

Occurrence of *Babesia felis* and *Babesia leo* in various wild felid species and domestic cats in Southern Africa, based on reverse line blot analysis

A.-M. Bosman^a, E.H. Venter^a and B.L. Penzhorn^a

^aDepartment of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag x04, Onderstepoort 0110, South Africa

Abstract

Reverse line blot (RLB) is a hybridization assay that can be used to detect various blood parasites and differentiate between them. Results, using the RLB, showed that *Babesia felis* and *Babesia leo* occurred as single or mixed infections in various felid species, but most frequently in domestic cats and lions, respectively. Prevalence of infection in free-ranging cheetahs in Namibia was low (7, 5%), whereas 50% of free-ranging lions in South Africa and Swaziland were infected. A large number (52, 9%) of samples tested positive only for *Babesia*, neither *B. felis* nor *B. leo*. This could be an indication of at least one further, as yet undescribed, *Babesia* species in felids.

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1. Introduction

Babesia is a tick-borne intracellular erythrocytic haemoprotozoan parasite of mammals, birds and reptiles (Bush et al., 2001 and Levine, 1985). Babesiosis, which is characterized by haemolytic anaemia that may be fatal if not treated (Gutierrez, 2000), is of great economic importance in countries where the specific tick vectors occur.

Babesia spp. have a wide host range in felids. Lingard and Jennings (1904; cited by Mangrulkar, 1937), the first authors to report a piroplasm in a cat, did not illustrate or describe their findings. *Babesia felis* from a Sudanese wild cat (*Felis ocreata*, syn. *F. sylvestris*) was described by Davis (1929) (Wilson and Reeder, 1993). Domestic cats experimentally infected with this parasite developed a parasitaemia in the absence of clinical signs (Davis, 1929). Stewart et al. (1980) described a large *Babesia* from domestic cats.

Other named species from felids are *Babesiella felis* that was described from a puma (*Felis concolor*) (Carpano, 1934), *Babesia cati* from an Indian wild cat (*Felis catus*) (Mudaliar et al., 1950), *Babesia herpailuri* from a jaguarundi (*Herpailurus yaguarondi*) (Dennig, 1967 and Dennig, 1969) and *Babesia pantherae* from a leopard (*Panthera pardus*) (Dennig and Brocklesby, 1972). Dennig and Brocklesby (1972) and Levine (1973) suggested that all small piroplasms from felids are synonyms of *B. felis*. *Babesia leo*, a small piroplasm isolated from lions (*Panthera leo*), is morphologically similar to *B. felis* found in domestic cats, but is serologically and phylogenetically distinct (López-Rebollar et al., 1999 and Penzhorn et al., 2001). Leopards and domestic cats could be experimentally infected with *B. leo* (López-Rebollar et al., 1999).

Unnamed small *Babesia*-like parasites were also reported from the North American “Bay lynx” (*Felis rufus*, probably the bobcat) (Wenyon and Hamerton, 1930), Indian leopard (*Panthera pardus fusca*) (Short, 1940), lions (Barnett and Brocklesby, 1968) and cheetahs (*Acinonyx jubatus*) (Averbeck et al., 1990). The small piroplasms found in a caracal (*Felis caracal*) were found to be closely related to *B. felis* (Penzhorn et al., 2001). Babesiosis in domestic cats was first recognised as a clinical disease in South Africa in 1937 (Jackson and Dunning, 1937 and McNeil, 1937). The parasite involved, morphologically resembled *B. felis*, but was named *Nuttallia felis* var. *domestica* (Jackson and Dunning, 1937). Babesiosis in domestic cats is regarded as an important disease in certain parts of South Africa, particularly in the coastal areas of the Western Cape, Eastern Cape and KwaZulu-Natal Provinces (Jacobson et al., 2000). Infections found in inland areas are mostly in cats that had accompanied their owners on holiday to the coast, but an endemic focus occurs along the eastern escarpment at Kaapschehoop, Mpumalanga Province (Penzhorn et al., 1999).

