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Detection of *Babesia* species in domestic and wild Southern African felids by means of DNA probes

by

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Submitted in partial fulfillment of the requirements for the degree of Magister Scientiae, (Veterinary Tropical Diseases) in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria

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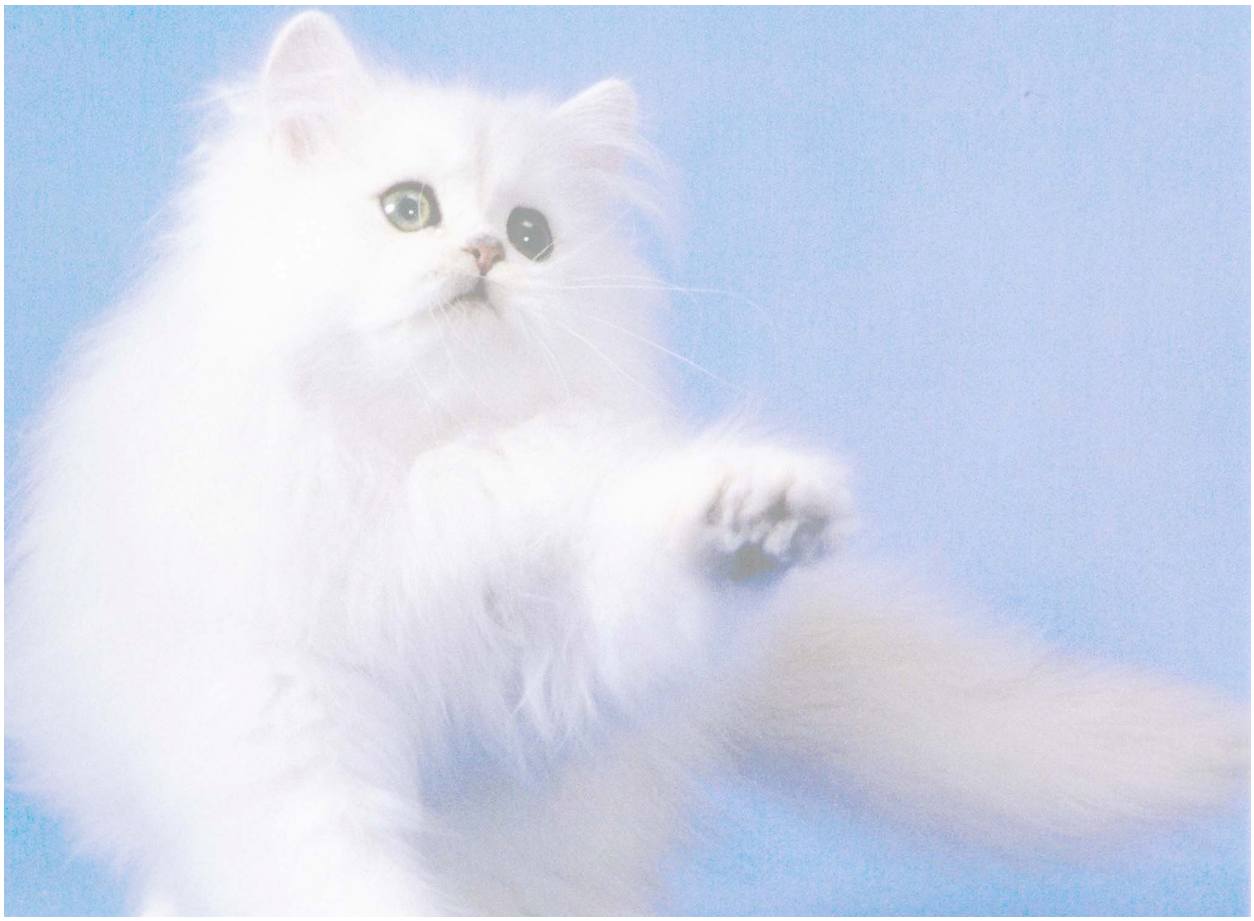
2010



"Thousands of years ago, cats were worshipped as gods. Cats have never forgotten this."

- Anonymous-

DECLARATION

I declare that this dissertation hereby submitted to the University of Pretoria for the degree of Magister Scientiae (Veterinary Tropical Diseases) has not previously been submitted by me for the degree at this or any other University, that it is my own work in design and in execution, and that all material contained herein has been duly acknowledged.

Signed:

Date:

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation and gratitude to:

University of Pretoria, National Research Foundation and **Feline Research Fund Trust** for sponsoring this study.

Professors Banie Penzhorn and Estelle Venter for their guidance, patience and their belief in me.

My colleagues in the Department of Veterinary Tropical Diseases, for their support and encouragement.

My family and friends, thanks for their support and encouragement.

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SUMMARY

Detection of *Babesia* species in southern African felids by means of DNA probes

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Feline babesiosis, first described in domestic cats in South Africa in 1937, is regarded to be of great importance in the coastal regions although isolated cases also occur on the eastern highlands of Mpumalanga Province. *Babesia felis* (described from domestic cats) and *B. leo* (described from lions) are the two best characterised *Babesia* species in felids. These two parasites are morphologically similar when examined under a light microscope, but are serologically and genetically distinct. In this study the prevalence of these two *Babesia* species in various wild and domestic felid species was determined. A total of 358 samples were tested using the reverse line blot hybridization (RLB) assay. This assay makes it possible to simultaneously detect and differentiate between blood parasites using DNA probes. The RLB consists of three basic steps, the first being amplification of the variable region (V4) in the 18S rRNA gene using genus-specific primers where one is labelled with biotin. This is followed by a blotting step, where the amplicons are hybridized to oligonucleotides bound to a nitrocellulose membrane. The third and last step is the detection of the hybridized amplicons by using chemiluminescence reagents. This assay is a screening tool utilizing the variable (V4) region in the 18S rRNA gene to detect and differentiate between blood parasites. A new *B. felis*-specific DNA probe was developed to use in the RLB assay. Results demonstrated that these two parasites not only occur in the felid species from which they have been described, but also in other felid species. *Babesia microti* was also detected in various felid species, while *B. rossi* was detected in 1 of the lion samples. Two hundred and twelve samples tested positive for *Babesia* spp., of which only 54.24% of the samples reacted with the genus-specific probe. This indicates the presence of a novel *Babesia* or *Theileria* species or variant of a species.

CHAPTER 1

1.1 INTRODUCTION

There is evidence that domestic cats were already living in close relationship with humans prior to 7000 BC, where they most likely scavenged for food at early settlements (http://asci.uvm.edu/bramley/CATNDOG.html). Today, domestic cats, either kept or feral, are widespread across most parts of the world. Cats were even deliberately introduced to remote, previously uninhabited, oceanic islands, for instance Marion Island in the sub-Antarctic region, to control rat populations. With the exception of Australia and Antarctica, all continents are inhabited by an array of indigenous cat species (Case 2003).

Felids can be infected by various micro-organisms such as viruses, bacteria, fungi and protozoa. The infection rate is influenced by host factors (immune response of host; undefined heritable resistance factors; maternal immunity; age at time of exposure; concurrent illness; nutritional state), environmental factors (population density; sanitation; ventilation; accumulation of excretions; interchange of animals between different populations) and agent factors (virulence; dose; route of infection) (Pedersen 1988).

Known protozoan diseases that can occur in felids are: *Babesia*, a tick-borne haemaprotozoan that occurs in various vertebrate spp. including cats and dogs. Babesiosis is a fatal disease in cats in South Africa, and has only been reported sporadically in the rest of the world (Jacobson *et al*. 2000). Toxoplasmosis, a zoonotic disease, is usually not a threat to the cat but can cause fatal disease to human foetuses and immuno-compromised persons. *Toxoplasma gondii,* the causative agent of toxoplasmosis, completes its life cycle in the cat and oocysts are the infective form that occurs in cat faeces. Cats are rarely infected with *Trypanosoma, Leshmania* and *Hepatozoon* spp. although these parasites have been poorly studied in cats (Shaw *et al*. 2001). Cytauxzoonosis is characterized by a short course of illness and is usually fatal. This tick-borne blood parasite occurs in the United States and is classified in the family Theileriidae. The causative agent is *C. felis*. This parasite was assigned to the genus *Theileria* (Wagner 1976) and significant serological relationship could be found between *C. felis* and *B. felis* (from South Africa) and *T. taurotragi* (South Africa). Infection studies showed that domestic cats and bobcats (probably a “Bay lynx" (*Felis rufus*) are susceptible to *C. felis* infection, but sheep were not found to be susceptible (Uilenberg *et al*. 1987). Evidence exists that *Ehrlichia* spp. can occur in cats and it was also shown that cats are susceptible to *E. phagocytophila* (Shaw *et al*. 2001). Enteric protozoan infections are limited to *Gardia, Pentatrichomonas, Entamoeba* and *Balantidium*, which are all important parasites in human health (Pedersen 1988; Greene 1990; Case 2003).

Further arthropod-transmitted parasites and bacteria of cats, such as *Bartonella* spp., *Rickettsia* spp., *Coxiella* spp., *Yersinia* spp. and *Francisella* spp., can be of importance from a human-health perspective (Shaw *et al*. 2001).

Very little is known about the prevalence of *Babesia* spp. in felids, i.e. members of the family Felidae. Although various piroplasms have been reported in domestic and wild felids, relatively few have been named and reports are often mere documentations of organisms seen on blood smears. Knowledge of the occurrence of *Babesia* spp. in free-ranging, captive and domesticated cat species is generally restricted to information pertaining to *B. felis* and *B. leo*.

Traditionally, identification of *Babesia* parasites depended on studying their morphology on blood smears (Schoeman *et al*. 2001), as well as serology (Morzaria *et al*. 1977; Jacobson *et al*. 2000). Using morphology as an identification method can be very misleading, however, as many species, e.g. *B. felis* and *B. leo*, are morphologically indistinguishable, but serologically distinct (Lopez-Rebollar *et al*. 1999; Penzhorn *et al*. 2001). Serological evidence *per se* is insufficient for species characterization. The reverse line blot (RLB) hybridization assay (Gubbels *et al*. 1999; Nijhoff *et al.* 2005), a nucleic-acid-based technique, allows the simultaneously detection and differentiation between species of haemoparasites in blood, organs and ticks. The RLB technique was 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), cattle (Brígido *et al*. 2004), and antelope species (Nijhof *et al*. 2005; Oosthuizen *et al*. 2008).

The aim of this study was to determine the prevalence of *B. felis* and *B. leo* in wild and domestic felids in southern Africa using the RLB assay. This entailed developing a probe specific to *B. felis*, screening of various blood and other specimens from a variety of cat species for the presence of *Babesia* parasites and searching for possible other and unknown *Babesia* species.

1.2 LITERATURE REVIEW

1.2.1 The position of Babesia spp. in the phylum Apicomplexa

Babesia species are intra-erythrocytic parasites belonging to the phylum Apicomplexa, the name of which is derived from two Latin roots: “apex” meaning “the top” and “complexus” meaning infolds referring to the set of organelles at the tip of the spindle-shaped sporozoite**.** The Apicomplexa are a group of organisms characterised by a complex intracellular life cycle and include protozoan organisms such as *Babesia* spp*., Theileria* spp, *Cytauxzoon* spp, *Plasmodium*, *Cryptosporidium* and *Toxoplasma* spp. These organisms are potential parasites of vertebrate animals, including humans. They can also cause severe diseases in animals (tick-bite fever, East Coast fever) and humans (malaria) world-wide, especially in the savannah and tropical regions (http://bioweb.uwlax.edu/zoolab/Lab-2a/Protozoa-Apicomplexans.htm**).**

Feline babesiosis in domestic cats, caused by *Babesia felis*, seems to be confined to South Africa (Jacobson *et al.* 2000). However, no evidence has been reported on the occurrence of a closely related piroplasm, *Cytauxzoon felis*, in South Africa. This piroplasm infects wild and domestic felids in the USA (Wagner 1976; Glenn *et al.* 1982).

1.2.2 Transmission and replication

One of the typical features of apicomplexan parasites, which are transmitted by vectors, is the existence of specific cellular stages in their transmission between their hosts. The gametocyte is the transmission stage from vertebrate host to tick vector, while the transmission stage from the tick to the host is called sporozoites. Chauvin *et al*. (2009) stated that these stages are poorly understood due to the lack of knowledge of parasite development, insufficient amount of parasite material, and the absence of known specific makers.

Fleas from the family Pulicidae and ticks from the family Ixodidae are commonly recognised as vectors of feline diseases (Shaw *et al*. 2001; Tabar *et al.* 2008). Ticks are the only known vectors of *Babesia* spp. and transmission occurs by the bite of a tick, most probably of the family Ixodidae (Neitz 1956; Horak *et al.* 1987; Hoskins 1991; Bush *et al.* 2001). The vector for *Babesia* species in felids is not known (Jacobson *et al*. 2000; Penzhorn *et al*. 1999, 2001, 2004). In a phylogenetic study (Penzhorn *et al*. 2001) in which babesias from felids were compared to other related *Babesia*, *Theileria* and *Cytauxzoon* spp., *B. felis* was grouped with *B. microti*, suggesting that they have the same mode of transstadial transmission by tick vectors, most probably of the family Ixodidae.

