was conducted (Takahashi et al., 1993; Oyamada, Esaka, Kudo, and Yoshikawa 1996).

Our study suggests that the specific serological diagnosis of gnathstomiasis nipponica with *G. doloresi* antigen is difficult. The dot-ELISA test using *G. nipponicum* antigen and other serological methods remain to be developed. In the present case we failed to extirpate migratory worms because new creeping eruptions appeared after the biopsy. However, the treatment with thiabendazole seemed to be effective, although it is not clear whether the cure was due to the drug or to the spontaneous death of the migrating larvae.

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Two Species of Canine *Babesia* in Australia: Detection and Characterization by PCR

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ABSTRACT: The haemoprotozoan *Babesia canis* has been recognized in Australia for many years, and a second, smaller species has recently been discovered. Amplification and sequencing of a partial region of the 18S small subunit ribosomal RNA (rRNA) gene enabled detection and characterization of the large and small canine babesiae of Australia for the first time. Isolates from northern Australia were genetically characterized to be 99% homologous to *Babesia canis vogeli*, confirming previous speculation about the subspecies of *B. canis* endemic to Australia. The partial 18S rRNA gene sequence amplified from isolates obtained in southeastern Australia was genetically identical to *Babesia gibsoni*, a species not previously known in Australia. The polymerase chain reaction (PCR) used was shown to be specific to *Babesia* and had a high sensitivity, detecting DNA at a parasitemia of approximately 0.0000027%. This study also reports the first known detection and characterization of *B. canis* DNA in *Rhipicephalus sanguineus* ticks using PCR.

Babesiosis is an emerging tick-transmitted disease of both veterinary and medical significance, resulting from intraerythrocytic infection by species of *Babesia* (Homer et al., 2000; Kjemtrup and Conrad, 2000). Clinical signs and pathogenesis of the disease are variable and depend on the species or strain of the parasite and the immune status of the infected animal. The canine *Babesia* have traditionally been recognized as belonging to 2 morphologically distinct species, the large *B. canis* and the small *B. gibsoni*. Recently, the phylogenetic classification of these canine piroplasms has received renewed attention, and it is now believed that multiple species exist worldwide (Carret et al., 1999; Zahler, Rinder, Zweygarth et al., 2000).

At least 3 species of small canine piroplasm have been described (Kjemtrup et al., 2000). Those genetically characterized as *B. gibsoni* have been reported throughout Asia (Patton, 1910; Fowler et al., 1971; Farwell et al., 1982; Zahler, Rinder, Zweygarth et al., 2000) and parts of the United States (Anderson et al., 1979; Birkenheuer et al., 1999). Genotypically distinct, yet morphologically indistinguishable, from these are the small piroplasm species found in dogs from northern Spain, which have been given the name *Theileria annae* (Zahler, Rinder, Schein et al., 2000; Camacho et al., 2001). A third species has been found in the blood of dogs in California and remains unnamed (Kjemtrup et al., 2000). Genetically uncharacterized small piroplasms also have been found in dogs in Italy (Casapulla et al., 1998) and northern Africa (Botros et al., 1975).

Three subspecies of *B. canis* are now recognized, *B. canis canis*, *B. canis rossi*, and *B. canis vogeli* (Uilenberg et al., 1989; Zahler et al., 1998; Carret et al., 1999). Furthermore, it has been suggested that each of these is distinct enough to assume species status on the basis of genetic characterization (Carret et al., 1999). *Babesia canis canis* is transmitted by *Dermacentor reticulatus* ticks in Europe, and *B. canis rossi* is transmitted by *Haemaphysalis leachi* in southern Africa (Uilenberg et al., 1989). *Babesia canis vogeli* is transmitted by *R. sanguineus* and is distributed throughout various tropical and subtropical countries, including Australia.

Within Australia, it was originally believed that *B. canis* was the only endemic species reported to cause disease throughout northern and

semitropical regions (Hill and Bolton, 1966; Irwin and Hutchinson, 1991). These large piroplasms were shown to be experimentally transmitted by *R. sanguineus* ticks (Hill, 1966), and using information regarding *B. canis* subspecies vector specificity (Uilenberg et al., 1989), it was later inferred that the Australian strain was likely to be *B. canis vogeli* (Irwin and Hutchinson, 1991). *Babesia canis* also has been reported in dingoes (*Canis familiaris dingo*) (Callow, 1984); these wild canids may represent a potential reservoir of infection for domestic dogs. Recently, *B. gibsoni* was identified in the blood of 3 dogs in southeastern Australia (Muhlnickel et al., 2002).