The mode of transmission of babesiosis in felids is unknown (Jacobson et al., 2000 and Penzhorn et al., 1999). In a phylogenetic study in which felid babesias were compared to other related *Babesia*, *Theileria* and *Cytauxzoon* species, *B. felis* was grouped with *Babesia microti*, suggesting that they have the same mode of transstadial transmission by tick vectors (Penzhorn et al., 2001).

Currently, identification of *B. felis* is done primarily on its morphology on blood smears and serology (Futter and Belonje, 1980, Jacobson et al., 2000, López-Rebollar et al., 1999, Penzhorn et al., 2001 and Schoeman et al., 2001), but this is unsatisfactory.

Nucleic-acid-based techniques are more sensitive for identifying small piroplasms. One such technique is the reverse line blot (RLB) hybridization assay (Gubbels et al., 1999). It can simultaneously detect the genus and differentiate between the species of haemoparasites in blood, organs and ticks. This assay can screen 43 samples simultaneously and is a useful tool in the characterization of blood parasites. The RLB technique has been successfully used for the detection and characterization of *Theileria* and *Babesia* species in blood specimens from horses (Nagore et al., 2004a), sheep (Nagore et al., 2004b) and cattle (Brígido et al., 2004).

Although the morphology of *B. felis* and *B. leo* parasites is adequately described and their full-length 18S rRNA gene sequences are available, very little is known about their relationship to other reported *Babesia* parasites in felid species. The first aim of this study was to investigate the prevalence of *B. felis* and *B. leo* in various felid species in South Africa and the second to ascertain whether further, as yet uncharacterised, *Babesia* species occur.

In order to distinguish between various felid *Babesia* spp. a probe specific to *B. felis* was developed to use in conjunction with the existing *Cytauxzoon felis* (unpublished), *B. leo* (unpublished) and other *Babesia* and *Theileria*-specific probes (Gubbels et al., 1999).

2. Materials and methods

2.1. Samples

2.1.1. Collection

Suspected *Babesia*-positive blood samples from captive cheetahs and lions from South Africa, as well as from domestic cats were submitted to the Biotechnology Laboratory, Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, for routine diagnostics. Felid blood specimens submitted to the Clinical Pathology Laboratory of the same faculty, and found to harbour piroplasms, were also forwarded to the Biotechnology Laboratory for further processing. In addition, blood specimens from free-ranging cheetahs from Namibia and free-ranging lions from Swaziland and South Africa, collected during routine survey, as well as blood specimens from one free-ranging leopard, free-ranging black-footed cats (*Felis nigripes*), servals (*Felis serval*) and caracals, were also screened for the presence of *Babesia* species. All blood samples were collected in EDTA, and stored at -20°C .

2.1.2. Controls

Blood specimens from a lion serologically positive for *B. leo* and a domestic cat serologically positive for *B. felis* were used as positive controls. These specimens were also RLB positive for *B. leo* and *B. felis*, respectively. Blood from a domestic cat that tested negative for *Babesia* parasites using serology and the RLB, as well as water were used as negative controls.

2.2. DNA extraction

DNA was extracted from whole blood using the commercially available QIAamp[®] DNA Mini Kit (Qiagen, Southern Cross Biotechnologies, South Africa), according to the manufacturer's instructions.

2.3. PCR amplification

The PCR was performed as described by Gubbels et al. (1999) using primers (RLB-F-5'-GAGGTAGTGACAAGAAATAACAATA-3' and RLB-Rbiotin-5'-TCTTCGATCCCCTAACTTTC-3') that amplified a 460–520 bp fragment in the V4 variable region of the 18S rDNA of *Theileria* and *Babesia* species. A reaction mixture consisting of Platinum Quantive PCR Supermix-UDG (Invitrogen, The Scientific Group, South Africa), 20 pM of each primer (Isogen, The Netherlands) and 2.5 µl of 70–100 ng purified DNA to a final volume of 25 µl was used. A touch down PCR programme was followed, starting with 3 min at 37 °C; 10 min at 94 °C; and 10 cycles of 94 °C for 20 s, 67 °C for 30 s, 72 °C for 30 s with decreasing of the annealing temperature after every second cycle by 2 °C for five times. These cycles continued until the annealing temperature reached 57 °C. Finally, 40 cycles of 94 °C for 20 s; 57 °C for 30 s and 72 °C for 30 s were performed in a 9600 Perkin-Elmer thermocycler (Applied Biosystems, South Africa). The PCR amplicons were verified using agarose gel electrophoresis before it was analysed by RLB hybridization.