The development of *Babesia* spp. in the tick and the vertebrate host is not synchronized and various stages of development can be seen in a blood sample at the same time (Mehlhorn *et al*. 1984; Bush *et al.* 2001; Chauvin *et al*. 2009).

1.2.2.1 Life cycle of the Babesia parasite in the tick

The parasites are ingested by ticks during a blood meal. Many of the parasites are destroyed during digestion, but the pre-gametocyte stage of the parasite survives and undergoes further development (Mehlhorn *et al*., 1984). The pre-gametocytes transform into gametocytes. Elongated bodies appear after a view hours. These are believed to be gamonts and are so-called “ray bodies” (“Strahlenkörper”). In the lumen of the tick’s digestive tract these gametes fuse to form zygotes, which penetrate the midgut cells and are transformed to ookinetes. Ookinetes are a motile stage that appears to be haploid. In this stage meiosis occurs, which indicates the beginning of sporogony in the Apicomplexa life cycle. These ookinetes escape from the epithelium cells to invade the body tissue of the tick, i.e. the ovaries of the female tick, resulting in transovarial transmission. Kinetes can also penetrate the salivary glands of the tick and transform to sporozoites, which represent the infectious stage of the parasite (Chauvin *et al*. 2009).

1.2.2.2 Life cycle of the Babesia parasite in the vertebrate host

Sporozoites are transferred to the mammalian host during the bite of a tick. It appears that sporozoite formation in the salivary glands of the tick is only initiated after the vector starts to feed on a vertebrate host (Neitz 1956; Levine 1971, 1985, 1988; Friedhoff 1988; Bush *et al.* 2001).

In contrast to *Plasmodium* or *Theileria* spp., *Babesia* parasites directly enter the erythrocytes of the host, where after the sporozoite is called a trophozoite. Binary fission takes place resulting in two merozoites. Cell lysis takes place and merozoites invade new erythrocytes. These merozoites reinitiate the replication cycle by invading further erythrocytes. Occasionally the trophozoites change into pre-gametocytes that are the infection stage of the parasite that will infect the tick (Chauvin *et al*. 2009).

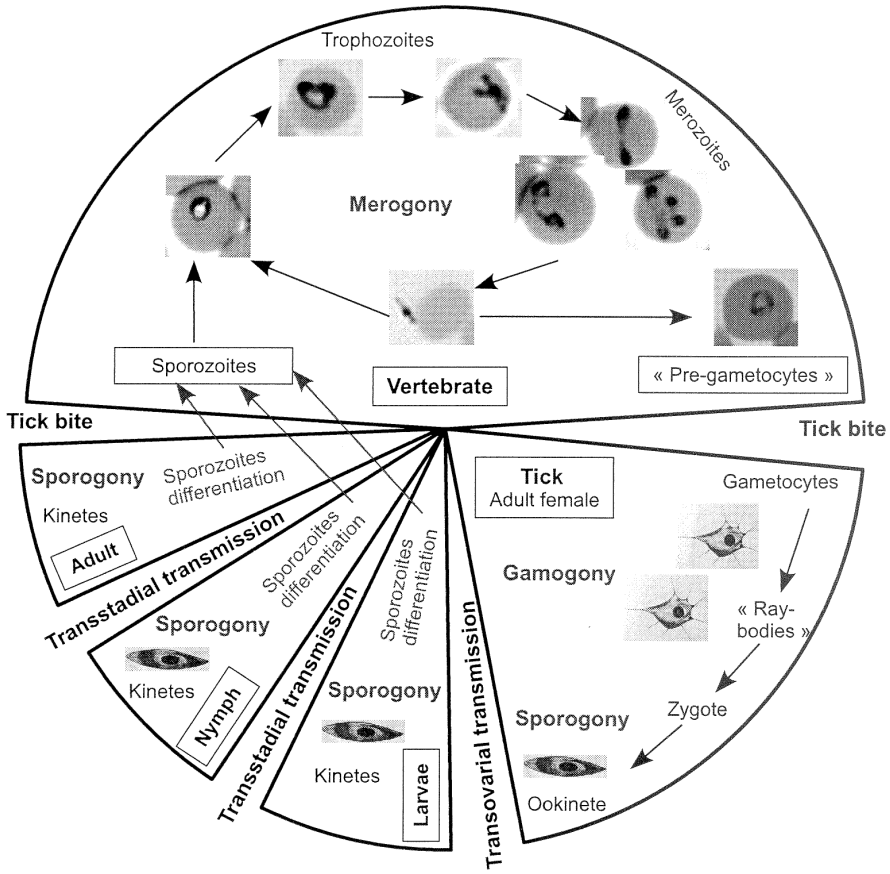


Figure 1 Schematic illustration of the life cycle of *Babesia* species (Chauvin *et al*., 2009)

1.2.3 Morphology

Intra-erythrocytic babesias are classified as “large” or “small”. *Babesia* parasites in dogs, for example, are classified into either the “large” group, namely *B. canis*, *B. rossi* and *B. vogeli*, and the “small” group, namely *B. gibsoni*, *B. conradae* and "*Theileria annae*" (Matjila *et al.* 2008).

Classification of babesias has been based primarily on the host from which the parasite is recovered as well as on their morphology in erythrocytes (Neitz 1956; Futter and Belonje 1980a; Levine 1988; Bush *et al*. 2001). Using morphology as a method of identification needs a practised eye, taking in consideration that *Babesia* parasites have a complex life cycle and the various life stages, which differ morphologically, can occur in the blood sample simultaneously.

Pre-gametocytes occur in the host and are the infective stage from the host to the tick. In the tick they are known as gametocytes and are difficult to differentiate using light microscopy but small differences are visible under the electron microscope (Chauvin *et al*. 2009). This stage develops into gametocytes. After these gametocytes are ingested by the tick, and arrow-head-shaped ray bodies appear. Gamete fusion takes place and elongated zygotes appear. These zygotes are 8 to 10 µm in length with a spike-like arrow head. The arrow-headed zygote is equipped to penetrate the midgut cell membranes. The ookinete, the next stage in the life cycle of *Babesia* spp. in the tick, has lost the arrow-head structure.

The stages in the vertebrate host include sporozoites and trophozoites that are round or ovoid, and merozoites that are pyriform (pear-shaped and occur in pairs) and joined at the extremities (Futter and Belonje 1980a; Jacobson *et al.* 2000; Chauvin *et al*. 2009).

Except for *B. herpailuri*, the intra-erythrocytic stages of felid *Babesia* spp. are small, pleomorphic, and difficult to differentiate morphologically (Dennig 1967; Futter and Belonje 1980b; Lapin and Caplin 1990; Jacobson *et al.* 2000). The reported size of *Babesia* spp. in felids varies from 1.5 µm to 3 µm (McNeil 1937; Futter and Belonje 1980a; Lopez-Rebollar *et al.* 1999), although Stewart *et al*. (1980) reported on a large *Babesia* in domestic cats. This was only a morphological finding and never investigated in detail. The size of *B. felis* tropozoites is 1–1.2 x 2.2–2.5 µm. The morphology of *Babesia* parasites in felids can be described as pleomorphic, the parasites being round or irregularly circular, with a faint blue cytoplasm and dark red chromatin. Maltese crosses (four pear-shaped merozoites in a cruciform shape) are occasionally seen. In most cases erythrocytes contain only one parasite, but in severe infections two parasites may occur which are at different stages of development. Elongated forms and large pyriform parasites are infrequently present (Dennig 1967; Futter and Belonje 1980b; Lapin and Caplin 1990; Leger *et al.* 1992; Jacobson *et al.* 2000).

1.2.4 Host range

Babesia primarily infects mammals, but a few are known to be parasites of birds and reptiles (Olsen 1986; Bush *et al.* 2001). Hosts include rodents, carnivores, swine, sheep, cattle, horses and primates (Olsen 1986; Bush *et al.* 2001). Some commonly known species in animals are *B. felis* (domestic cats), *B. leo* (lions), *B. canis* and *B. gibsoni* (dogs), *B. caballi* (horses), *B. bigemina* and *B. bovis* (cattle), *B. motasi* (sheep) and *B. trautmanni* (swine) (Olsen 1986).

It is believed that *B. felis* has a wide host range within the cat family (Futter and Belonje 1980a; Jacobson *et al*. 2000; Schoeman *et al.* 2001; Penzhorn 2006) and feline babesiosis is regarded as an important disease in certain parts of South Africa, particularly in the coastal areas (Jacobson *et al*. 2000).

1.2.4.1 Babesia species reported in wild free-ranging and captive felid species

The first piroplasm species in felids was described from a Sudanese wild cat (*Felis ocreata*, syn. *F. sylvestris*; Wilson and Reeder 1993) by Davis (1929), who named the parasite*, Nuttalia felis.* Davis also artificially infected domestic cats by subinoculation of blood from the wild cat. Although parasitaemia developed, the cats remained clinically normal.

Mudaliar *et al*. (1950) reported on a parasite in an Indian wild cat. The parasitaemia was very low. As the parasite did not resemble *B. felis* morphologically, they named it *Babesia cati*. Levine (1973) listed *Babesiella felis, Nuttallia felis* var. *domestica* and *Babesia cati* as synonyms for *Babesia felis*.

A small piroplasm was reported in the North American "Bay lynx" (*Felis rufus*, probably the bobcat) (Wenyon and Hamerton 1930). *Babesiella felis* was described in a captive puma (*Felis concolor*) in the Cairo Zoo (Carpano 1934). A small *Babesia* was also described from an Indian leopard (*Panthera pardus fusca*) (Shortt 1940). Dennig and Brocklesby (1972) suggested that the above-mentioned parasites should be considered to be *B. felis.*

Babesia herpailuri (Dennig 1967, 1969) and *Babesia pantherae* (Dennig and Brocklesby 1972) were recovered from a jaguarondi (*Herpailurus yaguarondi*) and a leopard (*Panthera pardus*), respectively.

Piroplasms were also reported in lions (*Panthera leo*) in Kenya and Uganda and it is believed that the famous lioness, Elsa, died of babesiosis (Barnett and Brocklesby 1968). *Babesia leo* has been described from lions by Lopez-Rebollar *et al*. (1999) and Penzhorn *et al*. (2001). *Babesia leo* is morphologically similar to *B. felis* but serological studies (Lopez-Rebollar *et al*. 1999) and phylogenetic studies (Penzhorn *et al*. 2001) showed that *B. leo* is a diistinct *Babesia* species. Lopez-Rebollar *et al.* (1999) infected a domestic cat with lion blood that was infected with *B. leo.* Blood smears were negative for any *Babesia* parasite for 42 days. The cat was splenectomized at that stage and parasites appeared on blood smears 8 days later. The cat showed only a slight anemia and

temperature and appetite of the animal remained normal. Blood from this animal was tested serologically for *B. felis* antibodies but no *B. felis* antibodies were detected only antibodies against *B. leo*. Phylogenetic analysis of the 18S rRNA gene placed this parasite in the same clade as *B. felis* but on a separate branch. These data support the serological results to define *B. leo* as a species in its own right.

Further undescribed piroplasms in wild felids were reported from cheetahs (*Acinonyx jubatus*) (Averbeck *et al.* 1990) and a caracal (*Felis caracal*) (Penzhorn *et al,* 2001). Phylogenetic analysis of the 18S rRNA of the piroplasm found in the caracal showed that this parasite is closely related to *B. leo* and *B. felis,* but this finding was not further explored. Butt *et al*. (1991), Rotstein *et al*. (1999) and Yabsley *et al*. (2008) reported on *Babesia* species in samples collected from "panthers" (*P. concolor coryi*) (Florida, USA) and cougars (*P. concolor*) (Texas, USA). Small piroplasms have been reported from wild Iberian Lynx (*Lynx pardinus*) and Mongolian Pallas cats (*Otocolobus manul*), respectively (Ketz-Riley *et al.* 2003; Luaces *et al.* 2005).

1.2.4.2 Babesia species reported in domestic felids

Lingard and Jennings (1904; cited by Mangrulkar 1937), were apparently the first authors to report finding piroplasms in blood smears of a domestic cat. They did not illustrate or describe their findings. Feline babesiosis in domestic cats was described for the first time in South Africa in the Stellenbosch area, by Jackson and Dunning (1937). They considered the parasite to be similar to *B. felis*, but due to its pathogenicity in domestic cats, referred to it as *Nuttallia felis* var. *domestica*. McNeil (1937) also reported babesiosis in domestic cats in the Cape Town area. The latter two reports were published simultaneously in the same journal.