Various diagnostic techniques have been used to detect the presence of *Babesia* sp. infection, with differing levels of success. Detection of *Babesia* sp. parasites is usually achieved using microscopic examination of blood smears, but this technique is limited because of low sensitivity and the difficulty of distinguishing morphologically similar strains and species (Krause et al., 1996). The immunofluorescent antibody test also is used to assess exposure to *Babesia* sp. infections in dogs; however, it has poor specificity as a result of antigen cross-reactivity (Yamane et al., 1994). It also fails to identify current infection. The use of alternative techniques, such as polymerase chain reaction (PCR), has become necessary to detect and identify *Babesia* sp. infections effectively and has been reported in numerous recent studies (Carret et al., 1999; Ano et al., 2001; Fukumoto et al., 2001). It is hoped that these molecular techniques will provide a solution to the issues of low sensitivity and specificity exhibited by the more traditional diagnostic methods. Furthermore, DNA sequence analysis can provide taxonomic information when morphological characteristics of different parasites are identical. In this study, we used a genus-specific PCR to detect and characterize *Babesia* sp. in canine blood and *R. sanguineus* ticks by amplifying and sequencing a portion of the 18S ribosomal RNA (rRNA) gene. This is the first report of molecular characterization of the Australian canine *Babesia* sp. parasites and the first time PCR was used to detect canine *Babesia* sp. in Australia.

Sixty-four samples of venous blood were collected from 34 dogs of various breeds and ages with anemia or thrombocytopenia (or both) that were presented to veterinary clinics in northern Australia and from 3 dogs in Victoria (southeastern Australia). Twelve dogs were known to be infected with *Babesia* sp. through microscopic observation of the parasites in blood smears made from peripheral blood samples. Blood was prevented from clotting by addition of ethylenediamine-tetraacetic acid (EDTA) and was frozen at -20 C until analysis. An isolate of sonicated whole *B. canis* parasite originating from north Queensland was used as a positive control sample (Irwin, 1989).

DNA was isolated from 200-μl aliquots of EDTA blood using a QIAamp® DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The primers PIRO A1 (5′ AGGGAGCC-TGAGAGACGGCTACC 3′) and PIRO-B (5′TTAAATACGAATGC-CCCCAAC 3′) were used to amplify an approximately 450-bp region of the 18S rRNA gene. The reverse primer PIRO B was used previously by Carret et al. (1999) and the forward primer PIRO A1 was developed to amplify most *Babesia* species using sequence information from GenBank. One microliter of extracted DNA was added to a 24-μl reaction mixture comprising 0.625 units of HotStarTaq™ DNA Polymerase (QIAGEN), 200 μM of each deoxynucleoside triphosphate, 12.5 pmol of each primer, and 2.5 μl of 10× PCR buffer (containing 15 mM MgCl$_2$) (QIAGEN). Amplification was performed using a GeneAmp PCR System 2400 Thermal Cycler (Perkin–Elmer, Foster City, California). An initial activation step at 95 C for 15 min, 62 C for 1 min, and 72 C for 2 min was followed by 35 cycles of amplification (94 C for 30 sec, 62 C for 20 sec, and 72 C for 30 sec) and a final extension step of 7 min at 72 C. The PCR products were electrophoresed on a 1% agarose gel and purified using an UltraClean™ Gelspin DNA Purification Kit (MO Bio Laboratories, Inc., Sohlana Beach, California) according to manufacturer's instructions.

Products of PCR were sequenced using an ABI Prism™ Dye Terminator Cycle Sequencing Kit (Applied Biosystems [ABI], Foster City, California). The sequenced products were analyzed using SeqEd v.1.0.3 (ABI), compared with sequence data available from GenBank™ using the BLAST 2.1 program (http://www.ncbi.nlm.nih.gov/BLAST/), and aligned using CLUSTAL X (Thompson et al., 1997).

