0.125 µM TaqMan® MGB™ probe and 2.5 µl of target DNA. The cycling conditions included an initial denaturation at 95 °C for 20 s, followed by 45 amplification cycles of 95 °C for 1 s and 60 °C for 30 s. Optimized singleplex assays were combined into the MqTbBb multiplex format to detect both parasites simultaneously. The multiplex assay contained each primer pair at a concentration of 0.25 µM, and the probes TbicqPr677 and BbicqPr602 at 0.125 µM each.

2.4. Efficiency, sensitivity and specificity

The analytical sensitivity of each singleplex assay was determined using synthetic GeneArt Strings DNA Fragments (ThermoFisher Scientific™, South Africa) containing the targeted 18S rRNA gene fragment from either *T. bicornis* or *B. bicornis*. The DNA concentration of each synthetic fragment was quantified using an Xpose™ spectrophotometer (Trinean, Belgium) and adjusted to 10 ng/µl before preparing a ten-fold serial dilution ranging from 10^{10} to 10^{1} copies/µl. Based on the calculated copy numbers, the genomic equivalent per µl of DNA was converted into % parasitemia (% equivalent parasitized erythrocytes; PE), assuming an average of 7.35×10^{6} red blood cells per µl of rhinoceros blood (Miller et al., 2015). The qPCR amplification of the standard dilution series ($1.55\times10^{\circ}$ to 1.55×10^{-7} % equivalent PE) was repeated in triplicate and on three separate occasions. The data generated from each of the nine runs were used to calculate linear regression equations of the quantification cycle (Cq) against the log % equivalent PE, from which the efficiency of the assay was determined (Bustin et al., 2009).

For the multiplex assay (MqTbBb), equimolar amounts of each of the *T. bicornis* and *B. bicornis* GeneArt strings, at 10 ng/µl respectively, were mixed before preparing a ten-fold dilution series ranging from 10^{6} to 10^{-1} copies/µl. Quantitative PCR amplifications of the standard dilution series ($1.55\times10^{\circ}$ to 1.55×10^{-7} % equivalent PE) were repeated six times in triplicate. The resulting mean quantification cycle (Cq) values from the six replicate runs were plotted against the log % PE to generate a linear regression equation, from which the amplification efficiency of the MqTbBb assay could be determined. To determine the limit of detection (LOD) of the assay, probit regression analysis was performed using the Statistical Package for the Social Sciences (SPSS Statistics v25, IBM Analytics, USA). A two-fold serial dilution, covering the non-linear range of the assay, starting at 1.55×10^{-4} % equivalent PE, was prepared in four replicates. Each replicate series consisted of 15 dilutions and was tested across four independent experimental runs, yielding a total of 16 observations per dilution. The proportion of positive responses at each dilution was then analyzed using probit analysis to estimate the concentration (% equivalent PE) corresponding to a 95 % detection limit.

The analytical specificity of each qPCR assay was assessed by testing DNA from non-target protozoan parasites that included *Theileria equi, Theileria taurotragi, Theileria velifera, Babesia bigemina, Babesia caballi* and *Babesia microti*. Each run included two negative controls: DNA extracted from a rhino sample that was consistently negative for piroplasms using the reverse-line blot hybridization assay, and a no-template control (NTC).

2.5. Detection of T. bicornis and B. bicornis using the MqTbBb qPCR assay

The effectiveness of the MqTbBb assay was assessed by screening 223 field samples for the presence of *T. bicornis* and *B. bicornis*.

2.6. Comparison of the RLB hybridization and the MqTbBb assays for detecting *T. bicornis* and *B. bicornis*

The Kappa coefficient was used to determine the level of agreement between the RLB assay and the MqTbBb assay in detecting *T. bicornis* and *B. bicornis*, respectively.

3. Results

3.1. RLB detection of T. bicornis and B. bicornis in field samples

Of the 223 samples screened using RLB, 96 % (213/223) tested positive for piroplasms. This included 96 % (97/101) of black rhino (*D. bicornis*) samples and 95 % (116/122) of white rhino (*C. simum*) samples, all of which hybridized to the *T. bicornis* probe. None of the samples hybridized to the *B. bicornis* probe; however, 75 % (76/101) of black rhino and 32 % (39/122) of white rhino samples hybridized to the Babesia catch-all probe. Ten (4 %) samples tested negative (Fig. 1).

3.2. Analytical specificity, efficiency and limit of detection of the MqTbBb qPCR assay

The *in silico* specificity of the qPCR primers and probe targeting the V4 hypervariable region of the 18S rRNA gene was assessed using BLASTn analysis (S1). The *T. bicornis* forward primer (TbicqFwd653) and probe (TbicqPr677), each exhibited 100 % sequence identity and coverage exclusively to *T. bicornis* 18S rRNA sequences in GenBank. However, due to high sequence homology among *Theileria* species, a species-specific reverse primer could not be designed. Consequently, the reverse primer (TbicqRev739) shows 100 % sequence identity and coverage with the 18S rRNA gene sequences of both *T. bicornis* and *T. equi*. Similarly, the *B. bicornis* primers showed 100 % identity to all

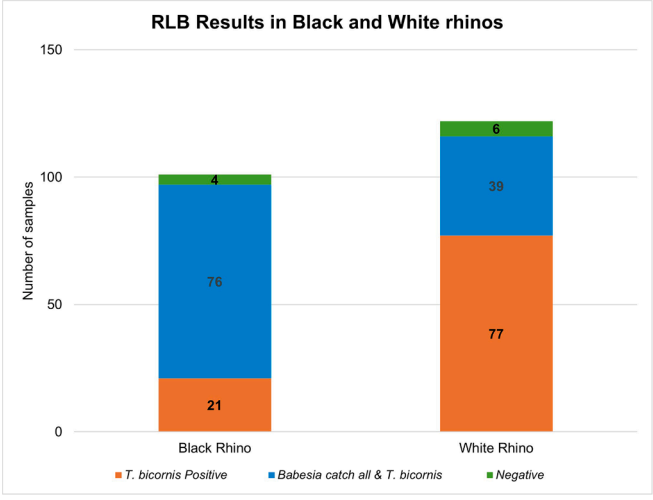

Fig. 1. Stacked bar chart summarising RLB results for blood samples from black rhinos (*D. bicornis*) and white rhinos (*C. simum*), indicating samples positive for *T. bicornis* (orange), positive for the *Babesia* catch-all probe and *T. bicornis* (blue), and samples that tested negative (green).

known 18S rRNA sequences in GenBank. The BbicqPr602 probe also matched all sequences, except for isolate MT903301.1, which has a single nucleotide mismatch. This minor variation (95 % identity) is unlikely to affect qPCR detection.

