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Identification of a Novel *Babesia* sp. from a Sable Antelope (*Hippotragus niger* Harris, 1838)▽

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Babesiosis in a sable antelope (*Hippotragus niger* Harris, 1838) was first reported in 1930; the parasite was named *Babesia irvinesmithi*. Recently, specimens from an adult sable that presented with a sudden onset of disease and that subsequently died during immobilization were submitted for molecular characterization. Microscopic examination of thin blood smears revealed the presence of small piroplasms. DNA was extracted from blood samples; the V4 variable region of the 18S rRNA gene was amplified and analyzed using the reverse line blot (RLB) assay. Amplicons did not hybridize with any of the *Babesia* or *Theileria* species-specific probes present on the blot and hybridized only with a *Babesia* or *Theileria* genus-specific probe, suggesting the presence of a novel species. The full-length 18S rRNA gene sequence was obtained and aligned with published sequences of related genera, and phylogenetic trees were constructed. Sequence similarity analyses indicated that a *Babesia* species, designated *Babesia* sp. (sable), was present. The sequence showed its highest similarity to *B. orientalis* and to an unnamed *Babesia* species previously detected in bovine samples. The latter was later established to be *Babesia occultans*. A *Babesia* sp. (sable)-specific RLB oligonucleotide probe was designed and used to screen 200 South African sable samples, but so far, no other sample has been found to be positive for the presence of *Babesia* sp. (sable) DNA. In summary, we identified a novel piroplasm parasite from a sable antelope that died from an unknown illness. While the parasite was observed in blood smears, there is no direct evidence that it was the cause of death.

The sable antelope (*Hippotragus niger* Harris, 1838) is regarded as a rare species, and the World Conservation Union (IUCN) Red List of Threatened Species lists it as "lower risk" and "conservation dependent" (http://www.iucnredlist.org). Declining numbers could, however, lead to a "threatened" listing in the near future. Four subspecies of sable antelope are recognized: common or southern (*Hippotragus niger niger*), Zambian (*H. n. kirkii*), eastern (*H. n. roosevelti*), and giant or Angolan (*H. n. variani*) sable antelope (see the American Zoo and Aquarium Association Antelope Taxon Advisory Group website [www.antelopetag.com]). Of these, the giant sable antelope (*H. n. variani*) of central Angola is classified as "critically endangered" on the IUCN Red List of 2007 (http://www.iucnredlist.org) and is listed in appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (7). Sable antelope occur in scattered populations in dry open woodlands and medium-tall grass savannas in eastern and southern Africa. Some populations have been established outside the historic range of the species, while breeding populations are held in a number of zoos throughout the world. Declining numbers of sable antelope can be attributed to a combination of disease, drought-caused food shortages, habitat loss and degradation, and subsistence hunting (http://www.arkive.org/species/GES/mammals/Hippotragus_niger/).

African antelope host a variety of intraerythrocytic organisms, some of which are benign, while others are pathogenic in wildlife and/or domestic animals. *Theileria* spp. (29), *Babesia* spp. (24, 26, 36, 38), and *Anaplasma* spp. (10, 36) from sable antelope have previously been reported. The first fatal case of babesiosis in a sable was reported in 1930, with the death of a sable in the Johannesburg Zoological Gardens, six weeks after arrival (24). *Babesia*-like parasites were observed in the blood smears, and the postmortem examination showed typical liver and spleen lesions characteristic of bovine babesiosis. The disease was diagnosed as babesiosis and was probably due to reduced resistance following the change of environment and close confinement. Interestingly, this is also the first record of an antelope dying from piroplasm infection. The parasite was subsequently named *Babesia irvinesmithi* (25). Only three further cases of babesiosis in sable antelope have since been recorded (26, 36, 38). These findings were based on microscopic examinations of blood smears and postmortem examinations. McInnes and colleagues (26) showed that the parasite was smaller than *Babesia bigemina* and *Babesia major* but similar in size to *Babesia bovis*. Hove and colleagues (13) presented circumstantial evidence of *B. bigemina* infection in a sable antelope. They collected engorged *Rhipicephalus evertsi evertsi* nymphs from a sable antelope that had died of acute babesiosis and allowed them to molt. The resulting adults were then fed on bovines, which became infected with *B. bigemina*, as confirmed by the indirect fluorescent antibody test and PCR. This suggests that the immature ticks may have become

