‘*Babesia gibsoni*’ of dogs from North America and Asia belong to different species

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SUMMARY

18S rDNA sequences from 4 isolates of *Babesia gibsoni* originating from Japan, Malaysia and Sri Lanka were compared with a previously published, 0·5 kb portion of the 18S rDNA from a *B. gibsoni* isolate from California, USA, and with the corresponding 18S rDNA sequences of other *Babesia* spp. Distance, parsimony and maximum likelihood analyses showed almost identical genotypes among the small canine *Babesia* from Asia, but an unexpectedly distant genetic relationship to that from the USA. While the American isolate segregated together with *B. equi*, the Asian isolates showed a close relationship to *B. divergens* and *B. odocoilei*. These results indicate that small *Babesia* of dogs originating from North America and Asia belong to different, genetically distantly related species.

Key words: *Babesia gibsoni*, dog, *Theileria*, genetic species differentation, 18S rDNA, new canine species.

INTRODUCTION

Small babesia infecting dogs are generally attributed to *Babesia gibsoni* mainly based on the assumption that no other small *Babesia* spp. parasitize dogs (Conrad *et al*. 1991; Casapulla *et al*. 1998). Small *Babesia* spp. cause diseases in dogs whose clinical manifestations are variable and mainly characterized by anaemia (Groves & Dennis, 1972), fever, lethargy, anorexy and splenomegaly (Yamane, Conrad & Gardner, 1993). These clinical signs are, however, not necessarily indicative of small *Babesia* but also occur with a large *Babesia* sp. of dogs, *B. canis* (Taboade & Merchant, 1991). The parasites generally considered as *B. gibsoni* are pleomorphic and appear most commonly as individual ring forms or piriform bodies of a size between 1 and 2·5 μm (Conrad *et al*. 1991). However, within the group of small *Babesia*, different species are often similar in morphology. For example, *B. gibsoni* has a strong similarity to *B. equi* (Purnell, 1981), a horse parasite which has recently been redescribed as *Theileria equi* (Mehlhorn & Schein, 1998), and cannot be reliably differentiated from *B. microti* based on Giemsa-stained blood smears (Conrad *et al*. 1992).

Serological tests, for example the commonly used immunofluorescence antibody test, do not distinguish between species of *Babesia* in dogs, because considerable cross-reactions occur between *Babesia* spp. (Yamane *et al*. 1994). It is therefore not known whether all small *Babesia* of the dog indeed belong to the same species. The existence of more than one small canine *Babesia* sp. is not unlikely. Following the first description of *B. gibsoni* in India (Patton, 1910), small *Babesia* were found in dogs in many countries in Asia as well as in America, Africa and Europe (Yamane *et al*. 1993). Finally, heterogeneity of small canine *Babesia* may also be inferred from inconsistent results of therapy studies (Yamane *et al*. 1993).

In order to investigate the possibility of more than one species of small *Babesia* in dogs, a molecular biological approach was applied to compare small *Babesia* of dogs originating from different Asian countries and the USA by sequence analysis of the nuclear small subunit 18S rRNA gene.

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MATERIALS AND METHODS

Babesia *isolates*

B. gibsoni isolates originating from different geo-

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graphical locations were used. Isolate 'Japan 1' was obtained from a naturally infected dog from Osaka, Japan in 1997. Isolate 'Japan 2' originated from a naturally infected dog in Nagasaki, Japan, in 1973 and was maintained in dogs since then. Isolate 'Malaysia' originated from a naturally infected dog in Malaysia. Isolate 'Sri Lanka' was taken from a dog living in Germany which had acquired the infection in Sri Lanka.