Both TaqMan® MGB™ qPCR assays successfully detected and differentiated between *T. bicornis* and *B. bicornis*. These assays exhibited amplification efficiencies of 98 % for *T. bicornis* (87 bp amplicon) (Fig. 2a) and 100 % for *B. bicornis* (51 bp amplicon) (Fig. 2b). Regression analysis of the multiplex MqTbBb qPCR assay indicated that the linear range of detection extended from 1.55 to 1.55×10^{-6} % PE for *T. bicornis* and 1.55 to 1.55×10^{-7} % PE for *B. bicornis*. Both targets showed strong linearity ($R^2 > 0.99$) and amplification efficiencies, 98 % for *B. bicornis* and 100 % for *T. bicornis*, which were comparable to those observed in the singleplex assays (Fig. 2c).

The MqTbBb qPCR assay was highly specific, showing no amplification from DNA extracted from a piroplasm-free rhinoceros or other protozoal parasites, including *T. equi, T. velifera, T. taurotragi, T. mutans, B. microti, B. bigemina* and *B. caballi* (Fig. 3).

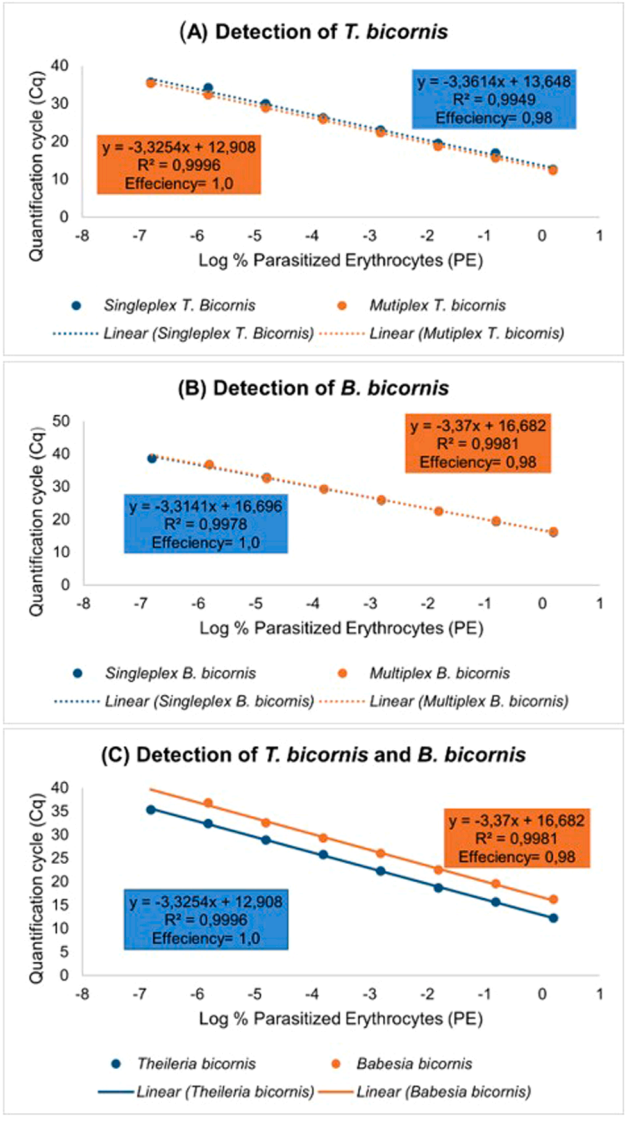

Fig. 2. Linear regression for the quantification of *T. bicornis* and *B. bicornis* parasite DNA. Quantification cycle (Cq) values were plotted against the log % equivalent parasitized erythrocytes (PE) from a 10-fold serial dilution series. DNA from *T. bicornis* and *B. bicornis* was mixed in equimolar amounts, corresponding to a range from 1.55 to 1.55×10^{-7} % equivalent PE. Comparisons are shown between the singleplex and multiplex qPCR linear regressions for quantifying (A) *T. bicornis* and (B) *B. bicornis*. (C) Linear regression for quantifying both *T. bicornis* and *B. bicornis* parasite DNA using the multiplex MqTbBb qPCR assay.

Probit analysis revealed that the 95 % limit of detection (LOD) of the assay for detecting *T. bicornis* was 1.00×10^{-6} % equivalent PE [95 % Confidence Interval (CI): 6.04×10^{-7} to 2.22×10^{-6} % equivalent PE] (Fig. 4), corresponding to a quantification cycle (Cq) of 33. As the target concentration decreased, the assay's sensitivity in detecting *T. bicornis* declined from 81 % at a concentration of 6.06×10^{-7} % equivalent PE to 25 % at 3.79×10^{-8} % equivalent PE. Similarly, for *B. bicornis*, the 95 % LOD was observed at 6.27×10^{-6} % equivalent PE [95 % CI: 3.30×10^{-6} to 2.02×10^{-5} % equivalent PE], with a corresponding Cq of 34. The sensitivity of the assay in detecting *B. bicornis* also decreased from 81 % at a concentration of 4.43×10^{-6} % equivalent PE to 13 % at 7.58×10^{-8} % equivalent PE (Fig. 4). The mean Cq values and coefficient of variation (CV) for both parasites are summarized in Table 3. The intra- and inter-run standard deviations (SD) for *T. bicornis* and *B. bicornis* were low, indicating high assay precision. The maximum CV observed between the replicate runs was 7.03 % for *T. bicornis* and 6.81 % for *B. bicornis*. Although the CV was slightly higher for the *T. bicornis* assay, the values reflect minimal variation within and between runs. Furthermore, the CV values decreased at concentrations approaching the assay's detection limit (Table 3).