Semiengorged *R. sanguineus* ticks were collected from 2 dogs in northern Australia from which blood samples were concurrently obtained. Ticks were macerated using a scalpel blade, and DNA was ex-
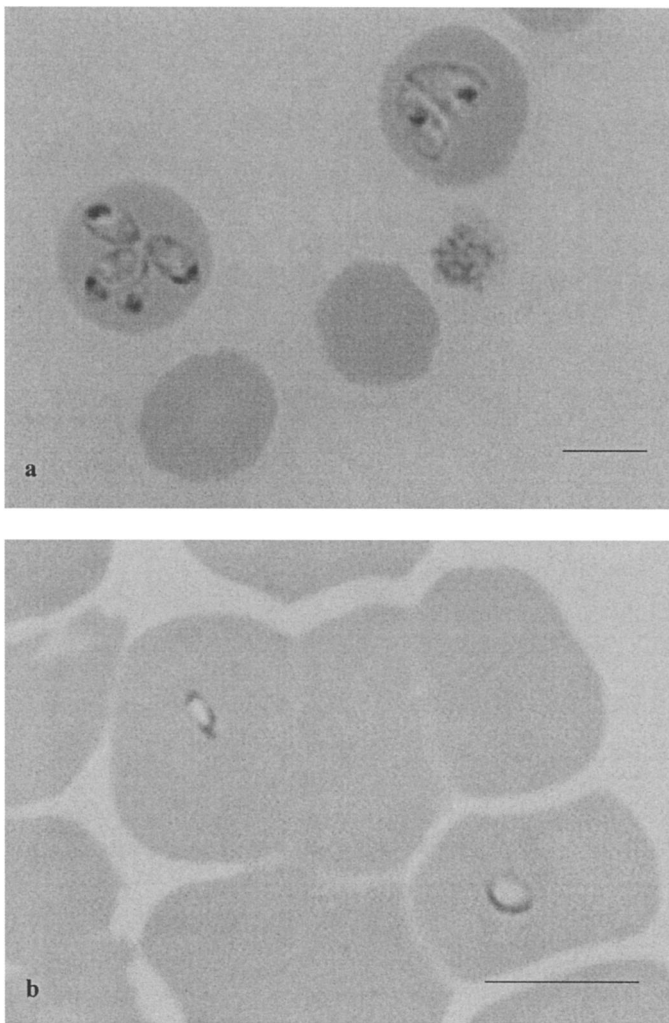

FIGURE 1. Light microscope view of canine erythrocytes infected with *Babesia canis vogeli* (**a**) and *Babesia gibsoni* (**b**). Bar = 5 μm.

tracted using a QIAamp® DNA Mini Kit (QIAGEN) according to the manufacturer's instructions. The extracted DNA was amplified and sequenced according to the protocol previously described for blood.

The detection limit of the PCR was estimated using a system of serial dilutions of parasitized blood in normal, uninfected canine blood. A venous blood sample with a *Babesia* sp. parasitemia of 2.72% (calculated according to Read and Hyde, 1993) was diluted 1:10 in canine blood known to be free from *Babesia* infection by microscopy and by its lack of exposure to tick vectors. This process was repeated until a 1×10^{-7} dilution was obtained. DNA was extracted and amplified from each dilution according to the protocol previously described.

Babesia specificity was confirmed by BLAST searching of the PIRO A1 and B primers (http://www.ncbi.nlm.nih.gov/BLAST/). This also was used to ensure that the primers would not amplify human DNA or DNA from other blood-borne canine parasites and bacteria. The primers also were tested against a variety of other pathogens potentially found in canine blood, including *Ehrlichia canis*, *E. platys*, *Rickettsia rickettsia*, *Bartonella vinsonii*, *Neospora caninum*, *Toxoplasma gondii*, *Trypanosoma cruzi*, and *Dirofilaria immitus*. Sixteen blood samples also were obtained from clinically normal dogs in New Zealand, a country reportedly free from canine babesiosis, as an added negative control.