2.4. Reverse line blot hybridization

2.4.1. *B. felis*-specific probe

This probe was developed in collaboration with the Department of Tropical Medicine, Utrecht University, and Isogen, Maarssen, The Netherlands. GenBank accession numbers for the 18S rRNA gene sequences used to develop the *B. felis* species-specific probe were as follows: AF078815 (*Theileria mutans*); AF078816 (*T. spp.*, MSD); AF097993 (*T. velifera*); L02366 (*T. parva*); L19082 (*T. taurotragi*); M64243 (*T. annulata*); U97047 (*T. buffeli*, type A); Z15105 (*T. equi*); AF175300 (*Babesia gibsoni*, Japan); AF205636 (*B. gibsoni*, USA); AF244911 (*B. leo*); AF244912 (*B. felis*); L19077 (*B. bovis*); L19079 (*B.*

canis); U09833 (*B. microti*); U16369 (*B. odocoilei*); X59604 (*B. bigemina*, gene A); Z48751 (*B. divergens*); and L19080 (*Cytauxzoon felis*). These sequences were aligned using MUTALIN on-line interface (<http://www.bioinformatics.vg>). The variable regions of these sequences were flanked by the sequences of the two PCR primers that were used for amplification. A sequence representing *B. felis* was chosen in this variable region between the two amplification primers. For the use in the RLB assay, a *B. felis* probe (5'-TTATGCTTTTCCGACTGGC-3') was synthesized with an N-terminal *N*-(trifluoroacetamido)hexyl-cyanoethyl,*N,N*-diisopropyl phosphoramidite [TFA]-C₆ amino linker (Isogen, Maarsse, The Netherlands).

2.4.2. Preparation of the membrane

A Biotodyne C blotting membrane (Pall Biosupport, Ann Arbor, USA) was activated with 16% 1-ethyl-3-(3-dimethyl-amino-propyl) carbodiimide (EDAC) (Sigma–Aldrich, South Africa) at room temperature (18 °C). The membrane was washed for 2 min with distilled water and placed in a MN45 mini blotter (Immunetics, Cambridge, UK). The *B. felis* probe (800 pM) together with other *Theileria* and *Babesia* species-specific probes (Gubbels et al., 1999) was covalently linked to the membrane by an incubation period of 1 min at room temperature. The membrane was inactivated with 100 mM NaOH for 10 min at room temperature. The inactivated membrane was washed with 2× SSPE (360 mM NaCl, 20 mM NaH₂PO₄, 2 mM EDTA [pH 8.4]) containing 0.5% sodium dodecyl sulphate (SDS). The membrane was stored in 20 mM EDTA, pH 8, at 4 °C.

2.4.3. Hybridization

The method described by Gubbels et al. (1999) was used. A volume of 10 µl of the PCR product was added to 2× SSPE/0.1% SDS, and denatured for 10 min at 96 °C. After denaturation the sample mixture was “snap cooled” on ice. The denatured PCR products were applied to the pre-prepared Biotodyne C blotting membrane containing the new *B. felis* probe and other *Babesia* and *Theileria* species-specific probes and hybridized for 60 min at 50 °C (Gubbels et al., 1999). PCR products that did not hybridize were washed away using 2× SSPE/0.5% SDS washing buffer at 50 °C. The membrane was incubated for 30 min at 42 °C in peroxidase-labelled streptavidin (Roche Diagnostics, South Africa) following another washing step using 2× SSPE/0.5% SDS washing buffer at

42 °C. The detection of the probe-PCR-streptavidin complex is based on chemiluminescence. ECL detection fluid (AEC-Amersham) was added to the membrane and the membrane was exposed to an X-ray film (Hyper film; AEC-Amersham, South Africa). The X-ray film was photographically developed to visualize the hybridization complex.