3.3. Quantitative detection of T. bicornis and B. bicornis in field samples

A total of 223 black and white rhino samples were screened across 13 locations in KNP using the MqTbBb qPCR assay. *Theileria bicornis* was detected as a single infection in 179 samples (80 %), with Cq values ranging from 16.5 (8.3×10^{-2} % equivalent PE) to 34.1 (4.18×10^{-7} % equivalent PE). Co-infections with both *T. bicornis* and *B. bicornis* were identified in 40 samples (18 %), while *B. bicornis* was never detected as a single infection. The remaining four samples (2 %) tested negative for both parasites.

White rhinos were predominantly infected with *T. bicornis* (121/122; 99 %) (Fig. 5), while co-infections with both *T. bicornis* and *B. bicornis* were more frequently observed in black rhinos, particularly at Malelane and Skukuza, occurring in 40 % of samples (40/101). However, single infections with *T. bicornis* were still identified in 58 black rhino samples (57 %). Malelane recorded the highest total number of sampled rhinos ($n = 44$), followed by Tshokwane ($n = 34$), with both sites showing high prevalence of *T. bicornis*. Negative results were rare, accounting for only four individuals, predominantly among black rhinos ($n = 3$) at Crocodile Bridge, Tshokwane, and Stolznek, with only one white rhino being negative from Malelane (Fig. 6).

Chi-square analyses were performed in SPSS to evaluate the association between parasite prevalence and host characteristics (sex, age class, species). No significant association was observed between sex and *T. bicornis* infection status ($\chi^2 = 0.061$, df = 1, $p = 0.806$), with nearly identical infection rates in females (98.0 %) and males (98.4 %) (Fig. 7). Similarly, there was no significant association between sex and *B. bicornis* infection ($\chi^2 = 0.041$, df = 1, $p = 0.839$), with both sexes showing infection rates ranging of 17.7 % and 18.4 %.

When comparing infection prevalence across age categories (adult, sub-adult and juvenile), no statistically significant differences were observed for either *T. bicornis* ($\chi^2 = 2.105$, df = 2, $p = 0.349$) or *B. bicornis* ($\chi^2 = 4.971$, df = 2, $p = 0.083$). Although *B. bicornis* prevalence was the highest in juveniles (31.3 %) compared to sub-adults (13.3 %) and adults (17.2 %), this difference was not statistically significant (Fig. 7).

While no significant difference in the prevalence of *T. bicornis* between black and white rhinoceroses was observed ($\chi^2 = 1.451$, df = 1, $p = 0.228$), *B. bicornis* infection was found to be prevalent only in black rhinos (40 %), with this difference being highly significant ($\chi^2 = 58.879$, df = 1, $p < 0.001$) (Fig. 7).

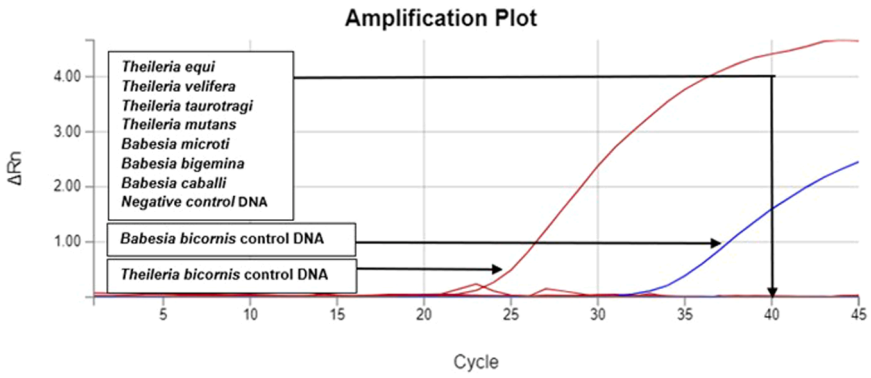

Fig. 3. Detection of *T. bicornis* and *B. bicornis* positive control DNA using the MqTbBb qPCR assay, indicated by an increase in the fluorescence signal [*T. bicornis* (red) and *B. bicornis* (blue)]. No increase in fluorescence was observed from DNA isolated from a piroplasm-free rhinoceros (negative control DNA) and from other protozoal parasites, including *T. equi, T. velifera, T. taurotragi, T. mutans, B. microti, B. bigemina* and *B. caballi.*

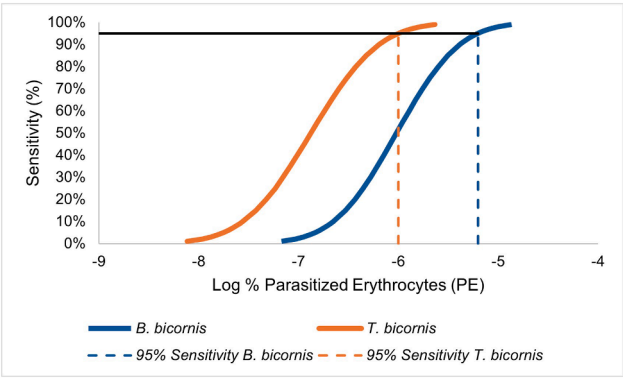

Fig. 4. Probit analysis of a twofold dilution series of *T. bicornis* (orange) and *B. bicornis* (blue) DNA mixed in equimolar concentrations and tested using the MqTbBb qPCR assay. The calculated %PE spans the non-linear range of the assay (1.55×10^{-4} % equivalent PE to 3.79×10^{-8} % equivalent PE), and the 95 % detection limits for each analyte are indicated.

3.4. Comparison of the MqTbBb and the RLB hybridization

A comparison was conducted between the MqTbBb qPCR assay and the RLB assay for detecting *T. bicornis* and *B. bicornis*. Of the 223 samples tested, 219 were positive for *T. bicornis* using the MqTbBb qPCR, while the RLB assay detected 213 positive samples. The agreement between the two assays for detecting *T. bicornis* was 97 % (217 out of 223 samples), resulting in a Cohen's kappa (κ) value of 0.560, which indicates moderate agreement (Table 4A).