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infected while engorging on the sable antelope. It should be borne in mind, however, that large *Babesia* spp. are typically transmitted transovarially, and there exists the possibility that the progenitors of the ticks involved may have fed on infected cattle. Unfortunately, Hove and colleagues (13) did not mention the origin of the sable antelope, i.e., whether there were cattle in the area.

Babesia occultans, which causes a benign form of cattle babesiosis in South Africa, was described by Gray and De Vos in 1981 (9). They speculated that *B. occultans* originated from African antelope, but to date, this organism has not been identified in wildlife in South Africa. Little else is known about *B. occultans*, and no sequence data are available for comparison.

Wilson and colleagues (38) reported on a *Theileria*-like species which was associated with mortality in juvenile sable and roan (*Hippotragus equinus* Desmarest, 1804) antelope in South Africa and subsequently described it as a *Cytauxzoon* sp. Subsequently, synonymizing the genus *Cytauxzoon* with *Theileria* was suggested based on observations of blood parasites in sable antelope (36). Nijhof and colleagues (29) reported on a fatal case of theileriosis in translocated sable antelope in 2005 and described the parasite as *Theileria* sp. (sable). They also suggested, based on the close phylogenetic relationship between members of the genera *Theileria* and *Cytauxzoon*, that the taxonomic status of the etiological agents of theileriosis in African antelope species be clarified.

An adult male sable antelope, originating from a game ranch in Limpopo Province, South Africa, presented with a sudden onset of disease and subsequently died during immobilization. A study was undertaken to characterize the piroplasm that was detected in the sable antelope and to determine its phylogenetic relationship to *B. occultans* and other piroplasms previously identified in African antelope.

MATERIALS AND METHODS

DNA extraction. Blood samples, collected in EDTA, of the adult male sable antelope were submitted to our laboratory for molecular characterization. DNA was extracted from 200 μl of blood by using the QIAamp DNA extraction kit (Qiagen, Southern Cross Biotechnologies). Extracted DNA (labeled as Sable_2505) was eluted in 100 μl elution buffer and stored at 4°C until further analysis. *Babesia occultans* DNA was extracted from 200 μl of tissue culture stabilate (37).

RLB technique. The V4 variable region of the parasite 18S rRNA gene from sample Sable_2505 was amplified by PCR using the *Theileria* and *Babesia* genus-specific primers RLB F2 (5′-GAC ACA GGG AGG TAG TGA CAA G-3′) and biotin-labeled RLB R2 (5′-biotin-CTA AGA ATT TCA CCT CTG ACA GT-3′) as previously described (28, 29). The PCR products were analyzed using the reverse line blot (RLB) hybridization technique as described by Nijhof and colleagues (29). The commercial tick-borne disease detection–RLB kit (Isogen Life Sciences, Ijsselstein, The Netherlands) was used following the manufacturer's instructions.

Cloning and sequencing. Primers Nbab_1F (5′-AAG CCA TGC ATG TCT AAG TAT AAG CTT TT-3′) and Nbab_1R (5′-CTT CTC CTT CCT TTA AGT GAT AAG GTT CAC-3′) were designed for the amplification of a 1,600-bp fragment of the parasite 18S rRNA gene from the Sable_2505 and *B. occultans* samples. The amplification mixture contained 2.5 μl DNA (~75 ng), 12.5 μl Expand high-fidelity PCR master mix (Roche Diagnostics, Mannheim, Germany), 0.1 μM of each primer, and nuclease-free water to a total volume of 25 μl. For each of the samples, the reaction was duplicated four times to obtain a total volume of 100 μl. Amplification was performed in an automated thermocycler (Perkin-Elmer, Foster City, CA). The initial denaturing was performed at 94°C for 2 min followed by 40 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min. A final extension was at 72°C for 7 min, after which the products were stored at 4°C.