3. Results

The reaction of the *B. felis* probe was tested using 259 samples (Table 1) from various captive and free-ranging felid species, comprising 137 (52.9%) from cheetahs, 81 (31.3%) from lions, 27 (10.4%) from domestic cats and 14 (5.4%) from other felids such as black-footed cats, servals, caracals and a leopard.

Table 1.

Occurrence of *Babesia* infections in wild and domestic felids

Host	Number of samples tested	Positive	Only <i>Babesia</i> spp. (+)	Only <i>B. felis</i> (+)	Only <i>B. leo</i> (+)	Mixed infection <i>B. leo</i> and <i>B. felis</i>
Diagnostic:	149	89	49	29	6	5
Cheetah (captive)	97	63	42	18	3	0
Lion (captive)	25	12	2	3	3	4
Domestic cat	27	14	5	8	0	1
Survey:	110	38	17	5	15	1
Cheetah free range (Namibia)	40	3	0	3	0	0
Lion free range	56	28	12	1	14	1

Host	Number of samples tested	Positive	Only <i>Babesia</i> spp. (+)	Only <i>B. felis</i> (+)	Only <i>B. leo</i> (+)	Mixed infection <i>B. leo</i> and <i>B. felis</i>
(Swaziland; South Africa)						
Black-footed cat	8	5	5	0	0	0
Serval	3	1	0	1	0	0
Leopard	1	1	0	0	1	0
Caracal	2	0	0	0	0	0
Total	259	127	66	34	21	6

Unless stated otherwise, specimens are from free-ranging animals from South Africa.

The occurrence of *B. felis*, *B. leo* and unknown *Babesia* spp., based on RLB analysis, in felid blood specimens suspected of being *Babesia* positive, based on blood smear examination, is shown in Table 1. The prevalence of *Babesia* infections in free-ranging cheetahs from Namibia, and free-ranging lions from South Africa and Swaziland, is also shown in Table 1.

B. felis was detected in domestic cats, cheetahs, lions and a serval while *B. leo* was detected in lions, cheetahs, a domestic cat and a leopard. Mixed infections of *B. felis* and *B. leo* were found in lions and a domestic cat. *B. felis* and *B. leo* occurred in cheetahs, but not as mixed infections. A high number (43.3%) of samples from captive cheetahs hybridized only with the genus-specific probe for *Babesia* (Fig. 1).