In contrast, for *B. bicornis*, the MqTbBb qPCR assay detected 40 positive samples, while the RLB detected 115 positive samples based on hybridization to the *Babesia* genus-specific probe. As the identity of the RLB *Babesia* genus-positive products was not confirmed, these may represent *Babesia* species other than *B. bicornis*. Accordingly, direct comparison of the two assays cannot be used to draw firm conclusions regarding agreement in detecting *B. bicornis* specifically. Rather, the relatively low concordance (59 %, 132 out of 223 samples; Cohen's kappa (κ) = 0.200) likely reflects the broader specificity of the RLB probe, which detects multiple *Babesia* species compared with the species-specific qPCR assay (Table 4B).

4. Discussion

Translocating rhinos to suitable environments is critical for conservation efforts in Southern Africa. However, this translocation process carries inherent risks, including exposing naïve animals to new pathogens and potentially spreading novel infections to previously unaffected areas. Furthermore, stress associated with translocation has been implicated in rhino mortalities, often related to infections like babesiosis (Zimmermann et al., 2022).

In this study, the MqTbBb qPCR assay demonstrated the ability to detect low-level parasitemia, with detection limits of 10^{-6} % equivalent parasitized erythrocytes (PE). This high sensitivity is important for the early identification of asymptomatic infections, especially in conservation management situations where translocation or other stressors could trigger clinical disease. Furthermore, the assay showed excellent reproducibility, with inter- and intra-run coefficient of variation (CV) values below 7.1 %. There is also a strong linear correlation between parasite load and cycle threshold values ($R^2 > 0.99$), which further confirms the robustness of the MqTbBb qPCR assay for both research and

Table 3
The inter-run and intra-run variations for 12 twofold dilutions of *T. bicornis* and *B. bicornis* 18S rRNA fragments, mixed in equimolar concentrations, and prepared over the non-linear range of the MqTbBb qPCR assay.

	T. bicornis					*B. bicornis*				
Log PE	Total mean	Inter-run SD	Intra-run SD	Total SD	%CV	Total mean	Inter-run SD	Intra-run SD	Total SD	%CV
−4,1	28,01	0,17	0,35	0,46	1,63	31,86	0,28	0,37	0,47	1,49
−4,4	28,90	0,19	0,20	0,27	0,92	32,81	0,28	0,44	0,54	1,65
−4,7	29,78	0,13	0,13	0,17	0,57	34,13	0,27	0,46	0,60	1,76
−5,0	30,79	0,13	0,10	0,15	0,49	35,32	0,24	0,56	0,69	1,94
−5,3	31,83	0,08	0,36	0,34	1,07	36,34	0,84	0,85	1,18	3,24
−5,6	32,92	0,32	0,50	0,58	1,77	38,27	0,98	1,10	1,29	3,37
−5,9	34,02	0,39	0,98	0,96	2,84	39,38	1,99	1,60	1,32	3,36
−6,2	35,12	0,63	0,55	0,76	2,17	39,97	2,36	2,78	2,35	5,89
−6,5	35,43	0,27	0,57	0,65	1,84	>40	0,00	0,00	0,00	0,00
−6,8	36,37	0,88	0,81	1,14	3,14	>40	0,00	0,00	0,00	0,00
−7,1	37,34	0,94	2,59	2,63	7,03	39,51	0,00	2,69	2,69	6,81
−7,4	35,72	0,03	0,59	0,49	1,38	>40	0,00	0,00	0,00	0,00

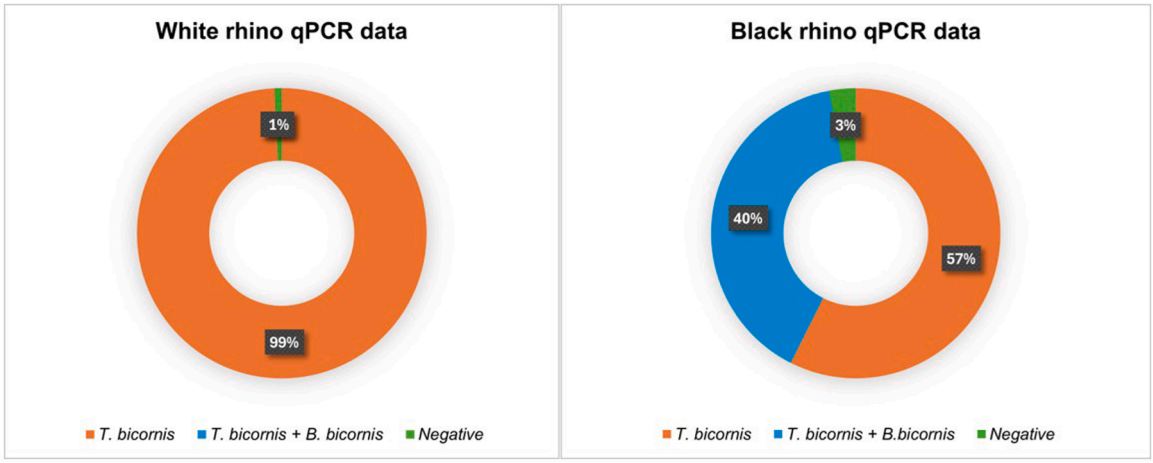

Fig. 5. Donut chart illustrating the distribution of qPCR results for *T. bicornis* and *B. bicornis* in (A) white rhino (*C. simum*) and (B) black rhino (*D. bicornis*) samples. The chart shows the proportion of samples that tested *T. bicornis*-positive (orange segment), co-infected with *T. bicornis* and *B. bicornis* (blue segment), and those that tested negative (green segment), as determined using the MqTbBb qPCR assay. A total of $n = 223$ blood samples were analyzed.