Babesia sp. trophozoites in the blood of dogs from northern Australia were observed using light microscopy as intraerythrocytic piroplasms, the morphology of which was consistent with previous descriptions (Hill and Bolton, 1966; Irwin and Hutchinson, 1991) (Fig. 1a). The protozoans were polymorphic; however, many were pyriform-shaped.
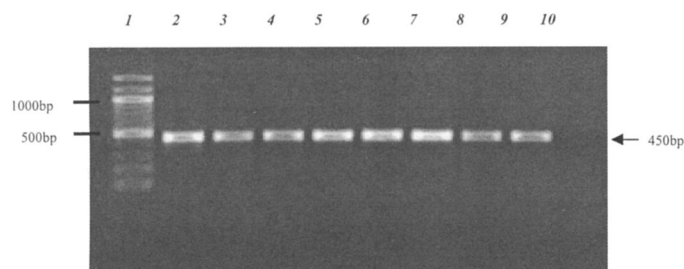
FIGURE 2.   Ethidium bromide–stained 1% agarose gel showing amplification of a 450-bp product for *Babesia*-positive samples (lane 1, molecular marker; lane 2, positive control; lanes 3–9, *Babesia*-positive samples; lane 10, negative control).

Trophozoites occurred singularly, paired, or as multiple organisms within individual red blood cells. The piroplasms ranged in size from 3.0 to 5.0 μm. *Babesia* sp. from the 3 dogs from Victoria were identified as singular or paired intraerythrocytic parasites. Free merozoites were occasionally observed. The parasites were polymorphic and ranged in diameter from 1 to 3 μm (Fig. 1b).

An approximately 450-bp product was amplified from the blood of 14 of the 34 dogs from northern Australia (Fig. 2). A similar-sized product was obtained from the blood of all 3 dogs from Victoria. Four of the 14 positive *Babesia* samples, in addition to the positive control sample from Queensland, were sequenced, and the partial 18S rDNA sequences showed 100% homology to one another (sequences submitted to GenBank, accession numbers AY102162 and AY102163). The sequences were 99% homologous to an Egyptian isolate sequence of *B. canis vogeli* obtained from GenBank (accession number AJ009769). These Australian isolates differed by 2 nucleotides within the Egyptian isolate and in both cases involved a pyrimidine substitution. The partial 18S rRNA gene of all three Victorian isolates were sequenced (submitted to GenBank, accession number AY102164) and showed an identical alignment with *B. gibsoni* (accession number AF271082).

Babesia sp. DNA was amplified from 1 of the semiengorged ticks originating from a dog from northern Australia that was also PCR positive for *Babesia* sp. The amplified product was sequenced and was identical to the *B. canis vogeli* genotype found in each of the canine blood samples from northern Australia.

The PCR used within this study was able to detect an estimated parasitemia of 0.0000027% (1 infected red blood cell in 4 million). None of the 16 blood samples from New Zealand contained amplifiable *Babesia* sp. DNA. The primers amplified DNA of *T. gondii* and *N. caninum*; however, the amplified products for these species could be differentiated based on size and were approximately 60 bp larger than the *Babesia* sp. products.

This study documents the first reported genetic characterization of the Australian strain of *B. canis*. On the basis of an amplified region of the 18S rRNA gene of the northern Australian isolates, it can be assumed that the Australian strain is *B. canis vogeli*. This confirms previous speculation by Irwin and Hutchinson (1991), who tentatively defined the Australian strain on the basis of pathogenicity and vector specificity. The amplified products from each of the large canine *Babesia* sp. isolates from northern Australia were found to be identical to one another and were 99% homologous with the Egyptian genotype of the same parasite. This suggests that there may be different genotypes of the subspecies *B. canis vogeli*; however, a paucity of available sequences of isolates from other geographic locations has prevented assessment of the degree of diversity among this subspecies. Additional research is required to assess the extent of differences between the various isolates of *B. canis vogeli*.