PCR products from the four duplicate reactions were pooled in order to minimize the possibility of obtaining sequence errors originating early in any one of the reactions. DNA amplicons were purified using the QIAquick PCR purification kit (Qiagen and Southern Cross Biotechnologies) and cloned into the pGEM-T Easy vector (Promega, Madison, WI) according to the manufacturer's instructions. For each sample, six recombinant plasmids were directly sequenced using the ABI BigDye Terminator cycle sequencing ready-reaction kit (PE Applied Biosystems), 350 ng plasmid DNA, and 3.2 pmol of primer. The primers used for sequencing were RLB F2, RLB R2, Nbab_1F, Nbab_1R, and the newly designed BT18S_2F (5′-GGG TTC GAT TCC GGA GAG GG-3′), BT18S_3F (5′-GGG CAT TCG TAT TTA ACT GTC AGA GG-3′), BT18S_4F (5′-CGG CTT AAT TTG ACT CAA CAC GGG-3′), and BT18S_4R (5′-CCC TCT CCG GAA TCG AAC CC-3′). Purified products were analyzed on an ABI3100 genetic analyzer at the Agricultural Research Council-Onderstepoort Veterinary Institute (South Africa) sequencing facility.

Phylogenetic analysis. The 18S rRNA gene sequence data were assembled and edited by using GAP4 of the Staden package (version 1.6.0 for Windows) (5, 33, 34). The assembled sequence was aligned with published sequences of related genera by using MAFFT (version 6) (15). The alignment was manually truncated to the size of the smallest sequence (*Theileria* sp. sable, 1,425 bp). Similarity matrices were constructed from the aligned sequence data by single distance using the two-parameter model of Kimura (16), which takes into account transitional and transversional substitution rates, as well as the Jukes and Cantor correction for multiple base changes (14). Phylogenetic trees were constructed by the neighbor-joining (30) and maximum-parsimony methods as implemented by the MEGA 3.0 software package (17). It was used in combination with the bootstrap method (1,000 replicates/tree for distance methods and 100 replicates/tree for parsimony methods) (8). The two different substitution models were used in order to identify clades supported by the highest possible bootstrap values.

Probe design. A *Babesia* sp. (sable)-specific RLB oligonucleotide probe (5′-GCG TTG ACT TTG TGT CTT TAG C-3′) with a melting temperature of 57.3°C (calculated empirically) was designed for use in the hypervariable V4 region of the 18S rRNA gene. The probe was synthesized containing an N-terminal *N*-(trifluoracetamidohexylcyanoethyl,*N*,*N*-diisopropyl phosphoramidite)-C_6 amino linker to facilitate covalent linkage to the membrane (Southern Cross Biotechnologies, South Africa) and prepared for use in the RLB assay as described by Nijhof and colleagues (29).

Nucleotide sequence accession numbers. The 18S rRNA gene sequence of the species designated *Babesia* sp. (sable) has been submitted to GenBank with accession number EU376016. The *Babesia occultans* 18S rRNA gene sequence is available under accession number EU376017.

RESULTS

The noted clinical signs exhibited by the adult sable antelope that presented with a sudden onset of disease were acute lethargy and recumbency. No postmortem examination was performed. The attending veterinarian reported that microscopic examination of thin blood smears revealed the presence of small piroplasms in red blood cells. No size estimate or detail on the morphology was recorded, and unfortunately, the blood smears are no longer available. RLB results indicated that the PCR products from Sable_2505 failed to hybridize with any of the *Babesia* or *Theileria* species-specific probes present on the blot but did hybridize with the *Babesia*/*Theileria* genus-specific probe, suggesting the presence of a novel species or variant of a species. Six different clones of the 18S rRNA gene were sequenced, and the resulting 1,551-bp sequences were identical. A BLAST search performed with this 18S rRNA gene sequence revealed no identical sequences in the public databases, and this parasite was therefore designated *Babesia* sp. (sable). The most closely related sequences, with approximately 97% identity, were from *B. orientalis* (accession number AY596279), identified in China from a water buffalo (*Bubalus bubalis* Linnaeus, 1758) (19), an unnamed *Babesia* species