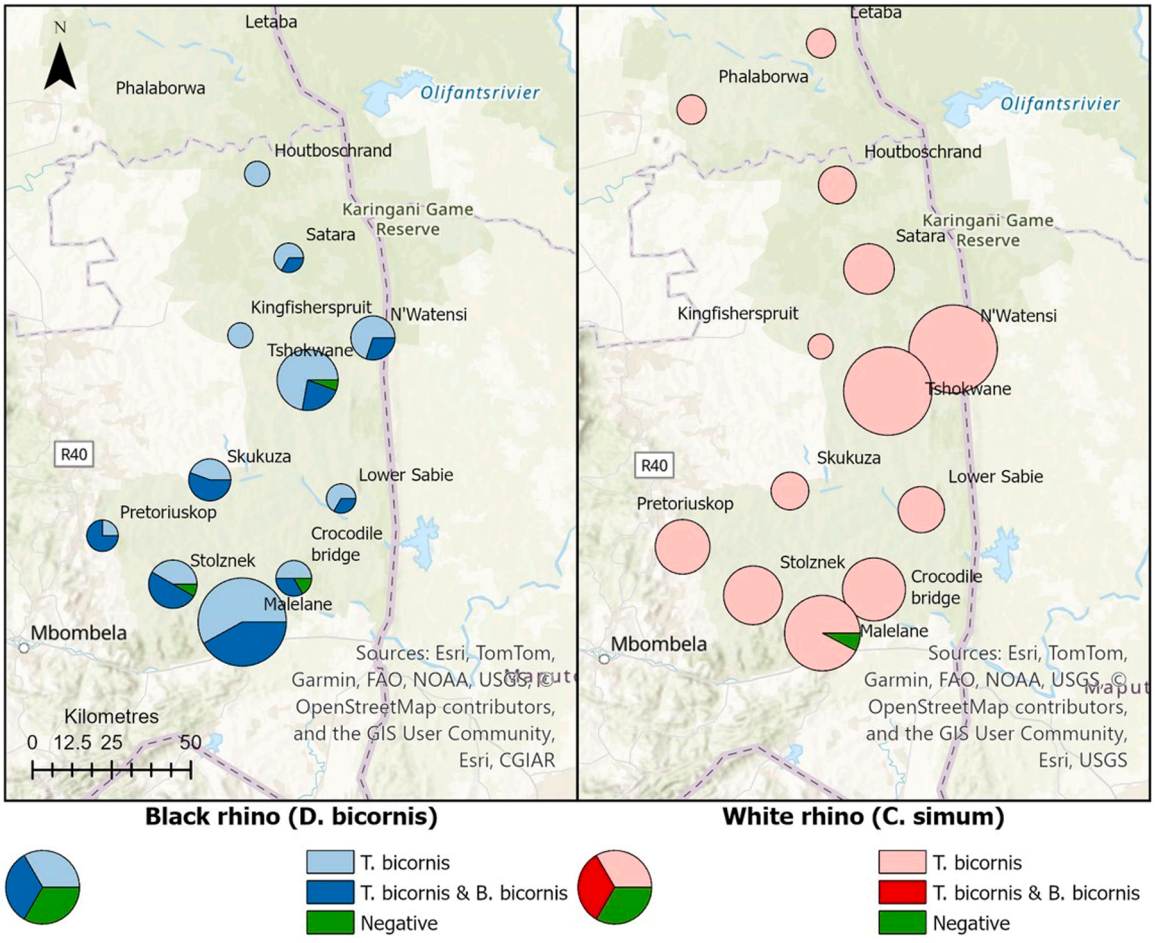

Fig. 6. Distribution of sampling sites and parasite detection results in black (*D. bicornis*) and white (*C. simum*) rhinos across Kruger National Park (KNP), South Africa. Each pie chart represents a sampling location, with the size of the chart proportional to the number of rhino samples collected at that site. For black rhinos (left panel), blue indicates samples positive for *T. bicornis*, dark blue indicates co-infection with *T. bicornis* and *B. bicornis*, and green indicates samples negative for both parasites. For white rhinos (right panel), light pink indicates *T. bicornis*-positive samples, red indicates co-infections with *T. bicornis* and *B. bicornis*, and green indicates negative samples.

diagnostic applications. The high sensitivity and reproducibility of the MqTbBb qPCR assay make it a valuable tool for the early identification of asymptomatic infections, particularly in conservation management situations where translocation or other stressors could trigger clinical disease. The strong linear correlation between parasite load and cycle threshold values supports its use not only as a diagnostic tool but also for

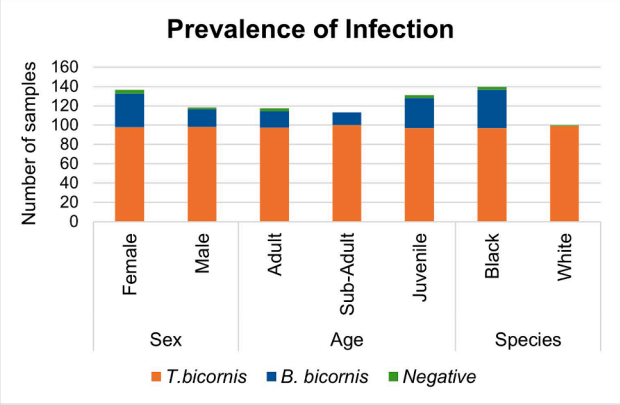

Fig. 7. Prevalence of *T. bicornis* and *B. bicornis* infections in rhinos, categorized by sex, age class, and species. Bars represent the number of samples per group, with orange indicating samples positive for *T. bicornis*, blue for *B. bicornis*, and green for samples negative for both parasites. Black rhinos showed a higher proportion of co-infections compared to white rhinos. Juveniles also had a relatively higher prevalence of *B. bicornis* infections.

Table 4
2 × 2 Contingency tables indicating the agreement level between the reverse line blot (RLB) and the MqTbBb qPCR assays in detecting (A) *T. bicornis* or (B) *B. bicornis* from the 223 rhino samples. For *B. bicornis*, RLB results are based on hybridization to the *Babesia* genus-specific probe.

A	*Reverse-Line Blot*			
		Positive	Negative	Total
MqTbBb qPCR T. bicornis	**Positive**	213	6	**219**
	Negative	0	4	**4**
	Total	**213**	**10**	**223**
Kappa= 0.560				
B	***Reverse-Line Blot***			
		Positive	**Negative**	**Total**
MqTbBb qPCR B. bicornis	**Positive**	32	8	**40**
	Negative	83	100	**183**
	Total	**115**	**108**	**223**
Kappa= 0.200				

quantitative monitoring of infection dynamics over time. Compared with previous molecular methods, which often lack sensitivity or quantitative accuracy, this assay provides a reliable approach for both research studies and practical disease management in rhino populations.