DNA isolation, amplification, cloning and sequencing

Using the QIAamp blood kit (Qiagen, Hilden, Germany) 200 µl aliquots of EDTA blood were processed according to the manufacturer's instructions. The upstream primer RIB-19 (CGG GAT CCA ACC TGG TTG ATC CTG C) was modified from the universal 'primer A' (Sogin, 1990) by reducing its annealing portion to match the anticipated melting temperature of RIB-20 and by changing the linker sequence. It targets a portion near the 5′ end of the 18S rDNA conserved between eukaryotes (Dams *et al*. 1988). The downstream primer RIB-20 (CCG AAT TCC TTG TTA CGA CTT CTC) at the 5′ end of the 18S was constructed to match a region conserved between *B. canis* (GenBank accession number L19079), *B. equi* (Z15105), *B. bigemina* (X59607), *B. caballi* (Z15104), *B. odocoilei* (U16369), *B. divergens* (U07885), *B. microti* (U09833), *B. rodhaini* (M87565) and *B. bovis* (L31922) as well as *Theileria parva* (AF013418), *T. sergenti* (AB016074) and *T. buffeli* (AB000272). Care was taken that the 3′ end, which is critical for the specificity of the primer (Sommer & Tautz, 1989), did not match mammalian DNA (human, K03432 and mouse, J00623). PCR amplifications were done using a 'hot start' technique in which 5 µl of the DNA solutions, 39·2 µl of H_2O, 1·8 µl of 50 mM $MgCl_2$ and 1 µl each for both primers (50 µM each) were overlain with 2 drops of mineral oil and denatured at 96 °C for 2 min. After cooling to 85 °C, a mixture of 5 µl of buffer (100 mM Tris–HCl, pH 8·3, 500 mM KCl), 0·5 µl of dNTP mix and 0·5 µl (5 U/µl) *Taq* DNA polymerase was added. The best signal to backround ratio was observed after 30 cycles with denaturation at 92 °C for 60 s, annealing at 54 °C for 60 s and extension at 72 °C for 90 s. Products were visualized in a 1·3% agarose gel containing 0·2 µg/ml ethidium bromide. PCR products of 1·7 kb were excised from the agarose gel and the DNA purified by silica gel adsorption (Geneclean; Bio 101, La Jolla, CA, USA). Using the *Bam*HI site of the primer RIB-19 and the *Eco*RI site of the primer RIB-20, the products were ligated into *Bam*HI- and *Eco*RI-cut pBluescript II SK(−) vectors (Stratagene, La Jolla, CA, USA). Clones were generated by transforming XL1-Blue cells (Stratagene) by electroporation and sequenced using a Sequenase 2.0 kit (United States Biochemicals Corp., Cleveland, OH, USA). For the 1·7 kb product, 2 external sequencing primers T3 and KS (Stratagene) positioned at the vector as well as 5 internal primers for the coding strand (RIB-24: GAC GGT AGG GTA TTG GC, RIB-26: TTG GAG GGC AAG TCT GG, RIB-22: ATG GTT AAT AGG AAC GG, RIB-28: AGG AAT TGA CGG AAG GG, and RIB-30: AGG CAA TAA CAG GTC TG) and 5 primers for the non-coding strand (RIB-23: TCT CCG GAA TCG AAC CC, RIB-25: TAC GCT ATT GGA GCT GG, RIB-27: CCT AAC TTT CGT TCT TG, RIB-21: TTT CCC CGT GTT GAG TC, and RIB-29: GCA TCA GTG TAG CGC GC) were used to obtain overlapping reads.

Sequence analysis

18S sequence alignments were obtained using the programs NALIGN and CLUSTAL in the PC/GENE software package (Intelligenetics, Mountain View, CA, USA) employing methods developed by Myers & Miller (1988) and Higgins & Sharp (1988). Computation parameters were set to an open gap cost of 10 and a unit gap cost of 10 for NALIGN and a K-tuple of 5, a gap penalty of 5, a window size of 10, and a filtering level of 2·5 for CLUSTAL. The alignments were readjusted manually. Likewise, consensus sequences were constructed manually by comparing aligned sequences. At least 2 clones from independent PCR amplifications were used to determine the individual isolate consensus sequences. All discrepant positions were resolved either by identity with all other isolates investigated (conserved position), or, in the case of a non-conserved position, by a third, independently generated clone. Mutations at a given position found in a single clone of a single isolate only were classified as polymerase errors or, indistinguishable from them, as rare genotypes, and were not included in the consensus sequence. Identities of consensus sequences were calculated from mutation rates by counting substitutions, deletions and insertions as one mutation each and dividing the sum by the average length of those sequences.

Phylogenetic analyses were based on a 518 bp portion of the 5′ end of the 18S rDNA for which the sequence of an American *B. gibsoni* isolate has been published in GenBank (accession number L13729) because no American isolate was available. This isolate, designated *B. gibsoni* '2Bg' (Thomford *et al*. 1994), originated from a naturally infected dog in Los Angeles County, and was subsequently passed through a splenectomized experimental dog. To determine the genetic relationship to other *Babesia* and *Theileria* spp. the corresponding 18S sequences of *B. canis* (GenBank accession number L19079), *B. equi* (Z15105), *B. bigemina* (X59607), *B. caballi* (Z15104), *B. odocoilei* (U16369), *B. divergens*

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(U07885), *B. microti* (U09833), *B. rodhaini* (M87565), *T. parva* (AF013418), *T. sergenti* (AB016074), and *Plasmodium falciparum* (M19172) were included in the alignment which may be obtained on request from the corresponding author. Phylogenetic trees were inferred using the PHYLIP phylogeny package version 3.5c (Felsenstein, 1993) employing distance matrix, maximum parsimony and maximum likelihood methods. For distance analysis, a neighbour joining tree was generated from a Kimura 2-parameter distance matrix with the algorithms DNADIST and NEIGHBOR. Maximum parsimony analysis was performed using the DNAPARS algorithm with gaps counted as one event each. Support for phylogenies derived from distance and parsimony algorithms was measured by bootstrapping over 1000 replicates with the programmes SEQBOOT and CONSENSE. The maximum likelihood analysis was done with the programme DNAML and the tree with the lowest natural logarithm (ln) likelihood score was chosen. Trees were drawn with the programme DRAWGRAM from the same PHYLIP package and using *Plasmodium falciparum* as the outgroup.