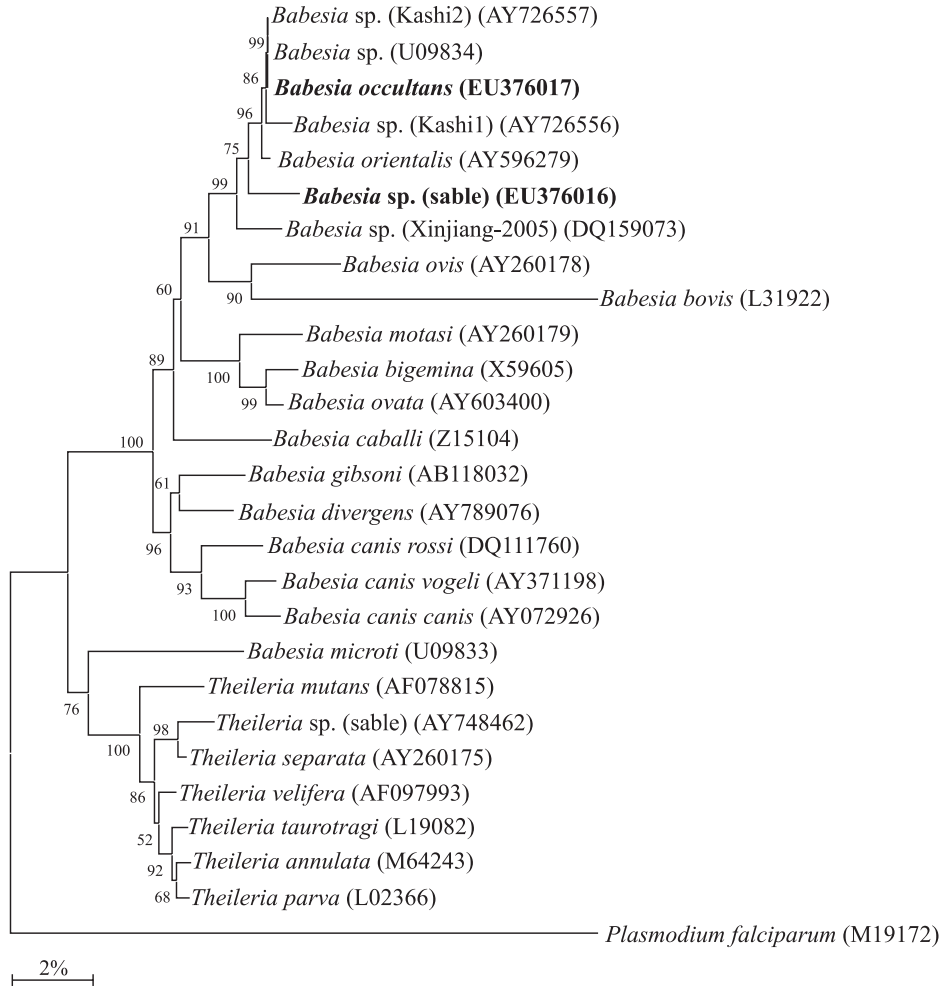

FIG. 1. Neighbor-joining tree showing the phylogenetic relationship of *Babesia* sp. (sable) with other *Babesia* and *Theileria* species based on 18S rRNA gene sequences. The GenBank accession numbers are indicated in parentheses. Branch lengths are proportional to the estimated genetic distance between the strains. The scale bar represents the percent nucleotide difference. Vertical lengths are not significant and are merely set for clarity. *Plasmodium falciparum* (M19172) was used as an outgroup.

(U09834) that was detected in a bovine in 1994 in South Africa (1), and *Babesia* sp. Kashi 2 (AY726557) (21), identified in bovines from China. Other highly similar sequences (with approximately 95% identity) were from *Babesia* sp. Kashi 1 (AY726556) also identified from a bovine in China (27) and *Babesia* sp. Xinjiang-2005 (DQ159073), which often leads to clinically inapparent infection in ovines (18).