The *in silico* analysis of the primer and probe sequences revealed that, except for the reverse primer for *T. bicornis* (TbicqRev739), all other sequences showed 100 % sequence identity to either the *T. bicornis* or *B. bicornis* 18S rRNA gene sequences available in GenBank. The TbicqRev739 reverse primer also showed 100 % identity to the *T. equi* 18S rRNA gene sequences, reflecting the highly conserved nature of this gene among *Theileria* species. This sequence similarity complicated the design of a species-specific reverse primer for *T. bicornis*. This is particularly relevant as previous studies in South Africa reported the detection of *T. equi* in both black and white rhinos using RLB (Govender et al., 2011; Zimmermann et al., 2021). Although cross-reactivity between RLB probe sequences was considered, it was ultimately excluded, and the findings were interpreted as dual infections with *T. bicornis* and *T. equi* (Zimmermann et al., 2021). However, in this current study, the MqTbBb qPCR assay demonstrated high specificity, as it did not amplify DNA from a panel of protozoan parasites, including *T. equi*, suggesting that despite sequence conservation, cross-reactivity was effectively avoided under the assay conditions.

A remarkably high prevalence of *T. bicornis* infection among both black (*D. bicornis*, 97 %) and white (*C. simum*, 99 %) rhinoceroses in KNP was reported in this study, with 96 % of sampled individuals testing positive for piroplasms using either the RLB-PCR assay or the MqTbBb qPCR assay. This prevalence was much higher than the 36.4 % reported in white rhinos sampled between 2003 and 2006 in the same park using the RLB-PCR assay (Govender et al., 2011). It also exceeds the prevalence of 23.7 % reported by Zimmermann et al. (2021) in black rhinos sampled from two *D. bicornis* subspecies across South African conservation areas. However, as the prevalence estimates are qPCR-based, they reflect the detection of parasite DNA and may not necessarily indicate the presence of viable parasites. Nonetheless, these findings support the hypothesis that *T. bicornis* may represent an endemic and potentially commensal infection in black and white rhino populations under natural conditions. However, it is important to consider that under stressful conditions, such as translocation, poor habitat, or co-infections, the parasite may become more pathogenic and contribute to stress-induced mortalities, highlighting a more complex host-parasite relationship.

A comparable *T. bicornis* prevalence of 49.12 % was reported in a study from Kenya, where a higher infection rate was observed in white rhinos (66 %) compared to black rhinos (43 %) (Otiende et al., 2015). However, in this study, a uniform infection rate between the two species suggests that in the KNP population, other factors may significantly impact parasite transmission. These factors include local environmental conditions such as vegetation, tick density and host contact rates, as well as ecological factors that include seasonal patterns and shared habitat use.

Furthermore, while both Govender et al. (2011) and Zimmermann et al. (2021) reported the presence of *T. equi* in rhino samples, *T. equi* was not detected in any of the samples in the present study. This discrepancy may be due to temporal variations in the circulation of *T. equi* over time, or geographic differences in the distribution of infected tick vectors or host exposure. This highlights the dynamic nature of parasite exposure and infection patterns in free-ranging rhino populations.

In the present study, *B. bicornis* was not detected using the species-specific RLB probe. However, previous studies detected *B. bicornis* in 5.8 % of black rhino samples, and hybridization to a *Babesia* genus-specific probe was reported in 14.1 % of samples (Zimmermann et al., 2021). The authors suggested the presence of a novel *Babesia* species or uncharacterized variants. In contrast, the current study found a significantly higher proportion of samples (52 %) hybridizing to the *Babesia* genus-specific probe, despite the absence of species-specific *B. bicornis* signals. This finding confirms the likelihood of undetected diversity within the *Babesia* species infecting rhinoceroses and emphasizes the need for further molecular characterization to identify potential novel species or genetic variants.

Conversely, the MqTbBb qPCR assay enabled the species-specific detection of *B. bicornis*, with 40 % prevalence in black rhinos and no detection in white rhinos (0 %, $p < 0.001$). This finding is consistent with previous reports of limited *B. bicornis* detection in white rhinos and suggests host-specific susceptibility, possibly influenced by differences in immune response, tick exposure or vector preferences (Otiende et al., 2015; Zimmermann et al., 2021). The high prevalence in black rhinos is particularly concerning, as latent parasitemia and co-infections may exacerbate stress-related morbidity during translocation or other intensive management activities.

Co-infections with both parasites were identified in 18 % of sampled rhinos, occurring most frequently in black rhinos (40 %). These findings highlight the uneven parasite distribution between species, likely influenced by immunological and ecological factors. For instance, the browsing behavior of black rhinos may increase their exposure to certain ticks compared to grazing white rhinos. Although the clinical significance of co-infections remains unclear, they may contribute to overall health burdens under stressful conditions such as capture, relocation or injury.

Consistent with prior studies, the results show no significant differences in the prevalence of *T. bicornis* or *B. bicornis* among animal age

categories or sex (Govender et al., 2011; Otiende et al., 2015). However, for *B. bicornis*, higher rates of infection, though not statistically significant (31.3 %), were observed amongst juvenile black rhinos. This finding differs from earlier reports on white rhinos, which indicated that peak infection rates occurred among sub-adults (Govender et al., 2011; Otiende et al., 2015). For juveniles, higher rates of infection could reflect waning maternal antibodies and the incomplete development of an acquired immune response.

Taken together, the uniform infection rates across different groups, combined with the high parasite prevalence in the absence of clinical signs, support the hypothesis of enzootic stability in the Kruger National Park. This suggests that ongoing exposure to infected vectors helps maintain a high background prevalence of the parasites without causing clinical disease.

The MqTbBb qPCR assay proved to be highly effective, offering greater specificity compared to the RLB-PCR assay. In particular, the low Cohen's kappa ($\kappa = 0.200$) observed between RLB and qPCR results for *Babesia bicornis* detection highlights the limited discriminatory ability of RLB, likely due to non-specific cross-reactivity of the *Babesia* catch-all probes. In contrast, the qPCR assay's design enabled precise species-level identification, thus reducing the risk of misclassification.

The occurrence of co-infections was notably higher among black rhinos (40 %) compared to the overall population (18 %), suggesting potential differences in vector exposure, susceptibility, or behavior. These findings underscore the need to better understand ecological and host-related factors that may influence parasite transmission dynamics within and between rhinoceros species.