RESULTS

Sequencing of 1646 bp PCR-amplified 18S rDNA between the 3′ ends of primers RIB-19 and RIB-20 demonstrated completely identical genotypes from the 2 Japanese isolates, 'Japan 1' and 'Japan 2', which were thereafter named 'Asia 1'. Additionally, the isolates 'Malaysia' and 'Sri Lanka' also had identical sequences and were named genotype 'Asia 2'. Between these 2 genotypes there was a difference of 1 mutation at position 101, corresponding to an identity of 99·9 %. The sequences of both genotypes have been submitted to GenBank (AF175300 and AF175301). With regard to the 0·5 kb portion of the 5′ end of the 18S rDNA , the identity between the 2 Asian genotypes was 99·2 % (1 mutation). This region of the Asian isolates was most similar to *B. canis* and *B. divergens* with a sequence identity of 97·2 % between 'Asia 1' and *B. canis* and between 'Asia 1' and *B. divergens*, each. In contrast, the identities between the Asian genotypes and the 'USA' genotype were distinctly less; 88·2 % between 'Asia 1' and 'USA' and 88·0 % between 'Asia 2' and 'USA'. The sequence of the 'USA' isolate was most similar to the sequence of *B. equi*, with an identity of 92·7 %, and reached identities of 91 % and 90·5 %, respectively, with *B. rodhaini* and *B. microti*.

Phlyogenetic analyses using distance (neighbour joining), parsimony and maximum likelihood methods demonstrated a segregation of the Asian and the American small *Babesia* of the dog in different clusters (Fig. 1). These analyses unanimously resulted in 2 groups. One of them included the isolate 'USA' together with *B. equi*, *B. rodhaini*, *B. microti*, *T. parva* and *T. sergenti*, while the other contained 'Asia 1', 'Asia 2', and the remaining *Babesia* spp. These 2 groups were strongly supported by high bootstrap values of 100 % in distance and parsimony analysis. For maximum likelihood the natural logarithm (ln) likelihood score was −2326.

DISCUSSION

Traditionally, species diagnosis in *Babesia* is based on morphology and an assumed host specificity. In the case of *B. gibsoni*, it has further been assumed that dogs are parasitized by only 1 small *Babesia* sp., called *B. gibsoni*. Whether this assumption is correct, cannot be answered by the diagnostic procedures used to date, including light microscopy and serology, since these tests lack specificity. Molecular biological methods using genotypic characters might therefore be a promising approach, because they introduce additional data to this discussion. In this context, rRNA genes have repeatedly been proven useful to clarify phylogenetic relationships. Nuclear small subunit 18S rDNA sequences have already been successfully used to investigate relationships between *Babesia* spp. (Conrad *et al*. 1992; Ellis *et al*. 1992; Allsopp *et al*. 1994; Mackenstedt *et al*. 1994; Thomford *et al*. 1994; Persing & Conrad, 1995; Armstrong *et al*. 1998).

The analysis of the 5′ 18S rDNA region used in this study and comparison with other *Babesia* spp. clearly showed that the genotypes of the Asian and the Californian isolates are distantly related. The Californian isolate had a much closer relationship to the *Theileria*-like species *B. equi*, which has recently been redescribed as *T. equi* (Mehlhorn & Schein, 1998) and grouped together with *T. parva* and *T. sergenti* while the Asian isolates were similar to the small species of the 'true' *Babesia* type, *B. divergens* and *B. odocoilei*, and to the large *Babesia* spp. *B. canis*, *B. caballi* and *B. bigemina*. Therefore it seems that the American parasites should be attributed to the genus *Theileria* while the Asian piroplasms are clearly *Babesia sensu stricto*. This result was confirmed with distance and parsimony, as well as with maximum likelihood algorithms. Furthermore, the comparison of the sequences of the 4 Asian isolates of different geographical origins (Japan, Malaysia and Sri Lanka) and from different years of natural infection (Japan 2 from 1973, Japan 1 from 1997) demonstrated only minimal intraspecific variation. Only 1 mutation was found within the entire 1646 bp 18S sequence (99·8 % identity regarding the 0·5 kb 5′ 18S rDNA region). This low intra-Asian variation, which contrasts to the clear distinctness to the American genotype (88·2 % identity in the 0·5 kb 5′ 18S rDNA) is further strongly indicative for the existence of different species.