A large unidentified *Babesia* was reported from an 8-year-old neutered Siamese cat from Zimbabwe that showed clinical signs of babesiosis (Stewart *et al*. 1981). Although the parasite was larger than *B. felis,* it was morphologically similar to *B. felis*. The cat has been treated with diminazine and a multi-vitamin mixture and in a day's time the temperature of the animal dropped. After 9 days the cat was eating well and its mucous membranes were of a normal colour.

A new subspecies of *B. canis* (*B. canis* subsp. *resentii*) in domestic cats in Israel was proposed by Baneth *et al*. (2004). They based their findings on sequencing data and phylogenetic evidence obtained from the internal transcribed spacer genes (ITS1 and ITS2) and 18S rRNA.

1.2.5 Geographical distribution

Babesia infects wild and domestic felids in South Africa (Jackson and Dunning 1937; McNeil 1937; Young 1975; Penzhorn *et al.* 1999) as well as in other countries, including France (Leger *et al.* 1992; Bourdeau 1996), Germany (Moik and Gothe 1997), Thailand (Jittapalapong and Jansawan 1993), Tanzania (Averbeck *et al*. 1990), Kenya (Barnett and Brocklesby 1968) and Zimbabwe (Stewart *et al.,* 1980).

Feline babesiosis is endemic along the coastal regions of the Western Cape, Eastern Cape and KwaZulu-Natal Provinces of South Africa (Jacobson *et al.* 2000). There are published data from Cape Town (Brownlie 1954), Bellville (Dorrington and Du Buy 1966), Stellenbosh (Jackson and Dunning 1937), Knysna (Robinson 1963), Port Elizabeth (Robinson 1963) and Durban (Penzhorn *et al*. 2001). If infection was found in an inland area it was from cats that had accompanied their owners on holiday to the coast. However, Penzhorn *et al*. (1999) reported on 18 cats at Kaapschehoop, Mpumalanga Province, that tested positive using the indirect fluorescent antibody test for *B. felis* These cats had not left the area. Occurrence of the parasite in felids is also seasonal, presumably depending on rainfall patterns that support the life cycle of a tick vector (Jacobson *et al.* 2000).

1.2.6 Diagnostic procedures

The diagnosis of babesiosis usually depends on the demonstration of parasites in erythrocytes in blood smears, a positive reaction with serology (IFA), or nucleic acid-based diagnostic procedures.

1.2.6.1 Microscopic examination

Blood smears for microscopic examination are prepared from fresh blood or blood collected into edetate acid (EDTA). Staining of smears is usually done by Giemsa and Wright's stains, which are commercially available. Giemsa stain is normally used for morphological differentiation of intracellular blood protozoa and Wright's stain for leukocyte counts as confirmation of animal's disease status (Lapin and Caplin 1990).

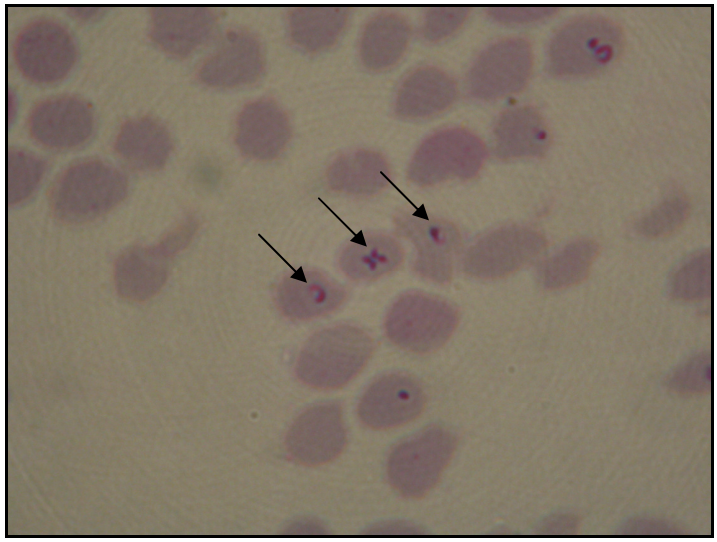


Figure 2 *Babesia felis* in erythrocytes. (Photo: by Dr MA Peirce, MP International Consultancy, United Kingdom)

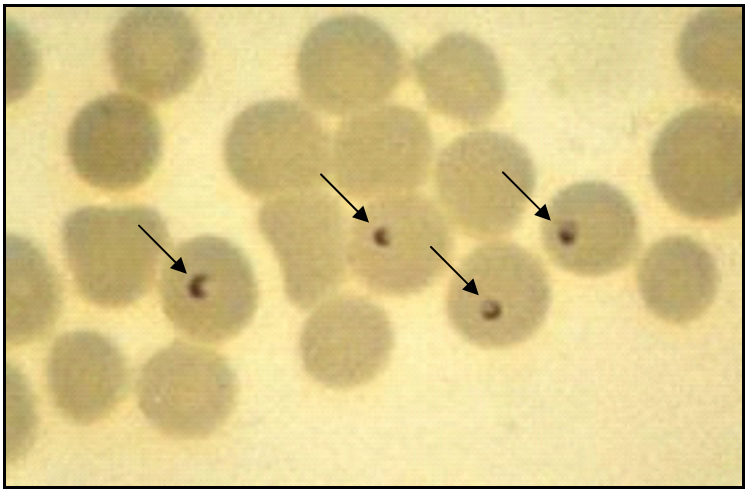


Figure 3 *Babesia leo* in erythrocytes. (Photo: Dr PT Matjila, Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, Onderstepoort, South Africa)

1.2.6.2 Serology

Sero-diagnosis of protozoal diseases involves the testing of serum for the presence of antibodies against the antigens of the parasite. Parasite-specific serum antibody testing has been used as a diagnostic assay of protozoal diseases. The advantage of serological testing is that it is rapid, reliable and easy. Disadvantages of serological tests are the availability of antigen / antibodies and the specificity of these agents (Lapin and Caplin 1990; Gutierrez 2000).

The indirect fluorescent antibody test (IFAT) is the most reliable and commonly used test to detect canine babesiosis (Kier and Green 1990; Lapin and Caplin 1990; Gutierrez 2000). An IFA was also specifically developed to detect *B. leo* in lions (Lopez-Rebollar *et al.* 1999).

The ELISA and dot-ELISA techniques for antibody detection have been developed but these tests are used for sero-epidemiological studies rather than for clinical diagnosis (Gutierrez 2000).

1.2.6.3 Nucleic-acid-based procedures

The use of molecular tools for the characterization of blood parasites and their hosts is becoming increasingly important to the field of veterinary parasitology (Prichard and Tait 2001; Zarlenga and Higgs 2001) and is leading to greater sensitivity and higher throughput in the identification of parasites (Criado-Fonelio 2007). The possibility of sequencing a whole genome serves as the basis for functional nucleic acid diagnosis and analysis of parasite species. The ribosomal RNA (rRNA) genes of protozoa are mainly used for diagnostic purposes (Bishop *et al.* 1992, 1995; Allsopp *et al*. 1993; Allsopp *et al.* 1994; Marsh *et al.* 1995; Prichard and Tait 2001). Nucleic acid procedures are standardized to extract and amplify DNA or RNA from blood, tissue, faeces, skin and vectors (Bishop *et al.* 1992, 1996; Prichard and Tait 2001; Zarlenga and Higgs 2001). The use of radioactive labeled probes was the precursor of nucleic-acid-based diagnostic techniques to detect protozoal rRNA in blood. Although this technique is very sensitive, it is expensive and needs a specialized laboratory and trained personnel to perform (Bishop *et al.* 1992; Figueroa and Beuning 1995).

The most commonly used molecular techniques are: conventional PCR; RLB; real-time PCR; isothermal amplification methods such as loop-mediate amplification (LAMP); nucleic-acid-sequence-based amplification (NASBA) and transcription-mediated amplification (TMA) (Criado-Fornelio 2007). The diagnostic assay of choice in this study is the RLB: this hybridization assay is a versatile diagnostic tool for simultaneous detection and differentiation of blood parasites in various kinds of specimens such as whole blood, organs, stained and unstained blood slides, blood spots on filter paper and ticks.

The RLB technique was described by Embury *et al*. (1987), who used radioactively labelled amplified products that targeted the beta A-globulin gene in the detection of sickle cell anemia. The next year Saiki *et al*. (1988) reported on a non-radioactive labeled RLB assay, and since 1988 various radioactive labeled RLB assays were designed to detect pathogens such as group A streptococci and *Borrelia burgdorferi* (Kaufhold *et al*. 1994; Rijpkema *et al*. 1995). The first RLB attempt was a dot-blot technique where species-specific probes (oligonucleotides) were added as spots to the membrane (Rijpkema *et al*. 1995). Gubbels *et al*. (1999) used a mini blotter apparatus to apply probes to the membrane for the detection and characterization of *Theileria* and *Babesia* species. This modification was used in this study (Chapter 2, Figure 5).

The first step in the RLB is the PCR amplification of a variable region of the 18S rRNA gene of the *Babesia* and / or the genus *Theileria*. One of these genus-specific primers contains a biotin molecule (label) for detection of the hybridization reaction. This amplicon is then applied to a nylon membrane that contains species-specific probes bound to it. These probes derived from the hypervariable region within the PCR product. Oligonucleotides are synthesised with an amino-linker. This linker binds the probe to the membrane. The PCR amplicons are then hybridized to the species-specific probes. Unbound PCR amplicons are washed away and streptavidin–peroxidase is applied to the membrane that will bind to the biotin-labelled primer. Chemiluminescence reagent is added and the hybridization complexes are visualized as black spots on an X-ray film after photographic development procedures (Chapter 3, Figure 8).

The RLB has since been successfully applied to detect and differentiate blood parasites from horses (Nagore *et al*. 2004a). sheep (Nagore *et al.* 2004b), cattle (Brígido *et al.* 2004), dogs and cats (Criado-Fornelio *et al*. 2007; Matjila *et al*. 2008), various antelope species (Nijhof *et al.* 2005; Oosthuizen *et al*. 2008) cougars and panthers (Yabsley *et al.* 2008).

1.3 JUSTIFICATION AND AIM OF THIS STUDY

From the literature it is evident that the molecular characterization of blood parasites in felids is not well described. Reports on the presence of various *Babesia*-like parasites in felids pose the question whether these morphologically similar parasites belong to the same species on molecular level. It is therefore hypothesised that piroplasms from the *Babesia* genus that occur in wild and domestic felids, are not necessarily *B. felis* and / or *B. leo.*

The aim of this study was to verify the prevalence of *B. felis* and *B. leo* in wild and domestic felids in southern Africa, using the RLB assay. This entails using the existing *B. leo* probe, developing a probe specific to *B. felis*, and screening various blood and other specimens from a variety of felid species for the presence of *B. felis* and / or *B. leo.*

CHAPTER 2

MATERIALS AND METHODS

2.1 SAMPLE COLLECTION

Whole blood (EDTA-buffered tubes) and blood on filter paper, stained and unstained blood smears and organs were collected from various felid species, including lions, cheetahs, caracals, tigers (*Panthera tigris*), black-footed cats (*Felis nigripes*), serval (*Leptailurus serval*) and domestic cats, from different regions in southern Africa (APPENDIX A to D). Samples from free-living felids were obtained from veterinarians who have an interest in wildlife and have access to various wildlife sanctuaries. Samples from domestic cats were also collected from private practitioners. In some cases blood stained blood smears accompanied the submitted specimen, these were microscopically examined prior to DNA extraction and the morphology of the parasite in the red blood cell was described. All samples were catalogued and aliquot into smaller volumes and stored at –20 °C.

The samples were divided in 4 main groups regarding the number of samples tested, namely cheetahs; lions; domestic cats and other felids. These samples were divided into diagnostic samples and survey samples. Suspected *Babesia*-positive blood samples from captive cheetahs and lions, as well as from domestic cats, were submitted for routine diagnostics. Felid blood specimens submitted to the Clinical Pathology Laboratory of the Faculty of Veterinary Science, University of Pretoria, and found to harbour piroplasms, were also forwarded for further processing. Survey samples were collected during surveys undertaken by researchers and staff in the Department of Veterinary Tropical Diseases; most of these samples are from free-ranging animals.

2.2 DNA EXTRACTION

DNA was extracted from 200 µl whole blood collected in EDTA, blood spots on filter paper and stained and unstained blood smears using the commercially available QIAamp® DNA Mini Kit (Qiagen, Southern Cross Biotechnologies, South Africa), according to the manufacturer's instructions. The concentration of the extracted DNA was determined using a spectrophotometer (Beckman Coulter™, DU® 530; Beckman Coulter, South Africa) and 70–100 ng of DNA was used in the PCR reaction.

2.3 POLYMERASE CHAIN REACTION

PCR was performed using primers that amplified a 460 to 520 bp fragment in the V4 variable region of the 18S rDNA of *Theileri*a and *Babesia* species (Gubbels *et al.* 1999; Nijhof *et al.* 2005). The position of the primers in the V4 variable region is illustrated in Figure 4.