5. Conclusion

The developed MqTbBb qPCR assay represents a significant advancement for wildlife disease monitoring by allowing the quantitative detection and differentiation of these hemoparasites prior to translocation events. This is particularly important for black rhinos that are more frequently infected with the potentially pathogenic *B. bicornis*. While these infections may remain clinically silent under normal conditions, stress can lead to immunosuppression, allowing subclinical parasitemia to progress into acute disease. Since stress-inducing interventions are often necessary in conserving rhinos, sub-clinical infections can trigger health crises, reduce translocation success rates or lead to unexpected mortality, thereby undermining conservation outcomes.

Future research should focus on determining the clinical relevance of *T. bicornis* and *B. bicornis* infections, particularly under conditions that may compromise host immunity. Effectively managing rhino translocations within Southern Africa requires a thorough understanding of tick-borne disease epidemiology. By identifying transmission vectors, enhancing baseline epidemiological data, and using advanced diagnostic assays, conservationists can develop informed strategies to safeguard the health and survival of rhino populations in new environments.

Animal health and compliance

Ethical approval was obtained from the University of Pretoria Animal Ethics Committee (AEC) and Research Ethics Committee (REC) [REC030–24]. Additionally, permission to perform research in terms of Section 20 of the Animal Diseases Act, 1984 (Act No 35 of 1984) was obtained from the Department of Agriculture, Land Reform and Rural Development (DALRRD) [Ref no 12/11/1/1/6 (5014 MG) and 12/11/1/1/6 (6691 KL)].

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CRediT authorship contribution statement

Naledi P. Sekgobela: Writing – original draft, Visualization, Methodology, Investigation. **Ilse Vorster:** Writing – review & editing, Investigation. **Milana Troskie:** Writing – review & editing, Investigation. **Melvyn Quan:** Writing – review & editing, Visualization. **David E. Zimmerman:** Writing – review & editing. **Ayesha Hassim:** Writing – review & editing, Supervision, Investigation. **Luis Neves:** Writing – review & editing, Supervision. **Raksha V. Bhoora:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2025.102552.

Data availability

Data will be made available on request.

References

Bhoora, R., Quan, M., Franssen, L., Butler, C.M., Van Der Kolk, J.H., Guthrie, A.J., Zweygarth, E., Jongejan, F., Collins, N.E., 2010. Development and evaluation of real-time PCR assays for the quantitative detection of *Babesia caballi* and *Theileria equi* infections in horses from South Africa. Vet. Parasitol. 168, 201–211. https://doi.org/10.1016/j.vetpar.2009.11.011.

Bhoora, R.V., Collins, N.E., Schnittger, L., Troskie, C., Marumo, R., Labuschagne, K., Smith, R.M., Dalton, D.L., Mbizeni, S., 2020. Molecular genotyping and epidemiology of equine piroplasmids in South Africa. Ticks Tick Borne Dis. 11, 101358. https://doi.org/10.1016/j.ttbdis.2019.101358.

Bigalke, R.D., Keep, M.E., Keep, K.J., Schoeman, J.H., 1970. A large *Babesia sp*. and a *Theileria*-like piroplasm of the square-lipped rhinoceros. J. S. Afr. Vet. Med. Assoc. 41, 292–294. https://doi.org/10.10520/AJA00382809_4457.

Brocklesby, D.W., 1967. A *Babesia* species of the black rhinoceros. Vet. Rec. 80, 484.

Buling, A., Criado-Fornelio, A., Asenzo, G., Benitez, D., Barba-Carretero, J., Florin-Christensen, M., 2007. A quantitative PCR assay for the detection and quantification of *Babesia bovis* and *B. bigemina*. Vet. Parasitol. 147, 16–25. https://doi.org/10.1016/j.vetpar.2007.03.031.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55 (4), 611–622. https://doi.org/10.1373/clinchem.2008.112797.

Clift, S.J., Collins, N.E., Oosthuizen, M.C., Steyl, J.C., Lawrence, J.A., Mitchell, E.P., 2020. The pathology of pathogenic theileriosis in African wild artiodactyls. Vet. Pathol. 57, 24–48. https://doi.org/10.1177/0300985819879443.

Department of Forestry, Fisheries and the Environment, 2024. Biodiversity Management Plan For Black Rhinoceros (*Diceros Bicornis*) and White Rhinoceros (*Ceratotherium Simum*) in South Africa. Government Gazette no. 50829 notice 4980. Available. https://www.gov.za/sites/default/files/gcis_document/202406/50829gon4980.pdf [Accessed 19 April 2025].

Emslie, R., 2020a. Ceratotherium simum. The IUCN Red List of Threatened Species 2020: E.T4185a45813880. https://doi.org/10.2305/IUCN.UK.2020-1.RLTS.T4185A45813880.en. Available:[Accessed 28 February 2024].

Emslie, R., 2020b *Diceros bicornis.* The IUCN red list of threatened species 2020: E. T6557a152728945. Available: 10.2305/IUCN.UK.2020-1.RLTS.T6557A152728945.en [Accessed 28 February 2024].

Govender, D., Oosthuizen, M.C., Penzhorn, B.L., 2011. Piroplasm parasites of white rhinoceroses (*Ceratotherium simum)* in the Kruger National Park, and their relation to anaemia. J. S. Afr. Vet. Assoc. 82, 36–40. https://doi.org/10.4102/jsava.v82i1.31.

Gubbels, J., De Vos, A., Van Der Weide, M., Viseras, J., Schouls, L., De Vries, E., Jongejan, F., 1999. Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. J. Clin. Microbiol. 37, 1782–1789. https://doi.org/10.1128/jcm.37.6.1782-1789.1999.

Hall, T.A., 1999. Bioedit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/nt. Nucleic Acids Symp. Ser. 41, 95–98.

Kim, C.M., Blanco, L.B.C., Alhassan, A., Iseki, H., Yokoyama, N., Xuan, X., Igarashi, I., 2008. Diagnostic real-time PCR assay for the quantitative detection of *Theileria equi* from equine blood samples. Vet. Parasitol. 151, 158–163. https://doi.org/10.1016/j.vetpar.2007.10.023.

