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Short communication

Genotypic variations in field isolates of *Theileria* species infecting giraffes (*Giraffa camelopardalis tippelskirchi* and *Giraffa camelopardalis reticulata*) in Kenya

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ABSTRACT

Recently, mortalities among giraffes, attributed to infection with unique species of piroplasms were reported in South Africa. Although haemoparasites are known to occur in giraffes of Kenya, the prevalence, genetic diversity and pathogenicity of these parasites have not been investigated.

In this study, blood samples from 13 giraffes in Kenya were investigated microscopically and genomic DNA extracted. PCR amplicons of the hyper-variable region 4 (V4) of *Theileria* spp. small subunit ribosomal RNA (18S rRNA) gene were hybridized to a panel of genus- and species-specific oligonucleotide probes by reverse line blot (RLB). Two newly designed oligonucleotide probes specific for previously identified *Theileria* spp. of giraffes found single infections in eight of the specimens and mixed infections in the remaining five samples. Partial 18S rRNA genes were successfully amplified from 9 samples and the PCR amplicons were cloned. A total of 28 plasmid clones representing the Kenyan isolates were analyzed in the present study and compared with those of closely-related organisms retrieved from GenBank. In agreement with RLB results, the nucleotide sequence alignment indicated the presence of mixed infections in the giraffes. In addition, sequence alignment with the obtained 18S rRNA gene sequences revealed extensive microheterogeneities within and between isolates, characterized by indels in the V4 regions and point mutations outside this region. Phylogeny with 18S rRNA gene sequences from the detected parasites and those of related organisms places *Theileria* of giraffes into two major groups, within which are numerous clades that include the isolates reported in South Africa. Collectively, these data suggest the existence of at least two distinct *Theileria* species among giraffes, and extensive genetic diversity within the two parasite groups.

1. Introduction

Piroplasms in Kenyan giraffes were first reported by Brocklesby and Vidler [1] who identified *Theileria*-like piroplasms in both the Maasai (*Giraffa camelopardalis tippelskirchi*) and reticulated (*Giraffa camelopardalis reticulata*) giraffes, and one *Babesia* sp. in reticulated giraffes. Recently, Oosthuizen et al. [2] reported potentially pathogenic species of *Babesia* and *Theileria* suspected of causing fatalities in young giraffes in South Africa. In their report, each animal was found to be infected with a unique parasite, as deduced from 18S ribosomal RNA (18S rRNA) gene sequences, and suggested that more piroplasms may occur in giraffes [2]. The tick vectors, as well as, the epidemiology and pathology associated with these organisms in East African giraffes are not yet known.

Extensive genetic variation has been reported among field populations of different *Theileria* spp. [3–9]. Bhoora et al. [5] and Salim et al. [6] reported the occurrence of numerous genotypes of *Theileria equi*, the causative agent of equine piroplasmosis in horses, across geographic regions within a single country. Several unique genotypes and variants associated with disease outbreaks have also been reported recently in *Theileria orientalis*, the casual agent of bovine piroplasmosis in Asia and Australia [10]. This genetic heterogeneity within *Theileria* genus is believed to among other factors, arises from sexual recombination during gametogony in the vector-ticks [11]. In the present study, we report sequence heterogeneity in field isolates of *Theileria* spp. of the giraffes from Kenya.

Thirteen giraffe blood samples in EDTA-coated tubes were provided by the Veterinary Department, Kenya Wildlife Service (KWS), Nairobi, Kenya (Table 1). Genomic DNA was extracted from 500 μl of frozen blood or 300 μl of fresh blood with the Wizard Genomic DNA purification Kit (Promega, Madison, USA). Primers RLB F2 [5′-GAC ACA GGG

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Table 1
Summary of animal hosts from which giraffe blood samples were obtained, and GenBank accession numbers of corresponding *Theileria* isolates identified in this study. Samples that were positive in both touch-down PCR and reverse line blot (RLB) but failed to yield visible bands with 18S rRNA PCR were not cloned (ND).