Sequences of genus-specific primers that were used to amplified the V4 region of the 18S rDNA:
a) RLB-F-5'-GAGGTAGTGACAAGAAATAACAATA-3' (Forward primer)
b) RLB-R biotin labeled -5'-TCTTCGATCCCCTAACTTTC-3' (Reverse primer)

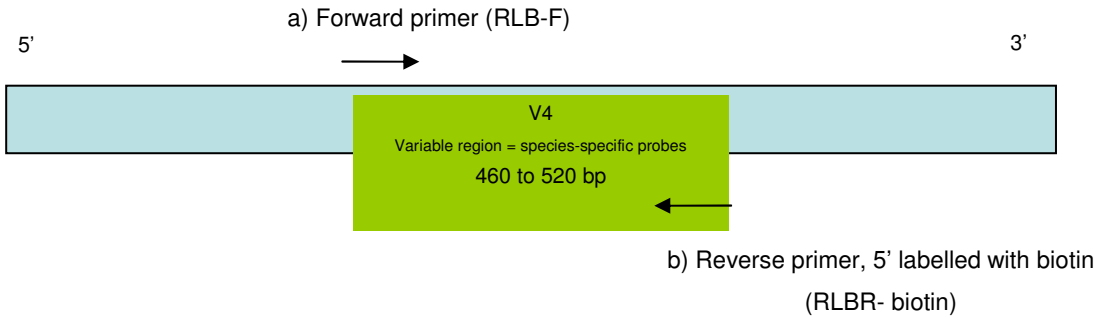


Figure 4 Schematic illustration of 18S rDNA gene (blue) showing the position of the primers (a and b) as well as the V4 variable region (green) where species-specific probes were developed.

A reaction mixture consisting of Platinum Quantitive PCR Supermix-UDG (Invitrogen, Celtic Biotechnology, South Africa), 20 pM of each primer (Operon, Southern Cross Biotechnologies, South Africa) and 2.5 µl purified DNA to a final volume of 25 µl was used. A touch-down PCR programme was followed, starting with 3 min at 37 ℃; 10 min at 94 ℃; and 10 cycles of 94 ℃ for 20 sec, 67 ℃ for 30 sec, 72 ℃ for 30 sec with decreasing of the annealing temperature after every second cycle by 2 ℃ for 5 times. These cycles continued until the annealing temperature reached 57 ℃. Finally, 40 cycles of 94 ℃ for 20 sec; 57 ℃ for 30 sec and 72 ℃ for 30 sec were performed in a Perkin Elmer 9600 Thermocycler (Applied Biosystems, South Africa). The PCR amplicons were verified using 2% agarose gel electrophoresis containing ethidiumbromide (10 mg / ml) before it was analysed by RLB hybridization.

2.4 REVERSE LINE BLOT HYBRIDIZATION

2.4.1 Preparation of nylon membrane with species-specific probes

A Biodyne C blotting membrane (Pall Biosupport, Ann Arbor, USA) was activated with 16% 1-ethyl-3-(3-dimethyl-animo-propyl) carbodiimide (EDAC) (Sigma-Aldrich, South Africa) at room temperature (18–24 °C) for 10 min. 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 (Table 1) were covalently linked with an N–terminal *N*–(trifluoracetamidohexyl-cyanoethyl, *N,N*-diisopropyl phosphoramidite [TFA])-C_6 amino linker, to the membrane by an incubation period of 1 min at room temperature. The membrane was then inactivated with 100 mM NaOH for 10 min at room temperature. The inactivated membrane was washed, with shaking, in 125 ml, 2 x SSPE (360 mM NaCl, 20 mM NaH_2PO_4, 2 mM EDTA [pH 8.4]) containing 0.5% sodium dodecyl sulphate (SDS) for 5 min at 60 °C and was directly used or sealed in a plastic bag and stored in 20 mM EDTA, pH 8, at 4 °C until used.

Table 1 The various genus- and species-specific probes used

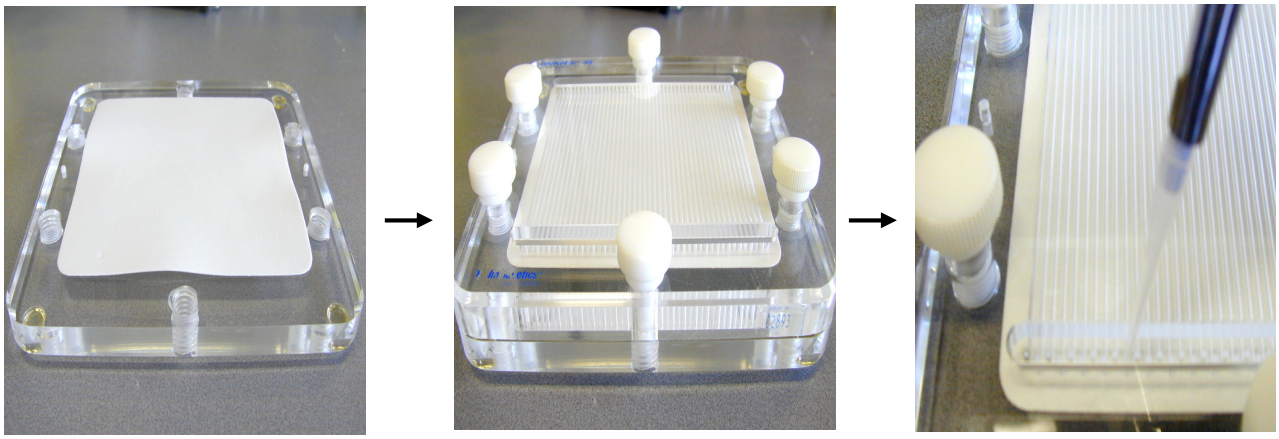
Probe name	Sequence 5' – 3'	Author
Theileria / Babesia genus-specific probe	TAA TGG TTA ATA GGA RCR GTT G	Nijhof *et al.* 2005 Gubbels *et al.* 1999
Babesia felis	TTA TGC GTT TTC CGA CTG GC	NEW
B. leo	ATCTTGTTGCCTTGCAGCT	Nijhof, personal communication
Cytauxzoon felis	CTGGTGATTTTTCGGTAT	Nijhof, personal communication
B. rossi	CGG TTT GTT GCC TTT GTG	Matjila *et al.* 2008
B. canis	TGC GTT GAC GGT TTG AC	Matjila *et al.* 2008
B. vogeli	AGC GTG TTC GAG TTT GCC	Matjila *et al.* 2008
B. gibsoni	TAC TTG CCT TGT CTG GTT T	Matjila *et al.* 2008
B. microti	GRC TTG GCA TCW TCT GGA	Gubbels *et al.* 1999
B. divergens	ACT RAT GTC GAG ATT GCA C	Gubbels *et al.* 1999
B. bovis	CAG GTT TCG CCT GTA TAA TTG AG	Gubbels *et al.* 1999
B. bigemina	CGT TTT TTC CCT TTT GTT GG	Gubbels *et al.* 1999
B. major	TCC GAC TTT GGT TGG TGT	Gubbels *et al.* 1999
B. caballi	GTT GCG TTK TTC TTG CTT TT	Gubbels *et al.* 1999
Theileria sp. Kudu	CTG CAT TGT TTC TTT CCT TTG	Nijhof 2005 Gubbels *et al.* 1999
T. mutans	CTT GCG TCT CCG AAT GTT	Gubbels *et al.* 1999
T. parva	GGA CGG AGT TCG CTT TG	Gubbels *et al.* 1999
T. taurotragi	TCT TGG CAC GTG GCT TTT	Gubbels *et al.* 1999
T. velifera	CCT ATT CTC CTT TAC GAG T	Gubbels *et al.* 1999
T. equi	TTC GTT GAC TGC GYT TGG	Gubbels *et al.* 1999
T. lestoquardi	CTT GTG TCC CTC CGG G	Gubbels *et al.* 1999

(Symbols indicate degenerate positions: R = A/G, W = A/T, K = G/T)

2.4.2 Hybridization

A volume of 20 µl of the PCR product, irrespective of concentration, was added to 2 x SSPE / 0.1% SDS to a final volume of 150 µl, and denatured for 10 min at 96 °C, and then "snap cooled" on ice. The denatured PCR products were applied to the pre-prepared Biodyne C blotting membrane (see 2.3.1) containing the species-specific probes and hybridized for 60 min at 50 °C. PCR products that did not hybridize were washed away using 2 x SSPE/ 0.5% SDS washing buffer for 10 min at 50 °C with shaking. This step was performed twice. The membrane was incubated for 30 min at 42 °C in peroxidase-labelled streptavadin (Roche Diagnostics, South Africa) following two washing steps using 2x SSPE/ 0.5% SDS washing buffer for 10 minutes at 42 °C with shaking. During all steps, including

incubation steps, the membrane must be covered with buffer at all times to avoid drying of the membrane.



A B C

Figure 5 Illustration of the loading procedure of denatured PCR amplicons onto a nylon membrane containing genus– and species-specific probes. (A) Put membrane on top of support cushion (Supplied with apparatus, preventing samples from spreading or leaking out of capillaries) onto the blotting apparatus. (B) Assembly of the apparatus by closing and screwing the apparatus together. (C) Apply the denatured PCR amplicons to the membrane using a micro pipette. When the membrane is prepared, probes are applied to the membrane in the same manner.

2.4.3 Analysis

The detection of the probe-PCR-streptavidin complex is based on chemiluminescence. ECL detection fluid (DNA Thunder™, Perkin Elmer, Separation Scientific, South Africa) was added to the membrane and the membrane was exposed to an X-ray film (X-OMAT™ Blue XB-1, Kodak, Separation Scientific, South Africa). The X-ray film was photographically developed to visualize the hybridization complex using developer (X-Ray – Developer: African X-Ray Industrial and Medical (Pty) Ltd, South Africa) and fixer (X-Ray – Fixer: African X-Ray Industrial and Medical (Pty) Ltd, South Africa).

2.4.4 Stripping of membrane

The membrane was stripped and used again for at least 10 times, if not dried out during storage or handling. The membrane was washed twice with pre-heated 1% SDS at 90 °C for 30 min under gentle shaking and then once with 20 mM EDTA for 15 min at room temperature under gentle shaking. The washed membrane was put into a plastic bag containing 20 mM EDTA (pH 8). The bag was sealed and stored at 4 °C until used.

2.5 DEVELOPMENT OF A DNA PROBE FOR THE DETECTION OF *BABESIA FELIS*

This probe was developed in collaboration with the Department of Tropical Medicine, Utrecht University, and Isogen, Maarssen, The Netherlands. Sequencing data from the 18SSU rDNA was used to develop the *B. felis*-specific probe. Sequencing data from different *Babesia* and *Theileria* species was obtained from GenBank (Table 2). These sequences were aligned using MUTALIN on-line interface (http://www.bioinformatics.vg), and Clustal X (Version 1.81 for Windows). The sequences were manually truncated to the same size and a unique sequence (5'-TTATGCTTTTCCGACTGGC-3') to *B. felis* was chosen. This sequence was tested for its uniqueness by using NCBI BLAST (http://www.ncbi/blast). The probe was synthesized with an N-terminal *N*-(trifluoracetamidohexyl-cyanoethyl, *N,N*-diisopropyl phosphoramidite [TFA])-C_6 amino linker (Isogen, Maarssen, The Netherlands). This new probe (Figure 6) was linked to a nylon membrane (as described in 2.3.1) together with other genus- and species-specific probes and the membrane was then used to screen various felid samples.