King'ori, E., Obanda, V., Chiyo, P.I., Soriguer, R.C., Morrondo, P., Angelone, S., 2019. Molecular identification of *ehrlichia, anaplasma, babesia* and *theileria* in African elephants and their ticks. PloSone 14, e022608. https://doi.org/10.1371/journal.pone.0226083.

Knight, M.H., Emslie, R.H., Smart, R., Balfour, D., 2015. Biodiversity Management Plan For the White Rhinoceros (*Ceratotherium Simum*) in South Africa 2015–2020. Department of Environmental Affairs, Pretoria. South Africa. Available: https://www.gov.za/sites/default/files/gcis_document/201512/39469gen1191.pdf#:~:text=The%20white%20(Ceratotherium%20simum)%20and%20black%20(Diceros,second%20largest%20land%20mammal%20after%20the%20elephant [Accessed 19 April 2025].

Mccullogh, B., Achard, P.L., 1969. Mortalities associated with capture, translocation, trade and exhibition of black rhinoceroses. Int. Zoo Yearb. 9, 184–191. http://www.rhinoresourcecenter.com/pdf_files/130/1300834356.pdf.

Mellya, R.V.K., Hopcraft, J.G.C., Eblate, E.M., Otiende, M., Chuma, I.S., Macha, E.S., Wambura, D., Kilbride, E., Mable, B.K., 2023. Genetic diversity of the Eastern black rhinoceros (*Diceros bicornis michaeli*) in Tanzania; implications for future conservation. *BioRxiv*. 2023.01.26.525648. https://doi.org/10.21203/rs.3.rs-2560054/v1.

Miller, M., Buss, P., Wanty, R., Parsons, S., Van Helden, P., Olea-Popelka, F., 2015. Baseline hematologic results for free-ranging white rhinoceros (*Ceratotherium simum*) in Kruger National Park, South Africa. J. Wildl. Dis. 51, 916–922. https://doi.org/10.7589/2015-03-081.

Mugera, G.M., Wandera, J.G., 1967. Degenerative polymyopathies in East African domestic and wild animals. Vet. Rec. 80, 410–413. https://doi.org/10.1136/vr.80.13.410.

Nijhof, A.M., Penzhorn, B.L., Lynen, G., Mollel, J.O., Morkel, P., Bekker, C.P.J., Jongejan, F., 2003. *Babesia bicornis sp. nov*. And *theileria bicornis sp. nov.*: tick-borne parasites associated with mortality in the black rhinoceros (*Diceros bicornis*). J. Clin. Microbiol. 41, 2249–2254. https://doi.org/10.1128/jcm.41.5.2249-2254.2003.

Nijhof, A.M., Pillay, V., Steyl, J., Prozesky, L., Stoltsz, W.H., Lawrence, J.A., 2005. Molecular characterization of *Theileria* species associated with mortality in four species of African antelopes. J. Clin. Microbiol. 43, 5907–5911. https://doi.org/10.1128/jcm.43.12.5907-5911.2005.

Otiende, M.Y., Kivata, M.W., Jowers, M.J., Makumi, J.N., Runo, S., Obanda, V., Gakuya, F., Mutinda, M., Kariuki, L., Alasaad, S., 2016. Three novel haplotypes of *Theileria bicornis* in black and white rhinoceros in Kenya. Transb. Emerg. Dis. 63, e144–e150. https://doi.org/10.1111/tbed.12242.

Otiende, M.Y., Kivata, M.W., Makumi, J.N., Mutinda, M.N., Okun, D., Kariuki, L., Obanda, V., Gakuya, F., Mijele, D., Soriguer, R.C., Alasaad, S., 2015. Epidemiology of *theileria bicornis* among black and white rhinoceros metapopulation in Kenya. BMC Vet. Res. 11, 4. https://doi.org/10.1186/s12917-014-0316-2.

Pfitzer, S., Oosthuizen, M., Bosman, A.M., Vorster, I., Penzhorn, B., 2011. Tick-borne blood parasites in nyala (*Tragelaphus angasii*, Gray 1849) from KwaZulu-Natal, South Africa. Vet. Parasitol. 176, 126–131. https://doi.org/10.1016/j.vetpar.2010.11.006.

Sibeko, K.P., Oosthuizen, M.C., Collins, N.E., Geysen, D., Rambritch, N.E., Latif, A.A., Groeneveld, H.T., Potgieter, F.T., Coetzer, J.A., 2008. Development and evaluation of a real-time polymerase chain reaction test for the detection of *Theileria parva* infections in Cape buffalo (*Syncerus caffer*) and cattle. Vet. Parasitol. 155, 37–48. https://doi.org/10.1016/j.vetpar.2008.03.033.

Troskie, M., De Villiers, L., Leisewitz, A., Oosthuizen, M.C., Quan, M., 2019. Development and validation of a multiplex, real-time PCR assay for *Babesia rossi* and *Babesia vogeli*. Ticks Tick Borne Dis. 10, 421–432. https://doi.org/10.1016/j.ttbdis.2018.12.004.

Wielgus, E., Mandinyenya, B., Wieprecht, S., Heurich, M., 2023. Bringing the black rhino back: key factors for reintroduction success. Glob. Ecol. Conserv. 48, e02756. https://doi.org/10.1016/j.gecco.2023.e02756.

Yam, J., Gestier, S., Bryant, B., Campbell-Ward, M., Bogema, D., Jenkins, C., 2018. The identification of *theileria bicornis* in captive rhinoceros in Australia. Int. J. Parasitol. Par. Wildl. 7, 85–89. https://doi.org/10.1016/j.ijppaw.2017.12.003.

Zimmermann, D., Vorster, I., Dreyer, C., Fowlds, W., Penzhorn, B., 2022. Successful treatment of babesiosis in a South-Western black rhinoceros (*Diceros bicornis bicornis*). J. S. Afri. Vet. Assoc. 93, 139–143. https://doi.org/10.36303/JSAVA.478.

Zimmermann, D.E., Penzhorn, B.L., Vorster, I., Troskie, M., Oosthuizen, M.C., 2021. *Babesia bicornis, Theileria bicornis*, and *Theileria equi* in metapopulations of two black rhinoceros (*Diceros bicornis*) subspecies in South Africa and their potential impact on conservation. Ticks Tick Borne Dis. 12, 101635. https://doi.org/10.1016/j.ttbdis.2020.101635.