To show whether *Babesia* sp. (sable) could in fact be *B. occultans*, we amplified, cloned, and sequenced the 18S rRNA gene from *B. occultans* tissue culture material (37). The *B. occultans* 18S rRNA gene showed 97% identity with that of *Babesia* sp. (sable) but was almost identical (99% identity) to *Babesia* sp. Kashi 2 (AY726557) and the unnamed *Babesia* species (U09834) detected in a bovine in 1994 (1).

Both neighbor-joining and maximum parsimony phylogenetic analyses were used to reveal the relationship among *Babesia* sp. (sable), *B. occultans*, and other *Babesia* and *Theileria* species. No significant changes in the topology of the trees or in the bootstrap values were found when using either the neighbor-joining or maximum-parsimony method. A representative tree obtained by the neighbor-joining method with the Kimura two-parameter distance calculation (16) is shown in Fig. 1. *Babesia* sp. (sable) lies in the *Babesia* sensu stricto clade, comprising *Babesia* spp. that are characterized by transovarial tick transmission, including *B. divergens*, *B. bigemina*, and *B. canis*. Within this clade, it forms a monophyletic group with *B. orientalis*, *Babesia* sp. Kashi 1, *Babesia* sp. Kashi 2, *B. occultans*, and the unnamed *Babesia* species (U09834) detected in a bovine in 1994 (1).

The newly designed *Babesia* sp. (sable)-specific RLB probe was shown to bind to its target sequence during RLB analysis. It was subsequently used to screen 200 sable antelope blood samples that originated from several different game farms in the Limpopo and Mpumalanga Provinces as well as from the Johannesburg Zoological Gardens. One sample originated from the same game ranch as the adult male sable. However, to date, no other sable sample has been found to be positive for the presence of DNA from this parasite species, indicating that it may represent a rare infection. Twelve roan antelope (*Hippotragus equinus*) samples and seven giraffe (*Giraffa camelop-*

ardalis) samples were also screened, but none were found to be positive for *Babesia* sp. (sable) DNA.

DISCUSSION

Wild ruminants such as the sable antelope, giraffe, and roan harbor *Babesia* species (6, 24, 26, 36, 38). Most of these animals carry the parasites as asymptomatic infections, with clinical babesiosis appearing only when the animals are stressed. The clinical signs observed are similar to those seen in domestic animals and include anemia, hemoglobinuria, pale to icteric mucous membranes, depression, and, in some cases, death. Unfortunately, little is known about the sable antelope from which *Babesia* sp. (sable) was isolated. All that is known is that the animal showed a sudden onset of disease and died during immobilization. Clinical signs noted by the attending veterinarian were acute lethargy and recumbency. It was also reported that microscopic examination of thin blood smears revealed the presence of small piroplasms in red blood cells. Unfortunately, these blood smears were not available to us, and the actual size of the piroplasms could not be determined.

Molecular techniques have proven very useful in the detection and identification of many hemoparasites and are based on species-specific PCR assays, many of which target the 18S rRNA gene. This gene has been used to identify and classify several previously unknown *Theileria* and *Babesia* parasites (4, 12, 28, 29, 31). An RLB assay based on species-specific sequences in parasite 18S rRNA genes has been developed for the simultaneous detection and identification of tick-borne parasites infecting cattle and small ruminants (3, 11, 32). In this study, the RLB assay indicated the presence of a novel species or variant of a species in the sable sample presented to us. The 18S rRNA gene sequence data showed that a *Babesia* sp. infection was present, and the organism was placed in the *Babesia* sensu stricto clade, confirming that it is a true *Babesia* sp. True *Babesia* spp. (e.g., *B. bigemina*, *B. divergens*, and *B. canis*) are characterized by transovarial transmission in the tick vector and division only in erythrocytes of the vertebrate hosts (2, 27). Within the *Babesia* sensu stricto clade, this organism formed a monophyletic group with *B. orientalis*, *Babesia* sp. Kashi 1, *Babesia* sp. Kashi 2, *B. occultans*, and the unnamed *Babesia* species (U09834) detected in a bovine in 1994.