Sample identification	Age (years)	Geographical origin	RLB screening	18S rRNA clone # (GenBank accession no.) [bp]
Giraffe #30	1	Garrisa, N.E.P.	Single infection	2(JQ928928)[1665], 5(JQ928927)[1361]
Giraffe #43	2	Garrisa, N.E.P.	Single infection	ND
Giraffe #44	5	Garrisa, N.E.P.	Mixed infection	1(JQ928926[a])[1433], 2(JQ928925)[1665], 4(JQ928924)[1672]
Giraffe #63	1.5	Garrisa, N.E.P.	Single infection	5(JQ928922[b])[1649], 6(JQ928921)[1665], 4(JQ928923)[1665]
Giraffe #64	2	Nairobi National Park	Single infection	ND
Giraffe #66	2	Nairobi National Park	Single infection	2(JQ928920)[1665], 3(JQ928919)[1653], 4(JQ928918[b])[1665], 6(JQ928917)[1665],
Giraffe #70	1.5	Nairobi National Park	Single infection	1(JQ928916)[1665], 6(JQ928914[b])[1644], 2(JQ928915)[1665]
Giraffe #81	Adult	Giraffe Centre, Nairobi	Mixed infection	1(JQ928913)[1665], 3(JQ928912)[1656], 6(JQ928911)[1665]
Giraffe #86	Adult	Giraffe Centre, Nairobi	Mixed infection	1(JQ928910)[1570], 3(JQ928909)[1665], 5(JQ928908)[1665], 6(JQ928907)[1614]
Giraffe #94	Juvenile	Giraffe Centre, Nairobi	Mixed infection	ND
Giraffe #104	Adult	Garrisa, N.E.P.	Single infection	ND
Giraffe #108	Adult	Garrisa, N.E.P.	Single infection	4(JQ928933[b])[1658], 1(JQ928934[b])[1664], 6(JQ928932)[1609]
Giraffe #115	Adult	Giraffe Centre, Nairobi	Mixed infection	6(JQ928929)[1665], 2(JQ928931[a])[1665], 3(JQ928930)[1184]

N.E.P.: North–Eastern Province. ND: No data.
[a] Isolates matching RLB probe *Theileria* sp. (giraffe) 2.
[b] Isolates matching RLB probe *Theileria* sp. (giraffe) 1.

AGG TAG TGA CAA G-3′] and biotin-labeled RLB R2 [5′-Biotin-CTA AGA ATT TCA CCT CTA ACA GT-3′] [12] were used to PCR amplify the V4 hypervariable region (V4) of parasite 18S rRNA gene from the giraffe DNA samples using a touchdown PCR protocol as described previously [13]. Genomic DNA from *Theileria parva*-infected cattle, Cape buffalo (*Syncerus caffer*) and eland (*Taurotragi orynx*) was used as positive controls at the PCR step for the subsequent RLB hybridization. The buffalo and eland positive controls were field samples from which *Theileria* sp. (buffalo) and *Theileria taurotragi*, respectively, had been previously detected while control genomic DNA for other *Theileria* spp. was not available. Sterile, deionized water was used as a negative control. Five μl of PCR products were electrophoresised through a 2% agarose gel, and stained with ethidium bromide prior to visualization under UV light.

To identify *Theileria* spp. occurring in giraffes, 18S rRNA gene sequences of *Theileria* spp. previously described in giraffes [2] were obtained from GenBank and used to design two species-specific probes, designated as *Theileria* sp. (giraffe) 1 and *Theileria* sp. (giraffe) 2. In addition to these two, probes specific for all *Theileria* and *Babesia* spp. (*Theileria*/*Babesia* catch-all), all *Theileria* spp. (*Theileria* catch-all), all *Babesia* spp. (*Babesia* 1 and 2) as well as several others that are specific for different *Theileria* spp. were included on the membrane for screening blood parasites (Table 2). Reverse line blot was conducted as detailed previously [19].

Table 2
List of oligonucleotide probes used in the RLB assay. GenBank accession numbers of *Theileria* spp. (giraffe) used to derive the newly designed probes are shown in parentheses.