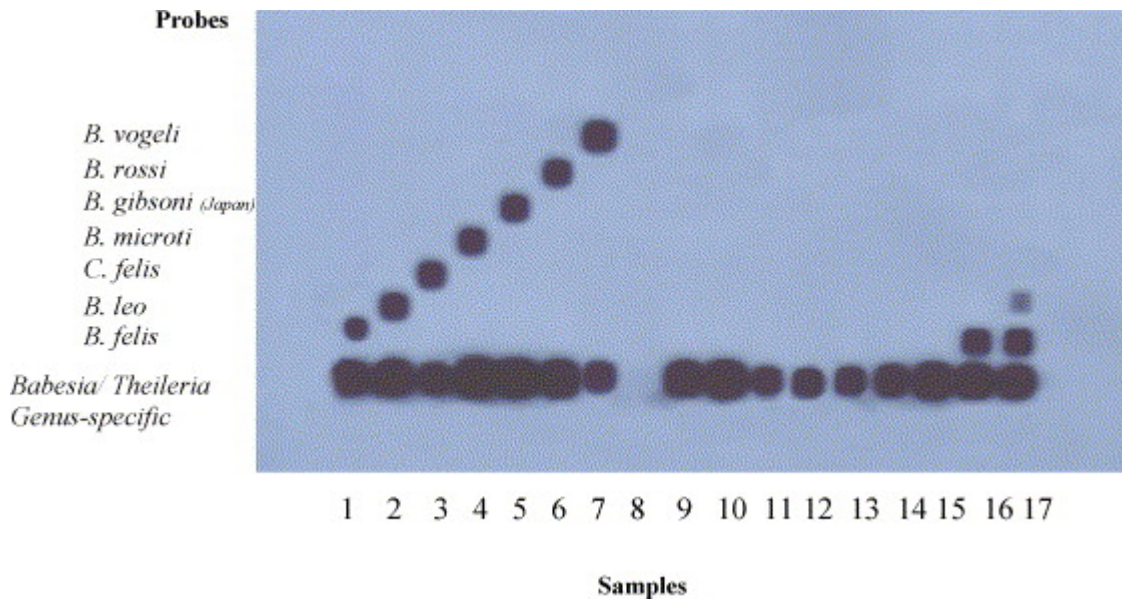


Fig. 1. Illustrated: positive RLB hybridization reaction. Samples 1–2 positive control samples: *B. felis* and *B. leo*; samples 3–7 represent positive DNA for the following parasites: *C. felis*, *B. microti*, *B. gibsoni* (Japan), *B. rossi*, *B. vogeli*; sample 8 is a negative control: water; 9–17 different felid samples tested: numbers 9–14 are samples from cheetahs in captivity that tested positive only with the genus-specific probe; number 16, a domestic cat that tested positive for *B. felis*; number 17, from a lion in captivity, showed a double infection of *B. felis* and *B. leo*.

A genus-specific hybridization reaction was also detected in a number of samples collected from domestic cats and lions.

4. Discussion

Probes exist for the detection of blood parasites in cattle, Cape buffaloes, several antelope species, dogs and horses, but there were only two probes for the detection of blood parasites in felids: one to detect *B. leo* and another to detect *C. felis*. In this study the 18S rRNA sequencing data were used to develop a *B. felis* probe to assist in the detection of *B. felis* infections and differentiate them from *B. leo* infections. This probe is specific for *B. felis* infection in felids.

This study showed that *B. felis* and *B. leo* occurred more frequently in the host from which they had initially been described, namely domestic cats and lions, respectively, but were also detected in other felid species. When using the RLB assay a number of samples

reacted only with the *Babesia/Theileria* genus-specific probe. This is an indication that further *Babesia/Theileria* parasites are present, but no probes exist as yet to identify them to species level.

These results indicate that identification of blood parasites using morphology alone can be misleading. The fact that these parasites infect various host species and can occur as mixed infections open a new understanding of these parasites and questions arise around the correlation between these results and previously described *Babesia*—like parasites in felids.

Blood parasites, morphologically similar to *B. felis*, seen in wild felids were previously believed to belong to that species. The numerous genus-specific signals found in cheetah samples in this study indicate that although these parasites are morphologically similar to *B. felis* their sequences in the 18S rRNA gene, where the *B. felis* probe was design, are different. This also applies for the genus-specific results that occurred in lion, black-footed cats, servals, caracals and a leopard. These findings questioned the suggestion by Dennig and Brocklesby (1972) and Levine (1973) that all these parasites are synonyms of *B. felis*.

The genus-specific signal can be an indication of one or more different *Babesia* parasites in felids. These taxonomic issues can only be resolved when more data are available and therefore, all these samples will be further analysed using sequencing and phylogenetic analysis.

Results from free-ranging cheetahs and lions were interesting. Only 7, 5% (3/40) of the free-ranging cheetahs were positive for *B. felis*. Namibia is a semi-arid country, and a low prevalence of a tick-borne infection is not unexpected. What is surprising, though, is that the three positive cheetahs were infected with *B. felis*, a parasite associated with domestic cats along the more mesic eastern and southern seaboard and eastern escarpment of South Africa (Jacobson et al., 2000 and Penzhorn et al., 1999). The vector of *B. felis* in domestic cats remains unknown.

Babesia infections were much higher (50%; 28/56) in free-ranging lions. These specimens were collected in northern KwaZulu-Natal Province, in Swaziland and in the Kruger National Park, areas with a higher rain fall than Namibia, and therefore

presumably more suitable for ticks.

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Corresponding author. Tel.: +27 12 529 8349; fax: +27 12 529 8312.