Very little is known about any of these parasites. *Babesia orientalis* causes water buffalo babesiosis and causes a tick-borne hemoparasitic disease that was described in 1997 by Liu and colleagues (20). The disease is characterized by fever, anemia, icterus, hemoglobinuria, and high mortality, and it causes severe economic losses (19). The authors showed that the tick vector of this parasite was *Rhipicephalus haemaphysaloides* and that the only host of *B. orientalis* was water buffalo. Unfortunately, the tick vector of the sable parasite identified in this study remains unknown.

Nothing is known about the unnamed *Babesia* species (U09834) that was detected in a bovine in 1994 in South Africa (1).

Babesia sp. Kashi 1 and *Babesia* sp. Kashi 2 were identified from nymphal and adult *Hyalomma anatolicum anatolicum* ticks, respectively, that were collected in the field in China (23). Molecular studies based on the 18S rRNA gene showed that the parasites detected from the two different tick stages (*Babesia* sp. Kashi 1 and *Babesia* sp. Kashi 2) were 99.7% identical, and the authors concluded that they were the same species. It is different from other *Babesia* species reported in China, including *B. bigemina*, *B. bovis*, *B. major*, and *B. ovata*, and represents a novel *Babesia* species in China (21). Other studies have shown that *H. a. anatolicum* can transmit this *Babesia* sp. transovarially but not transstadially (22). These authors recorded no apparent disease associated with this parasite.

Babesia sp. Xinjiang-2005 was originally detected from a batch of mixed *Rhipicephalus sanguineus* and *H. a. anatolicum* ticks from Kashi, Xinjiang Province, China, and was described as often leading to clinically inapparent infection in ovines (18).

Babesia occultans (9), the causative agent of a benign form of cattle babesiosis in South Africa, was originally obtained from a farm in northern Transvaal (now Limpopo Province), South Africa, in 1976 (35). It causes a mild disease in cattle and is transmitted transovarially by *Hyalomma marginatum rufipes* (9, 35). Since this species occurred at low numbers and did not seem to cause significant clinical reactions in normal animals, the specific name *Babesia occultans* sp. nov. was proposed (9). According to these authors, it is conceivable that *B. occultans* originated from African antelope. We therefore considered the possibility that *Babesia* sp. (sable), identified here as a new species, could in fact be *B. occultans.* However, the *Babesia* sp. (sable) 18S rRNA gene sequence was different from that of *B. occultans* obtained from tissue culture material. Interestingly, the *B. occultans* 18S rRNA gene sequence differed by only one base pair from the sequences of the unnamed *Babesia* species (U09834) and *Babesia* sp. Kashi 2. These sequence data thus give support for *Babesia* sp. (sable) being a novel species but suggest that the unnamed *Babesia* species (U09834) and *Babesia* sp. Kashi 1 and *Babesia* sp. Kashi 2 could possibly be *B. occultans*. While it has been reported that these parasites are transmitted by different *Hyalomma* species, the full vector range for *B. occultans* has not been elucidated. The reports of clinically inapparent or mild infection are certainly consistent for all three parasites.

In summary, in this study, a novel *Babesia* species, shown to be a member of the *Babesia* sensu stricto clade, was identified after isolation from a sable antelope. The parasite was named *Babesia* sp. (sable). It is tempting to propose that the parasite described in this study is *B. irvinesmithi*, first described by Martinaglia in 1930 (24), and responsible for fatal cases reported in sable antelope in the past. This remains speculative, however: although the parasite was observed in blood smears, there is no direct evidence that it was the cause of death. Unfortunately, to date, we have been unable to locate another sable antelope infected with this *Babesia* sp. which would enable us to describe the morphology of the parasite and to conduct infection studies. The tick vector of this parasite also remains unknown. Further research is required to show whether this was an isolated incident or whether this parasite in fact causes severe disease in sable antelope.

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