Table 2 **GenBank accession numbers of sequences used in the development of the *B. felis* probe**

GenBank accession number	Name of sequence
AF078815	*Theileria mutans*
AF078816	*T.* spp, MSD
AF097993	*T. velifera*
AF097993	*T. velifera*
L02366	*T. parva*
L19082	*T. taurotragi*
M64243	*T. annulata*
U97047	*T. buffeli,* type A
Z15105	*T. equi*
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*
L19080	*Cytauxzoon felis*

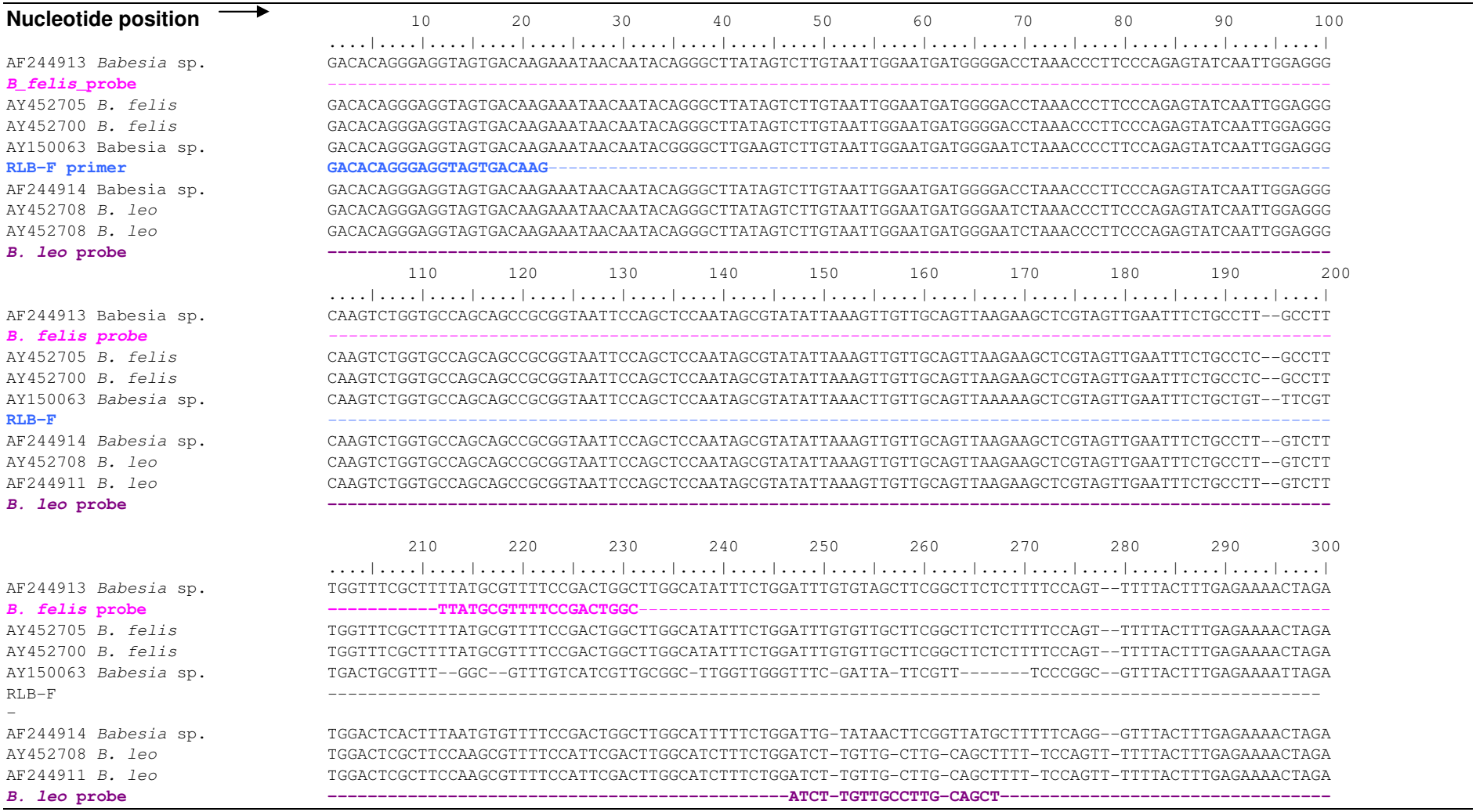


Figure 6 Positions of RLF-F primer and the newly developed *B. felis* probe in the *B. sp*, *B. felis* and *B. leo* sequences obtain from GenBank. Position of the *B. leo* probe is indicated. The position of RLF-R primer is not shown but is positioned at nucleotide 520 in the nucleotide sequence of *Babesia* and *Theileria* species.

2.6 CONTROLS

Controls were included to validate results obtained by both the PCR and RLB. A blood sample of a domestic cat suffering from babesiosis, confirmed on a blood smear and by serology, served as positive control for *B. felis*. Water and blood from a cat that tested previously negative on morphology, serology and RLB, were included as negative controls. These controls as well as a membrane control were applied together with the test samples to the pre-prepared membrane. The membrane control consists of plasmids containing fragments of DNA of which the sequences correspond to some of the species-specific probes on the membrane (TBD-RLB Manual: TBD-RLB kit, Isogen; Life Science; The Netherlands).

CHAPTER 3

RESULTS

A total of 385 felid samples were screened (Table 3). DNA was successfully extracted, amplified and PCR amplicons were tested prior to the RLB assay. This reaction indicates that either a *Babesia* or a *Theileria* spp. could be present (Figure 7). Results obtained (Table 3) showed only positive hybridization reactions with species-specific probes for *B. leo*, *B. felis*, *B. microti* and *B. rossi*. No sample tested positive for *C. felis* or any other blood parasite, as shown in Table 3.

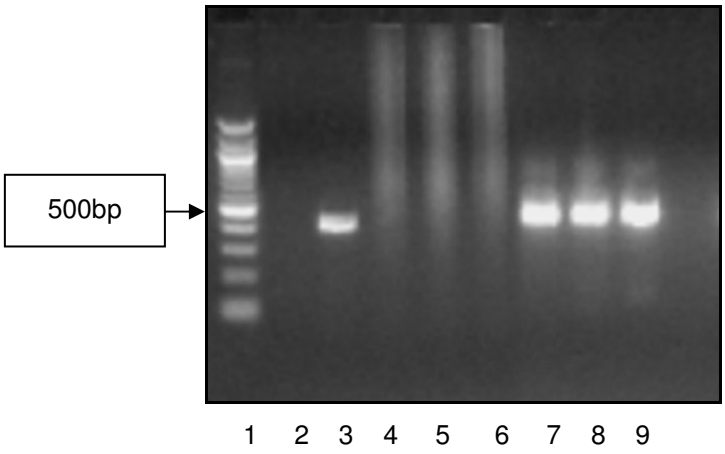


Figure 7 Illustration of an agarose gel with the PCR amplification products using primers that amplified a 460 to 520 bp fragment in the V4 variable region of the 18SSU rDNA of *Theileri*a and *Babesia* species (Nijhof *et al*., 2005). Lane 1 is a 100 bp DNA ladder (Fermentas, Inqaba, South Africa). Lane 2 is the negative control and lane 3 is the positive control. Lanes 3–9 are PCR amplicons. No amplification is evident in lanes 4–6 (Samples: BF124, BF17, BF196, APPENDIX B) and lanes 7–9 showed positive amplification reactions (Samples: BF223, APPENDIX C; BF32, APPENDIX A; BF147, APPENDIX B).

The reaction of the *B. felis* probe was tested using 358 samples from various felid species. The samples tested included 156 (40.5%) from cheetahs (APPENDIX A), 121 (33.8%) from lions (APPENDIX B), 89 (23.1%) from domestic cats (APPENDIX C) and 19 (4.9%) from other felids (APPENDIX D) such as black-footed cats, servals, caracals and a leopard. Twenty samples, 10 positive and 10 negative for *B. felis*, were allocated for specificity testing (APPENDIX C, marked"*") according to serological and microscopically findings. The newly developed probe showed to be specific to *B. felis*.

In total, 212 of the samples tested positive for a *Babesia* species (Table 3). Results varied from 115 (54.2%) samples that tested positive with the *Babesia / Theileria* genus–specific probe; 52 (24.5%) positive only for *B. felis*; 25 (11.8%) positive only for *B. leo;* 4 (1.9%) only for *B. microti* and 1 (0.47%) sample tested positive only for *B. rossi*. Various combinations of mixed infections occurred: 6 (2.83%) samples tested positive for *B. leo* and *B. felis*; and 4 (1.88%) samples tested positive for *B. felis* and *B. microti*. One sample, from a domestic cat, was infected with *B. felis*, *B. leo* and *B. microti*.

Babesia felis was detected in 22 cheetahs, 14 lions, 14 domestic cats and a serval. *Babesia leo* was detected in 3 lions, 17 cheetahs, 4 domestic cats and a leopard. *Babesia microti* was detected in 3 lions and 1 domestic cat. Mixed infections of *B. felis* and *B. leo*; *B. microti* and *B. leo; B. felis* and *B. microti; B. felis*, *B. leo and B. microti* were found in lions and domestic cats. *Babesia felis* and *B. leo* occurred in cheetahs (APPENDIX A), but not as mixed infections. No *B. microti* was detected in cheetahs.

A high number of felid samples hybridized only with the genus-specific probe for *Theileria / Babesia* (Gubbels *et al*. 1999; Nijhof *et al*. 2005), namely 54 cheetahs (APPENDIX A), 33 lions (APPENDIX B), 21 domestic cats (APPENDIX C), 1 serval (APPENDIX D; BF294) and 1 tiger (APPENDIX D; BF288).

A total of 90 survey samples were collected. *Babesia felis* was detected in 3 cheetahs (20 samples from Namibia; APPENDIX A), and 1 serval (n=4 collected; APPENDIX D). Five of nine black-footed cats tested positive for the genus-specific probe only; the others were negative. The only leopard sample tested was positive for *B. leo*. Sixty-four free-ranging lions were tested (APPENDIX B): 41 from South Africa (Kruger National Park and game parks in KwaZulu-Natal), 8 from Swaziland (Hlane Game Reserve), 11 from Namibia (Etosha National Park) and 4 from Botswana (various game reserves). Samples collected from lions in the Kruger National Park tested positive for *B. leo* (3 samples), 1 sample had a mixed infections of *B. leo* and *B. felis* and 3 samples tested positive only with the genus-specific probe. Of 11 samples collected on filter paper from lions in Etosha National Park, 7 tested positive – all with the genus-specific probe. No *Babesia* parasite was detected in the two caracal samples (n=2 samples; APPENDIX D).

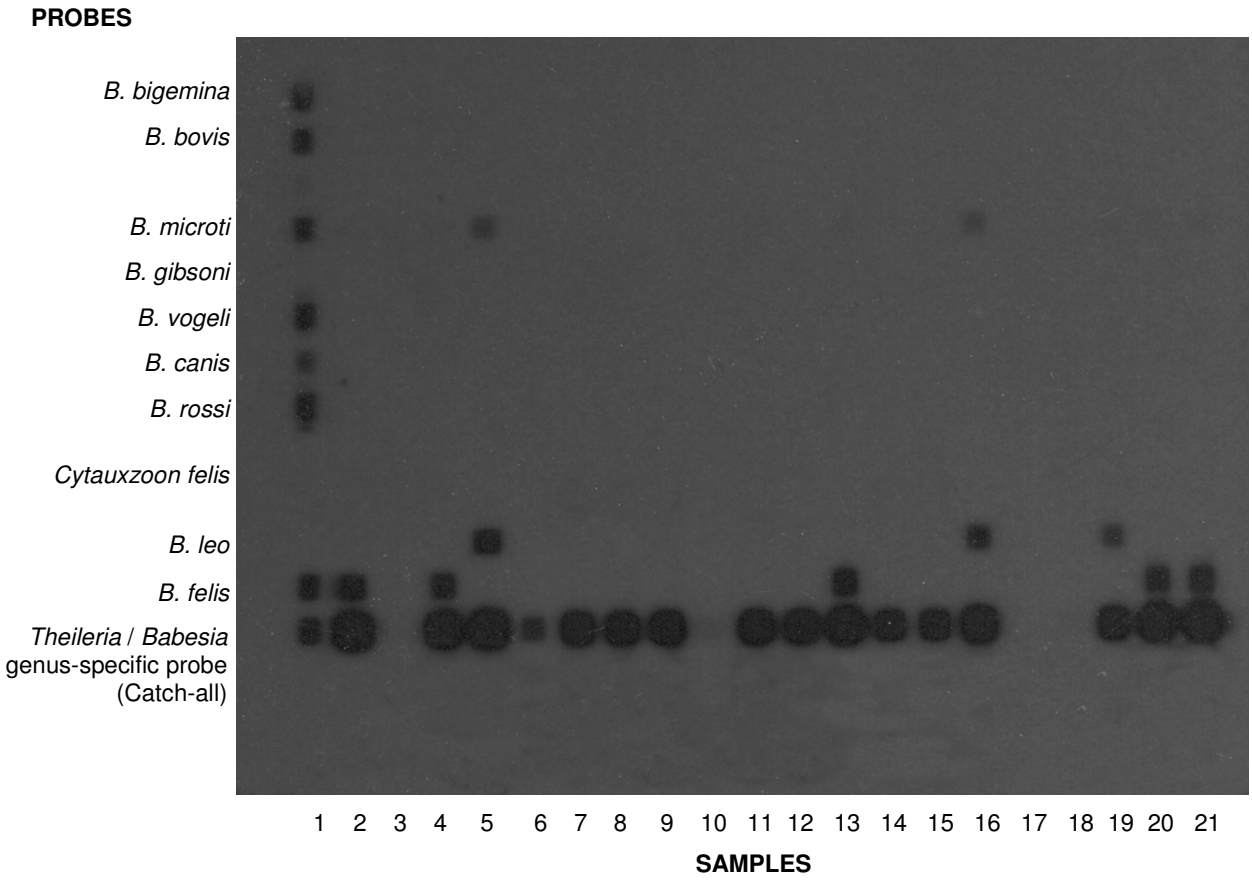


Figure 8 This figure illustrates a RLB analysis. Black spots indicate positive hybridization reactions. Lanes 1-3 are control samples: lane1 is the plasmid control; line 2 is the *B. felis* positive control; line 3 is water used as negative control; lanes 4-21 are samples: samples 5 and 16 showed mixed infection with *B. felis* and *B. microti* (BF220; BF269, APPENDIX C); lanes 4, 13, 20 and 21 (samples BF223, APPENDIX C; BF32, APPENDIX A; BF147, APPENDIX B, BF67) are positive for *B. felis*; samples 5, 16 and 19 (BF105, APPENDIX A; BF126, APPENDIX B; BF248, APPENDIX D) are positive for *B. leo.* The following lanes illustrates hybridization reactions for samples: Lanes 6-9 (samples BF107, 111, 112, 114, 115, 116: APPENDIX A), 11 (BF221, APPENDIX C), 14 (BF284, APPENDIX C) and 15 (BF294, APPENDIX D) reacted only with the genus-specific probe for *Babesia* and / or *Theileria;* Lanes 10 (BF124, APPENDIX B) 17 (BF196, APPENDIX B) and 18 (BF197, APPENDIX B) were negative samples.