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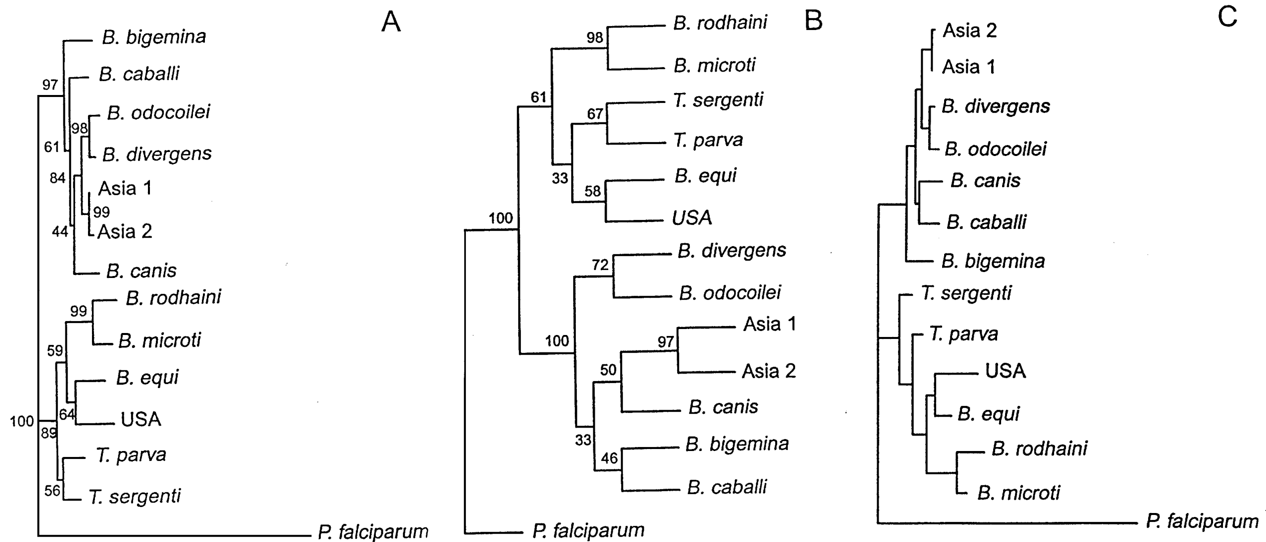

Fig. 1. Phylogenetic trees based on comparisons of 0·5 kb 5′ 18S rDNA sequence fragments of small *Babesia* from dogs (Asia 1, Asia 2, USA) with the corresponding genes from other *Babesia* and *Theileria* spp. The dendrograms were constructed with distance (A), maximum parsimony (B) and maximum likelihood methods (C) and *Plasmodium falciparum* as the outgroup. For distance and parsimony, the reliability of nodes is given as bootstrap values (percentages) for each node.

In light of this pronounced genetic distinctness, differences in phenotypic characters should be expected. However, morphologically, the small *Babesia* spp. of the dog are pleomorphic, but this is also true with respect to other small *Babesia* from other hosts. Intraerythrocytic quadruplet or 'Maltese cross' forms have been described as 'characteristic' for the American isolates (Conrad *et al*. 1991, 1992) and are also characteristic of *B. equi*, *B. microti*, *B. rodhaini* and *Theileria* spp. In contrast, the descriptions of these forms are notably missing from descriptions of Asian isolates (Patton, 1910; Rao, 1927; Groves & Dennis, 1972; Itoh, Higuchi & Kawamura, 1988; Namikawa, Sunaga & Kanno, 1988). Nor are they found in *B. canis*, *B. caballi*, or *B. bigemina*, again in accordance with the genotypic groupings. Whether this feature represents a taxonomic character, has still to be proven. For example in a small bovine *Babesia* sp., *B. divergens*, 2 merozoites are found in cattle erythrocytes, but 4 merozoites occur in erythrocytes of gerbils (*Meriones unguiculatus*) after division of the parasites (Böse, Schelp & Friedhoff, 1994).

Differences in pathogenesis and in susceptibility to chemotherapy might also be expected, but cannot be deduced from the literature. Finally, since development in lymphocytes has been shown for *B. equi* (Schein *et al*. 1981), such development should also be looked for in American isolates of small *Babesia* from dogs.

With regard to the distant relationship between the Asian and the Californian isolates of small canine piroplasms, it has to be concluded that they belong to different species. The Asian parasites clearly belong to the genus *Babesia*. Because the name *gibsoni* was first used for an Indian isolate by Patton (1910) it has priority for the Asian parasites. In contrast, the Californian parasites should be attributed to the genus *Theileria*. In case the identity of these parasites with an already existing species from other hosts cannot be demonstrated, a new species name is necessary.

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