Sequencing of the amplified region of the 18S rRNA gene also allowed for genetic confirmation of a small canine *Babesia* sp. recently reported in Australia by Muhlnickel et al. (2002). Based on the 18S rRNA gene, all 3 Victorian isolates were found to be identical to isolates of *B. gibsoni* from Oklahoma and North Carolina in the United States and from Okinawa in Japan. Zahler, Rinder, Zweygarth et al. (2000) have proposed that the isolates from Asia retain the name *B. gibsoni* on the basis of the description of these piroplasms in dogs in India by Patton (1910). It is implied that these Indian parasites are genetically

similar to those found throughout other regions of Asia. It can then be assumed that all small canine piroplasms that are genetically similar to the Asian genotypes also are allocated to the species *B. gibsoni*. This includes those from Oklahoma and North Carolina in the United States (Kjemtrup et al., 2000) and the Australian isolates described in this study. Further research is necessary to identify the genotype of isolates from other countries in order to allocate the correct name to each geographic isolate and help avoid further confusion.

Babesia gibsoni is a more pathogenic species than *B. canis vogeli* and is therefore of greater concern from a clinical perspective. Its discovery provides support for canine babesiosis being considered an emerging disease; however, this may have only been a result of increased awareness and better diagnostic techniques. The clinical signs and laboratory findings of *B. gibsoni* have a history of being inadvertently diagnosed as another form of anaemia such as idiopathic or immune-mediated haemolytic anaemia (Birkenheuer et al., 1999). Despite this it seems unlikely that this relatively virulent form of canine babesiosis would have remained undetected in Australia for a long period of time.

This is also the first report of the use of PCR to detect the presence of canine *Babesia* sp. DNA in *R. sanguineus* ticks. Molecular detection of pathogen DNA within ticks has been reviewed extensively by Sparagano et al. (1999); however, there are no reports of canine *Babesia* sp. DNA being extracted and amplified from ticks. The ticks used in this study were semiengorged, which made it difficult to assess whether they were themselves infected with *Babesia* parasites or whether the parasites were only present within the blood meal of the vector.

Ticks were not available from the dogs infected with *B. gibsoni* from Victoria, and it is therefore difficult to assess the vector responsible for transmission of these parasites in Australia. It has been reported that *B. gibsoni* can be transmitted by the ticks *Haemophysalis bipinosa*, *Haemophysalis longicornis*, and, possibly, *R. sanguineus* (Wozniak et al., 1997). Both *R. sanguineus* and *H. longicornis* are endemic in certain regions of Australia, thus increasing the transmission and distribution potential of *B. gibsoni*.

The use of PCR to detect the presence of canine *Babesia* sp. in Australia confirms that this technique is a highly sensitive and specific method of parasite detection. The advantage of using a genus-specific PCR is that multiple species, including novel genotypes, can be detected with the same set of primers. Although the PCR used in this study only detected *B. canis* and *B. gibsoni*, it has the potential to detect other *Babesia* species. Genetic sequencing can then be used to characterize each sample and can be further used to assess phylogenetic relationships between isolates. The PCR assay used in our study had the ability to detect a *Babesia* sp. infection with a blood parasitemia of 0.0000027%, which is comparable with previous reports (Fukumoto et al., 2001). It also exceeds the sensitivity of previously developed PCR assays for detection of *Babesia* species, which could amplify DNA from a sample with a parasitemia of 0.0001% (Ano et al., 2001) and 0.00008% (Roy et al., 2000). Despite a possible limitation in subclinical detection, PCR shows great promise as an effective means of detecting and characterizing canine *Babesia* sp. parasites and has the potential to be implemented in a clinical diagnostic laboratory situation.

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Cutaneous Trematode *Collyriclum faba* in Wild Birds in the Central European Carpathians

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ABSTRACT: The occurrence of cutaneous trematode *Collyriclum faba* in wild birds was monitored in the central European Carpathians from 1996 to 2001. A total of 5,414 birds, representing 86 species, was examined. *Collyriclum faba* was found at 7 sites (5 in Slovakia, 1 in Poland, and 1 in the Czech Republic), and prevalences at the sites varied from 1 to 16%. Ten species of passerine birds were infected: blackcap (*Sylvia atricapilla*) (16 positive/622 tested, 2% prevalence), black redstart (*Phoenicurus ochruros*) (2/25, 8%), chaffinch (*Fringilla coelebs*) (7/113, 6%), common blackbird (*Turdus merula*) (1/143, 1%), common redstart (*Phoenicurus phoenicurus*) (1/30, 3%), dipper