Table 3 A summary of the results obtained in this survey (2002-2007)

HOST	TOTAL SAMPLES TESTED	TOTAL POSITIVE	ONLY *Theileria/ Babesia* genus-specific	Positive hybridization reaction with species-specific probes							
				Single infections			Mixed infection				OTHER
				B. felis	***B. leo***	***B. microti***	***B. leo B. felis***	***B. leo B. microti***	***B. felis B. microti***	***B. felis B. leo B. microti***	
Diagnostic											
Tiger	3	2	1	0	0	0	0	1	0	0	0
Lion	65	48	21	13	3	3	4	3	0	0	*B. rossi*
Cheetah	76	76	54	19	3	0	0	0	0	0	0
Domestic cat	89	47	21	14	4	1	1	0	4	1	0
Survey											
Serval	4	2	1	1	0	0	0	0	0	0	0
Caracal	2	0	0	0	0	0	0	0	0	0	0
Black-footed cat	9	5	5	0	0	0	0	0	0	0	0
Leopard	1	1	0	0	1	0	0	0	0	0	0
Lion	56	28	12	1	14	0	1	0	0	0	0
Cheetah	40	3	0	3	0	0	0	0	0	0	0
TOTAL	**385**	**212**	**115**	**52**	**25**	**4**	**6**	**4**	**4**	**1**	**1**
		55.06 %	**54.24 %**	**24.52 %**	**11.79 %**	**1.88 %**	**2.83 %**	**1.88 %**	**1.88 %**	**0.47 %**	**0.47 %**
			Percentage samples that tested positive for a *Babesia* sp.								

CHAPTER 4

DISCUSSION

Nomenclature around babesiosis in felids is not very clear. Dennig (1967) proposed that feline babesias should be assigned to two groups: small babesias (*B. felis* and *B. cati*) and large babesias (*B. herppailuri* and *B. pantherae*). In contrast, Levine (1973) postulated that all babesias reported from felids are *B. felis*. This illustrated how little was known about the occurrence of *Babesia* species in various felid species.

In this study, 358 samples were screened with the objective of studying the occurrence of the two best characterised *Babesia* spp., *B. felis and B. leo*. The currently used detection assays for parasites in felids are based on morphology and IFA. These tests are not sensitive and therefore do not assist in identifying and differentiating babesias in cats. Molecular characterization of blood parasites becomes more and more important. The RLB is a molecular technique used to detect and differentiate between blood parasites in various animal species using PCR amplification in combination with blotting. This blotting technique was adapted to use DNA probes for the detection of blood parasites in cattle, Cape buffaloes, several antelope species, dogs and horses, but there were only two probes previously developed 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. Various felid samples were tested using the newly developed *B. felis* probe, together with an existing *B. leo*, *C. felis*, and other *Babesia* and *Theileria* species-specific probes (Chapter 2, Table 1). The *B. felis* probe showed to be specific and the RLB assay proved to be useful to screen a high number of samples. In some cases no amplification product could be visualized on an agarose gel, but a hybridization reaction was visualized on the RLB. It is speculated that this is due to low parasitaemia.

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. Test results also showed that these parasites can occur as single and mixed infections (Table 3). When using the RLB assay, a number of samples reacted only with the *Babesia* / *Theileria* genus-specific probe. This is an indication of a sequence difference in the V4 region and 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 opens 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 designed, are different. This also applies for the genus-specific results that occurred in lion, black-footed cats, servals, caracals and a leopard. These findings challenge 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, mainly from Namibia, were interesting. Only 7,5% (3/40) of the free-ranging cheetahs were positive for *B. felis*. Namibia is a semiarid country, and a low prevalence of a tick-borne infection is not unexpected (Penzhorn *et al.* 2008). 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; Penzhorn *et al*. 1999).

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 rainfall than Namibia, and therefore presumably more suitable for ticks. On the other hand, samples collected from lions in Namibia were positive on the genus-specific probe only. Blood parasites in lions and cheetahs have been reported before by Averbeck *et al*. (1990) from lions and cheetahs in the Serengeti National Park and the Ngorongoro crater, Tanzania. These studies were mere reports on morphological findings; parasites were not characterized further.

In some instances blood smears were submitted alone or with blood samples for RLB testing. These slides were examined for parasites. The parasites in the red blood cells on the slide did not show any difference in morphology and all parasites were “small” parasites as described in the literature review (Chapter 1).

Babesia microti, a blood parasite associated with infection of small rodents (Guitián *et al.* 2003; Tsuji *et al*. 2001), often occurs in various felid samples, as single and other times as mixed infections together with *B. felis* and *B. leo* with higher incidence among captive felids. The only explanation for this is that *B. microti* and *B. felis* and / or *B. leo* shared a similar tick vector. The vector of *B. felis* in domestic cats remains unknown and the high incidence of *B. microti* in mixed infections can underline the possibility that these parasites share a common vector. *Babesia microti* together with *B. duncani*,

B. divergens and *B. venatorum* are associated with human babesiosis. Although only 0.6% (13/212) of samples tested positive for *B. microti,* six domestic cats tested positive for *B. microti*. This finding may be important to human medicine where human babesiosis is regarded as an emerging disease and *B. microti* infection is regarded as the most common cause of human babesiosis. The first *B. microti* case in humans was described in 1957 (Vanier and Krause 2009). The geographical distribution includes North and South America, Europe, Asia, and Africa. Although humans are considered to be incidental hosts and dead-end hosts, transmission to other humans via blood transfusions is a public-health concern. Seventy cases of blood transfusion-transmitted *B. microti* with 12 associated deaths have been reported in the USA (Johnson *et al*. 2007). Bearing in mind that humans and domestic cats live in close association, this finding might be of importance to future human babesiosis studies.

A lion sample, collected from an unknown region in the Eastern Cape Province, South Africa, repeatedly tested positive for *B. rossi. Babesia rossi*, originally described in a side-striped jackal (*Canis adustos*) (Nutall 1910) occurs only in sub-Saharan Africa. Although it causes fatal disease in dogs, it may be a natural parasite of wild dogs (*Lycaon pictus*) and other indigenous canids (Matjila *et al*. 2008). The occurrence of *B. rossi* in lions can be explained by the presence of the vector: *Haemaphysalis elliptica* (previously misidentified as *H. leachi*), the known vector for *B. rossi* transmission (Uilenberg *et al*. 1989), that occasionally feeds on felids (Horak *et al*., 2009; unpublished data).

Results from this study open up a new understanding of *Babesia* parasites in felids but future investigations are still needed to solve the high incidence of “genus-specific-only” reactions. Future studies will include the analysis of the samples that reacted with the genus-specific probe alone. These samples (115 genus-specific only samples: 79 cheetahs; 76 lions; 47 domestic cats; 10 other felids) will be subjected to sequencing and phylogenetic analysis of the 18SSU rRNA gene to solve the sequence difference that occur in the V4 region where the probes have been developed.

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APPENDIX A

Samples collected from cheetahs in southern Africa

Sample identification number	Sample ID	Type of sample used for DNA extraction	RLB Results
BF4.1	950004; Tygerberg Zoo; *Haemobartonella.* pos	EDTA	Catch all
BF4.2	Haakneus brother, Tygerberg Zoo *Babesia* pos	EDTA	Catch all
BF4.3	Chinga; Tygerberg Zoo; pos	EDTA	Negative
BF4.4	Sha; Cheetah outreach; negative	EDTA	Negative
BF4.5	Charlie; Cheetah outreach; *Babesia* pos	EDTA	Negative
BF4.6	Nyana; Cheetah outreach; *Babesia* pos	EDTA	Negative
BF4.7	Shadow; Cheetah outreach; *Babesia* pos	EDTA	Negative
BF4.8	Haakneus; Tygerberg Zzoo; *Babesia* pos	EDTA	Catch all
BF4.9	Mawimbe; Tygerberg Zoo; unknown; cheetah	EDTA	Catch all
BF17	Eleanor; M286/73	EDTA	Catch all
BF18	Eleanor; M290/74	EDTA	Catch all
BF19	Eleanor; F303/72	EDTA	Negative
BF22	Eleanor; Namibia cheetah; AJ 259	Citrate	-
BF23	Eleanor; Namibia cheetah AJ 80	Citrate	-
BF24	Eleanor; Namibia cheetah AJ 19/99	Citrate	-
BF25	Eleanor; Namibia cheetah AJ 79	Citrate	-
BF26	Eleanor; Namibia cheetah AJ 81	Citrate	-
BF27	Eleanor; Namibia cheetah AJ 3	Citrate	-
BF28	Eleanor; Namibia cheetah AJ 2/01	Citrate	-
BF29	Eleanor; Namibia cheetah AJ 48/00	Citrate	-
BF30	Eleanor; Namibia cheetah AJ 21/99	Citrate	-
BF31	Eleanor; Namibia cheetah AJ 20/99	Citrate	-
BF32	Eleanor; Namibia cheetah AJ 47/00	Citrate	*B. felis*
BF33	Eleanor; Namibia cheetah AJ 70	Citrate	-
BF34	Eleanor; Namibia cheetah AJ 187	Citrate	-
BF35	Eleanor; Namibia cheetah AJ 132	Citrate	-
BF36	Eleanor; Namibia cheetah AJ 84	Citrate	-
BF37	Eleanor; Namibia cheetah AJ 133	Citrate	-
BF38	Eleanor; Namibia cheetah AJ 9	Citrate	-
BF39	Eleanor; Namibia cheetah AJ 261	Citrate	-

BF40	Eleanor; Namibia cheetah AJ 260	Citrate	-
BF41	Eleanor; Namibia cheetah AJ 138	Citrate	-
BF42	Eleanor; Namibia cheetah pp 04/01	Citrate	-
BF43	Eleanor; Namibia cheetah AJ 28/99	Citrate	-
BF44	Eleanor; Namibia cheetah AJ C	Citrate	-
BF45	Eleanor; Namibia cheetah AJ 4	Citrate	-
BF46	Eleanor; Namibia cheetah AJ 74	Citrate	-
BF47	Eleanor; Namibia cheetah AJ 240	Citrate	-
BF48	Eleanor; Namibia cheetah AJ 19	Citrate	-
BF49	Eleanor; Namibia cheetah AJ 56/00	Citrate	-
BF50	Eleanor; Namibia cheetah AJ 302	Citrate	*B. felis*
BF51	Eleanor; Namibia cheetah AJ 18	Citrate	-
BF52	Eleanor; Namibia cheetah AJ 18/99	Citrate	-
BF53	Eleanor; Namibia cheetah AJ 200	Citrate	-
BF54	Eleanor; Namibia cheetah AJ 255	Citrate	-
BF55	Eleanor; Namibia cheetah AJ 226	Citrate	-
BF56	Eleanor; Namibia cheetah AJ 244	Citrate	*B. felis*
BF57	Eleanor; Namibia cheetah AJ 256	Citrate	*B. felis*
BF58	Eleanor; Namibia cheetah AJ 228	Citrate	-
BF59	Eleanor; Namibia cheetah AJ 227	Citrate	-
BF60	Eleanor; Namibia cheetah AJ 225	Citrate	-
BF61	Eleanor; Namibia cheetah AJ 5	Citrate	*B. felis*
BF95	Eleanor; Cheetah; de Wildt; 4ft	EDTA	Negative
BF96	Eleanor; Cheetah; de Wildt; 14T	EDTA	Negative
BF97	Eleanor; Cheetah; de Wildt; Rufus blue top	EDTA	Negative
BF99	Eleanor; Cheetah; de Wildt; M449	EDTA	Negative
BF100	Eleanor; Cheetah; de Wildt; M450	EDTA	Negative
BF101	Eleanor; Cheetah; de Wildt; M451	EDTA	Negative
BF102	Eleanor; Cheetah; de Wildt; M448	EDTA	Negative
BF103	Eleanor; Cheetah; 1; 22/8	EDTA	Negative
BF104	Eleanor; Cheetah; 10; 27/8	EDTA	Catch all
BF105	Eleanor; Cheetah; 15; 27/8	EDTA	*B. leo*
BF106	Eleanor; Cheetah; 4; 27/8	EDTA	Negative
BF107	Eleanor; Cheetah; 7; 27/8	EDTA	Catch all
BF108	Eleanor; Cheetah; 3; 27/8	EDTA	Negative
BF109	Eleanor; Cheetah; 8; 27/8	EDTA	Negative
BF110	Eleanor; Cheetah; 5; 27/8	EDTA	Negative
BF111	Eleanor; Cheetah; 14; 27/8	EDTA	Catch all