Probe specificity	18S sequence (5′–3′)	Reference
Theileria/*Babesia* catch-all	TAATGGTTAATAGGARCRGTTG	Gubbels et al. 1999 [13]
Theileria catch all	ATTAGAGTGCTCAAAGCAGGC	Nagore et al. 2004 [14]
Babesia catch all 1	ATTAGAGTGTTTCAAGCAGAC	Bhoora et al. 2009 [4]
Babesia catch all 2	ACTAGAGTG TTT CAA ACAGGC	Brothers et al. 2011 [15]
Theileria parva	GGACGGAGTTCGCTTTG	Gubbels et al. 1999 [13]
Theileria taurotragi	TCTTGGCACGTGGCTTTT	Gubbels et al. 1999 [13]
Theileria sp. (buffalo)	CAGACGGAGTTTACTTTGT	Oura et al. 2004 [16]
Theileria annulata	CCTCTGGGGTCTGTGCA	Georges et al. 2001 [12]
Theileria buffeli	GGCTTATTTCGGWTTGATTTT	Gubbels et al. 1999 [13]
Theileria mutans	CTTGCGTCTCCGAATGTT	Gubbels et al. 1999 [13]
Theileria velifera	CCTATTCTCCTTTACGAGT	Gubbels et al. 1999 [13]
Theileria ovis	TTGCTTTTGCTCCTTTACGAG	Nagore et al. 2004 [14]
Theileria sp. (kudu)	CTCCATTGTTTCTTTCCTTTG	Nijhof et al. 2005 [17]
Theileria sp. (sable)	GCT GCA TTG CCT TTT CTC C	Oosthuizen et al. 2008 [18]
Theileria sp. (giraffe) 1 (FJ213582 & FJ213584)	TTATTTCTCCTTGACGAGTT	This work
Theileria sp. (giraffe) 2 (FJ213583)	CTCTTTGATGGGCTTTTG	This work

Partial 18S rRNA genes from giraffe DNA samples were PCR-amplified, cloned, and sequences edited manually as described previously [19]. Basic local alignment search tool (BLAST) [20] was used to search for sequences similar to the 18S rRNA gene sequences determined in the present study. A nucleotide sequence alignment of the new sequences and a number of related organisms obtained from the GenBank were constructed using CLUSTAL W [21] in Geneious Pro 5.5 [22] and the alignment trimmed to remove ambiguous sequence ends. Similarity matrices were performed using the two-parameter method of Kimura [23] alongside the Jukes–Cantor correction model for multiple base changes [24]. Phylogenetic trees were constructed using the neighbor-joining method [25] implemented by Geneious Pro 5.5 in combination with the bootstrap method at 1000 replicates/tree. GenBank accession numbers for the new sequences and corresponding animal sources are listed in Table 1.

Blood smears revealed the presence of haemoparasites in the erythrocytes of specimen #115 whereas the rest of the blood specimens were too haemolyzed for microscopy. Genomic DNA was successfully extracted from all samples. A touchdown PCR with primers targeting the V4 region of the 18S rRNA gene yielded visible bands with 12/13 (92%) of the giraffe-derived DNA samples and for the three positive controls while no contamination was detected in the water negative control (Fig. 1).

A total of sixteen generic and species-specific probes were included in the RLB assay. Among the positive controls, strong signals corresponding to *T. parva*, *Theileria* sp. (buffalo) and *T. taurotragi* were visible on the membrane while additional bands were also observed in the case of the buffalo and eland genomic DNA. In the study samples, strong and distinct RLB signals were obtained with the *Theileria*/*Babesia* catch-all and *Theileria* catch-all probes. RLB with oligonucleotide probes designed to detect *Theileria* spp. of giraffes showed that *Theileria* DNA was present in all 13 samples. Between these two newly designed probes, *Theileria* sp. (giraffe) 1 hybridized with PCR amplicons from a total of 11 giraffe samples (#30, 43, 44, 63, 64, 66, 70, 81, 86, 94 and 115) whereas the second probe, *Theileria* sp. (giraffe) 2, hybridized with 7 samples (44, 81, 86, 94, 104, 108 and 115). In addition, out of the 13 samples, 5 (44, 81, 86, 94 and 115) yielded signals with both new probes. No signals were detected with the water negative control or with the other species-specific probes present on the membrane (Fig. 1).

Subsequently, partial 18S rRNA genes were successfully amplified and cloned from nine giraffe specimens. In total, 28 sequences (1184–1672 bp) representing parasite isolates from Kenyan giraffes were