BF112	Eleanor; Cheetah; 13; 27/8	EDTA	Catch all
BF113	Eleanor; Cheetah; 16; 27/8	EDTA	Negative
BF114	Eleanor; Cheetah; 2; 27/8	EDTA	Catch all
BF115	Eleanor; Cheetah; 11; 27/8	EDTA	Catch all
BF116	Eleanor; Cheetah; 17; 27/8	EDTA	Catch all
BF123	Eleanor; Cheetah; 12; 27/8	EDTA	Negative
BF124	Eleanor; Cheetah; 9; 27/8	EDTA	Catch all
BF125	Eleanor; Cheetah; 6; 27/8	EDTA	Catch all
BF126	Eleanor; Cheetah; 18	EDTA	*B. leo*
BF127	Eleanor; Cheetah; 19	EDTA	Catch all
BF128	Eleanor; Cheetah; 21	EDTA	Catch all
BF129	Eleanor; Cheetah; 22	EDTA	Catch all
BF130	Eleanor; Cheetah; 23	EDTA	Negative
BF131	Eleanor; Cheetah; 24	EDTA	Negative
BF132	Eleanor; Cheetah; 25	EDTA	Negative
BF133	Eleanor; Cheetah; 26	EDTA	Catch all
BF134	Eleanor; Cheetah; 27	EDTA	Negative
BF135	Eleanor; Cheetah; 28	EDTA	Catch all
BF136	Eleanor; Cheetah; 29	EDTA	Catch all
BF137	Eleanor; Cheetah; 30	EDTA	Negative
BF138	Eleanor; Cheetah; 31	EDTA	Catch all
BF139	Eleanor; Cheetah; 32; F276; 24/9	EDTA	Negative
BF140	Eleanor; Cheetah; 33	EDTA	Catch all
BF141	Eleanor; Cheetah; 34	EDTA	Catch all
BF142	Eleanor; Cheetah; 35	EDTA	Catch all
BF143	Eleanor; Cheetah; 36	EDTA	Catch all
BF151	Cheetah; Katja; Clinpath F446	EDTA	Catch all
BF152	Cheetah; Katja; Clinpath M427	EDTA	Catch all
BF153	Cheetah; Katja; Clinpath 440	EDTA	Catch all
BF154	Cheetah; Clinpath; 1290; C2265	EDTA	*B. leo*
BF160	Cheetah; F388, 2812; Clinpath	EDTA	Catch all
BF161	Cheetah; M440; 2817; Clinpath	EDTA	Catch all
BF162	Cheetah; F390; 2813; Clinpath	EDTA	Catch all
BF163	Cheetah; 1615; F362; 2811, Cinpath	EDTA	Catch all
BF164	Cheetah; 1775; 3112; M295	EDTA	Negative
BF199	Cheetah; 1922; fel; 3342; Clinpath	Heparin	Negative
BF200	Cheetah; 2589; 4502; CA; Clinpath	EDTA (Very little)	Negative
BF227	Cheetah; 1915; F36; Clinpath	EDTA	Catch all

BF228	Cheetah; 1915; F370; Clinpath	EDTA	Catch all
BF229	Cheetah; 1915; M379; Clinpath	EDTA	Catch all
BF230	Cheetah; RLB 51/04; Golden Vet Lab	EDTA	Catch all
BF239	Cheetah; 1645; F425; 1; Clinpath	EDTA	Negative
BF240	Cheetah; 3138; 373; 2; Clinpath	EDTA	*B. felis*
BF241	Cheetah; 3139; M380; 3; Clinpath	EDTA	*B. felis*
BF242	Cheetah; 3140;4; Clinpath	EDTA	*B. felis*
BF243	Cheetah; 3141; 5; Clinpath	EDTA	*B. felis*
BF244	Cheetah; 3142; 6; Clinpath	EDTA	*B. felis*
BF245	Cheetah; 3143; 7; Clinpath	EDTA	Negative
BF246	Cheetah; Catch all; *B. felis*	EDTA	*B. felis*
BF247	Cheetah; 3145; 9; Clinpath	EDTA	*B. felis*
BF249	Cheetah; 3697; 54; Clinpath	EDTA	Negative
BF250	Cheetah; 3696; 53; Clinpath	EDTA	Negative
BF251	Cheetah; 3695; 52; Clinpath	EDTA	Negative
BF252	Cheetah; 3694; 51; Clinpath	EDTA	Negative
BF253	Cheetah; 3693; 50; Clinpath	EDTA	*B. felis*
BF254	Cheetah; 3692; 49; Clinpath	EDTA	*B. felis*
BF255	Cheetah; 3691; 48; Clinpath	EDTA	Negative
BF256	Cheetah; 3690; 47; Clinpath	EDTA	*B. felis*
BF257	Cheetah; 3689; 46; Clinpath	EDTA	*B. felis*
BF258	Cheetah; 3688; 45; Clinpath	EDTA	*B. felis*
BF259	Cheetah; 3701; 58; Clinpath	EDTA	*B. felis*
BF260	Cheetah; 3700; 57; Clinpath	EDTA	*B. felis*
BF261	Cheetah; 3699; 56?; Clinpath	EDTA	*B. felis*
BF262	Cheetah; Clinpath	EDTA	Negative
BF263	Clinpath; 1992; 3910; 71	EDTA	Catch all
BF264	Clinpath; 1992; 3911; 22	EDTA	Catch all
BF265	Path care; M474; 4626	EDTA	Catch all
BF266	Path care; M475; 4627	EDTA	Catch all
BF267	Path care; M481; 4626	EDTA	*B. felis*
BF268	Path care; M483; 4626	EDTA	*B. felis*
BF273	Clinpath; 2628	EDTA	Catch all
BF274	Clinpath; 2628	EDTA	Catch all

Survey animals

APPENDIX B

Samples collected from lions in southern Africa

Laboratory Identification	Sample ID	Type of sample used for DNA extraction	RLB Results
BF1	Lion cub (Prof Penzhorn)	EDTA	Negative
BF2	19/07/01; Krugersdorp game reserve Big lion cub; Rant-en-dal Vet	EDTA	Negative
BF3	19/07/01; Krugersdorp game reserve Tiny lion cub; Rant-en-dal Vet	EDTA	Negative
BF8	Alua; 1997/07/26; Kruger National Park; Lion	Giemsa-stained slide	Negative
BF9	Alua; 1997/07/26; Kruger National Park; Lion	Giemsa-stained slide	Negative
BF10	Alua; 1997/07/26; Kruger National Park; Lion	Giemsa-stained slide	Negative
BF15.1	Kruger Park lion	Unstained slide	Catch all
BF15.2	Kruger Park lion	Unstained slide	Catch all
BF20	Eleanor; Emma, Lion, Pta Zoo	EDTA	*B. felis, B. leo*
BF21	Eleanor; Moon, Lion, Pta Zoo	EDTA	*B. felis, B. leo*
BF69	Lion; Tweerivieren	Dart tip with blood	*B. rossi*
BF74	OVI; Lion cub; 17/7/97	Spleen	Negative
BF117	Eleanor; lion; female	EDTA	Negative
BF118	Eleanor; lion; Deon	EDTA	Negative
BF119	Eleanor; lion; Sassy	EDTA	Negative
BF120	Eleanor; lion; Kiara	EDTA	Negative
BF121	Eleanor; lion; Baby; Male	EDTA	Negative
BF144	Eleanor; lion; C Niveti; 8/4/02; Laeveld; 11/04/02	EDTA	*B.felis; B. leo*
BF145	Eleanor; lion; Dyasonx; 8/4/02; Laeveld; 11/04/02	EDTA	Catch all
BF146	Eleanor; lion; Methlwane; 8/4/02	EDTA	*B. leo*
BF147	Lion; Eleanor; lion; Black; dam C; 8/4/02	EDTA	*B. felis*
BF155	Lion; Clinpath; 1349; 2361	EDTA	Negative
BF157	Lion; Alua; 1997/07/06 (5xslide)	Giemsa-stained slide	Negative

BF166	Lion; Pathology-Utrecht students	Spleen	Negative
BF167	Lion; Joh Zoo; Prof Penzhorn	Spleen	Negative
BF168	Lion; Farra Joh Zoo; F; Utrecht Students	EDTA	Negative
BF169	Lion; Sahara; Joh Zoo, F; Utrecht students	EDTA	Negative
BF170	Lion, Jade; Joh Zoo; Utrecht students	EDTA	Negative
BF171	Lion; 1; Hlane; Swaziland; Utrecht Students	EDTA	*B. leo*
BF172	Lion; 2; Hlane; Swaziland; Utrecht Students	EDTA	*B. leo*
BF173	Lion; 3; Hlane; Swaziland; Utrecht Students	EDTA	*B. leo*
BF174	Lion; 4; Hlane; Swaziland; Utrecht Students	EDTA	*B. leo*
BF175	Lion; 5; Hlane; Swaziland; Utrecht Students	EDTA	*B. leo*
BF176	Lion; 6; Hlane; Swaziland; Utrecht Students	EDTA	*B. leo*
BF177	Lion; 7; Hlane; Swaziland; Utrecht Students	EDTA	*B. leo*
BF178	Lion; 8; Hlane; Swaziland; Utrecht Students	EDTA	*B. leo*
BF179	Lion; Swalu; Joh Zoo; Utrecht Students	EDTA	*B. leo*
BF180	Lion; 1; KNP lodge; Utrecht Students	EDTA	Catch all
BF181	Lion; 2; KNP lodge; Utrecht Students	EDTA	*B. leo*
BF182	Lion; 3; KNP lodge; Utrecht Students	EDTA	*B. leo*
BF183	Lion; 4; KNP lodge; Utrecht Students	EDTA	*B. leo*
BF184	Lion; 5; KNP lodge; Utrecht Students	EDTA	*B. leo;* *B. felis*
BF185	Lion; CDV +; Utrecht Students	EDTA	*B. felis*
BF186	Lion; Shani; Joh Zoo; Utrecht Students	EDTA	Negative
BF187	Lion, Vidor; Joh Zoo; Utrecht Students	EDTA	Negative
BF188	Lion; unknown; Utrecht Students	Spleen	*B. leo*
BF189	Lion, unknown; Utrecht Students	Spleen	Negative
BF190	Lion, KNP 13/11/03; Utrecht Students	EDTA	Negative
BF191	Lion, Cayla; Joh Zoo; Utrecht Students	Citrate	*B. felis*
BF192	Lion, Moon; Joh Zoo; Utrecht Students	Citrate	*B. leo*
BF193	Lion, Amber; Joh Zoo; Utrecht Students	Citrate	*B. leo*
BF194	Lion, Emma; Joh Zoo; Utrecht Students	Citrate	*B. leo;* *B. felis*
BF195	Lion, Jerimia; Joh Zoo; Utrecht Students	EDTA	T/B catch all; *B. felis*
BF196	Lion, Smudge; Joh Zoo; Utrecht Students	EDTA	Negative
BF197	Lion, Snake; Joh Zoo; Utrecht Students	EDTA	Negative
BF198	Lion, Joe-Joe; Joh Zoo; Utrecht Students	EDTA	Negative
BF201	Lion; Clinpath; 398, 688	EDTA	Negative
BF202	Lion; KZN; R236; Moritz	EDTA	Negative
BF203	Lion; KZN; R217; Moritz	EDTA	Negative

BF204	Lion; KZN; R232; Moritz	EDTA	*B. microti*
BF205	Lion; KZN; R235; Moritz	EDTA	*B. microti*
BF206	Lion; KZN; R234; Moritz	EDTA	*B. microti*
BF207	Lion; KZN; R244; Moritz	EDTA	Catch all
BF208	Lion; KZN; R245; Moritz	EDTA	Catch all
BF209	Lion; KZN; 1; Moritz	EDTA	Catch all
BF210	Lion; KZN; 2; Moritz	EDTA	Negative
BF211	Lion; KZN; 3; Moritz	EDTA	Negative
BF212	Lion; KZN; 4; Moritz	EDTA	Negative
BF213	Lion; KZN; 5; Moritz	EDTA	Negative
BF214	Lion; KZN; R238; Moritz	EDTA	Catch all
BF215	Lion; KZN; R239; Moritz	EDTA	Catch all
BF216	Lion; KZN; R242; Moritz	EDTA	Catch all
BF217	Lion; KZN; 6590218; Moritz	EDTA	Catch all
BF218	Lion; KZN; 62B28FA; Moritz	EDTA	Catch all
BF231	Lion; RLB 96/04; Thanda; 4313567913; Zvakanawild Vet; Moritz	EDTA	*B. leo;* *B. microti*
BF232	Lion; RLB 97/04; Thanda; 4313567913; Zvakanawild Vet; Moritz	EDTA	*B. leo;* *B. microti*
BF233	Lion; RLB 105/04; Thanda; 4313567913; 04-05-31-Lion1 “swazi” Zvakanawild Vet; Moritz	CITRATE	*B. leo;* *B. microti*
BF289	Lion, Golden Vet Lab, 175679	EDTA	Catch all
BF311	Lion; Prof P; Botswana	Unstained slide	Catch all
BF312	Lion; Prof P; Botswana	Unstained slide	Negative
BF313	Lion; Prof P; Botswana	Unstained slide	Negative
BF314	Lion; Prof P; Botswana	Unstained slide	*B. felis*
BF344	Lion; 2nd, P Caldwell; Prof P; E Lane	Filter paper	Negative
BF388	Lion; 2nd batch Moritz; KZN	EDTA	*B. felis*
BF389	Lion; 2nd batch Moritz; KZN	EDTA	Catch all
BF390	Lion; 2nd batch Moritz; KZN	EDTA	Catch all
BF391	Lion; 2nd batch Moritz; KZN	EDTA	Catch all
BF392	Lion; 2nd batch Moritz; KZN	EDTA	Catch all
BF393	Lion; 2nd batch Moritz; KZN	EDTA	*B. felis*
BF394	Lion; 2nd batch Moritz; KZN	EDTA	*B. felis*
BF395	Lion; 2nd batch Moritz; KZN	EDTA	*B. felis*
BF396	Lion; 2nd batch Moritz; KZN	EDTA	*B. felis*
BF397	Lion; 2nd batch Moritz; KZN	EDTA	*B. felis*

BF398	Lion; 2nd batch Moritz; KZN	EDTA	Catch all
BF399	Lion; 2nd batch Moritz; KZN	EDTA	Catch all
BF400	Lion; 2nd batch Moritz; KZN	EDTA	*B. felis*
BF401	Lion; 2nd batch Moritz; KZN	EDTA	Negative
BF402	Lion; 2nd batch Moritz; KZN	EDTA	*B. felis*
BF403	Lion; 2nd batch Moritz; KZN	EDTA	Catch all
BF404	Lion; 2nd batch Moritz; KZN	EDTA	Negative
BF405	Lion; 2nd batch Moritz; KZN	EDTA	*B. felis*
BF406	Lion; 2nd batch Moritz; KZN	EDTA	Negative
BF407	Lion; 2nd batch Moritz; KZN	EDTA	Catch all
BF408	Lion; 2nd batch Moritz; KZN	EDTA	Catch all
BF409	Lion; 2nd batch Moritz; KZN	EDTA	*B. felis*
BF410	Lion; 2nd batch Moritz; KZN	EDTA	Negative
BF411	Lion; Etosha; 22/11/2006	EDTA	Negative
BF412	Lion; Etosha; 22/11/2006	EDTA	Negative
BF413	Lion; Etosha; 22/11/2006	EDTA	Catch all
BF415	Lion; Etosha; 22/11/2006	EDTA	Catch all
BF416	Lion; Etosha; 22/11/2006	EDTA	Catch all
BF417	Lion; Etosha; 22/11/2006	EDTA	Negative
BF418	Lion; Etosha; 22/11/2006	EDTA	Catch all
BF419	Lion; Etosha; 22/11/2006	EDTA	Catch all
BF420	Lion; Etosha; 22/11/2006	EDTA	Catch all
BF421	Lion; Etosha; 22/11/2006	EDTA	Catch all
BF422	Lion; Etosha; 22/11/2006	EDTA	Negative
BF423	Lion; Etosha; 22/11/2006	EDTA	Negative
BF464	Moritz; USA student; 744587	EDTA	Negative
BF467	Moritz; USA student; 747880	EDTA	Negative

Survey animals

APPENDIX C

Samples collected from domestic cats in southern Africa

Laboratory Identification	Sample ID	Type of sample used for DNA extraction	RLB Results
BF5	Mrs Raven; Pelgrimsrus; Natali Coetzee; Sabie	Filter paper	*B. felis, B. leo*
*BF6	Mrs Raven; Pelgrimsrus; Natali Coetzee; Sabie	EDTA	*B. felis*
*BF7	Eleanor; B felis +	Unstained slide	*B. felis*
*BF62	OVI; Mysty; OP 036366; B. felis poss	EDTA	*B. felis*
*BF63	OVI; Mysty, B felis; 4/10/94	EDTA	*B. felis*
*BF70	OVI; Cat no 2; Blackie; 9/4/92	EDTA	Negative
BF72	OVI; Cat; Kurt; 11/10/94	Spleen	*B. odocoilei*
*BF75	OVI; Cat; Samtra; 29/9/94	Spleen	Negative
*BF76	OVI; Cat; Ginger; 29/9/94	Spleen	Negative
BF77	OVI; Cat 9; 23/03/93	Spleen	*B. odocoilei*
*BF78	OVI; Cat 7; 4/8/92	Spleen	Negative
*BF149	Sabie; Prof Penzhorn	Giems-stained slide	*B. felis*
*BF150	A231; Pathcare	Giemsa-stained slide	Negative
BF165	(19jr); 192, C3342; L Olivier Inclusion bodies in liymphocytes	EDTA	Negative
*BF219	60; Fel; 83; Clinpath	EDTA	*B. felis*
BF220	Prof Penzhorn	EDTA	*B. felis, B. microti*
BF221	94900693; Twelves; Virbac; Tshepo	EDTA	Catch all
*BF222	94916582; Huxon; Virbac; Tshepo	EDTA	Negative
*BF223	94916445; Mathewso; Virbac; tshepo	EDTA	*B. felis*
*BF224	94885100; Brown; Virbac; Tshepo	EDTA	Negative
*BF225	Fudge; Prof Penzhorn	EDTA	Negative
BF226	Aran Vet; Prof Penzhorn; “Rustenburg-kat”	Spleen; liver; lymph node	Catch all
*BF234	94959447; Virbac; Tshepo	EDTA	Negative
*BF235	95008146; Virbac; Tshepo	EDTA	Negative
BF236	95061393; Virbac; Tshepo	EDTA	Negative
BF237	Virbac; Tshepo	Unstained slide	Negative
BF238	Path Care; Lucia Lange; G92150830	EDTA	Catch all

BF269	Florida Hills Vet; Dr v Rijn; 168705	EDTA	*B. felis; B. microti*
BF270	PathCare; Houtbay (large piro)	EDTA	*B. felis; B. microti*
BF271	PathCare: Bredasdorp	EDTA	*B. felis*
BF272	PathCare: Hermanus; Jenkins	EDTA	Catch all
BF278	ClinPath: 2923; 5878	EDTA	Negative
*BF281	SAM 2:177716	EDTA	*B. felis*
BF282	PathCare: Nera King	EDTA	*B. felis*
BF283	Prof P, Lobetti: Aynur	EDTA	Negative
BF284	Path Care; Siamese-Lulu	EDTA	Catch all
BF285	Path Care; Pichachu	EDTA	Catch all
BF286	Path Care; Tiger	EDTA	Catch all
BF287	Lobetti	Filter paper	Negative
*BF297	Clinpath;630;1337	EDTA	*B. felis*
BF298	Path Care, Neeff, 650009527	EDTA	Negative
BF299	Clinpath, 706, 05c01531	EDTA	Negative
*BF305	Pathcare; Pheasant	EDTA	*B. felis*
BF306	Pathcare; CT 133073	EDTA	Negative
BF307	Pathcare; no 1; 694386350	EDTA	Negative
BF308	Pathcare; no 2; 694386351	EDTA	Negative
BF309	Pathcare; 650049913	EDTA	Negative
BF315	Feline Peney, Davey; Hillcrest (Vetcare)	EDTA	*B. felis,*
BF316	Thandi, Allborolight; Hillcrest (Vetcare)	EDTA	*B. felis, B. leo, B. microti*
BF317	Spark duPreez Hilcrest (Vetcare)	EDTA	*B. felis, B. microti*
BF318	Christmas Dabey, Hillcrest (Vetcare)	EDTA	*B. leo*
BF319	Heenie Duer, Hillcrest (Vetcare)	EDTA	Catch all
BF323	Tshepo;05; S01228	Stained slide	Negative
BF324	Puddin Coulon, Hilcrest	Filter paper	*B. felis*
BF325	Cork; Hillcrest	Filter paper	Catch all
BF333	Tshepo; Cleo	EDTA	Negative
BF336	Golden Vetlab; JB201886	EDTA	Negative
BF340	Mark Kallman	EDTA	Catch all
BF341A	BVH; R Lobetti	Filter paper	Catch all
BF342A	Andy; Tania Schoeman; Big	EDTA	Negative
BF347	Clinpath; 3612; 1685	EDTA	Negative
BF348	Clinpath; 1460; Fel3197	EDTA	Negative
BF350	Clinpath; 1518; Fel 3279	EDTA	Negative

BF351	Clinpath; 1689; Fel 3617	EDTA	Negative
BF352	Clinpath; 2362; Fel 1696	EDTA	Negative
BF353	Clinpath; 1625; Fel 3483	EDTA	Negative
BF354	Clinpath; JE 2388	EDTA	Negative
BF355	Clinpath; Fel 3467	EDTA	Negative
BF356	R17; 10/03/06	EDTA	Negative
BF357	R3; 10/03/06	EDTA	Negative
BF361	RR; 10/03/06	Filter paper	Negative
BF364	Vetpath	EDTA	Negative
BF370	Clinpath; Tshepo	EDTA	Negative
BF371	Clinpath, Tshepo	EDTA	Negative
BF372	Clinpath; Tshepo	EDTA	Negative
BF373	Clinpath; Tshepo	EDTA	Negative
BF374	Clinpath; Tshepo	EDTA	Negative
BF375	Clinpath; Tshepo	EDTA	Negative
BF461	W1612; Beytell	EDTA	*B. leo*
BF463	Tertius Gouws	Brain	Catch all
BF472	Prof Reyers; Poppie	EDTA	Catch all
BF473	Remo; MSD 232622 2093B	Filter paper	*B. leo*
BF474	Remo; JB 235458 2093D	Filter paper	*B. leo*
BF475	Remo; JB 244497 2093A	Filter paper	*B. felis*
BF476	Remo; Minx Edwards 2093C	Filter paper	Catch all
Cat01/2008	08IC0005; Hamer Vetdig.	EDTA	Catch all
Cat02/08	IDEX; Milana; RLB707	EDTA	Catch all
Cat03/08	Bartonella cat; Milana	EDTA	Catch all
Cat04/08	Bartonella cat; Milana	EDTA	Negative

APPENDIX D

Samples collected from felid species other than domestic cats, lions and cheetahs

Laboratory Identification	Sample ID	Type of sample used for DNA extraction	RLB Results
BF11	Witpootkat (*F. nigripes*; Pta Zoo; 07/01/1996 6M	Giemsa-stained slide	Catch all
BF12	Witpootkat (*F. nigripes*; Pta Zoo; 07/01/1996 3F	Giemsa-stained slide	Catch all
BF13	Witpootkat (*F. nigripes*; Pta Zoo; 07/01/1996 5M	Giemsa-stained slide	Catch all
BF6.1	Witpootkat (*F. nigripes*; Pta Zoo; 07/01/1996 1F	Giemsa-stained slide	Catch all
BF6.2	Witpootkat (*F. nigripes*; Pta Zoo; 07/01/1996 3F	Giemsa-stained slide	Negative
BF65	Witpootkat (*F. nigripes*; Pta Zoo; 07/01/1996 2F	Giemsa-stained slide	Negative
BF66	Witpootkat (*F. nigripes*; Pta Zoo; 07/01/1996 6M	Giemsa-stained slide	Catch all
BF67	Serval; Dr McGee	Giemsa-stained slide	*B. felis*
BF68	Serval; Dr McGee	Giemsa-stained slide	Negative
BF73	OVI; Caracal; Tammy; 17/7/97	Spleen	Negative
BF148	Caracal; Prof Penzhorn	Unstained slide	Negative
BF158	*F. nigripes*; 1996/01/07 (5xslide)	Giemsa-stained slide	Negative
BF159	Serval; Dr McGee (2xslide)	Giemsa-stained slide	Negative
BF248	Leopard; Kruger Park; Prof Penzhorn	EDTA	*B. leo*
BF279	Bengal Tiger, Pta Zoo: Leila	EDTA	Negative
BF280	Bengal Tiger, Pta Zoo: Male	EDTA	*B. leo;* *B. micro*ti
BF288	Tiger, Golden Vet, Rhino & ElephantPark,175733	EDTA	Catch all
BF290	African wild cat; Golden Vet Lab.	EDTA	Negative
BF294	Serval; Prof Penzhorn, Milana; 446962545B	Serum clot	Catch all

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