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Source: Journal of Wildlife Diseases, 44(1) : 168-171

Published By: Wildlife Disease Association

URL: <https://doi.org/10.7589/0090-3558-44.1.168>

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Morphology and Genetics of a *Babesia* Isolate from *Capreolus capreolus*

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ABSTRACT: A *Babesia* isolate that was morphologically distinct from *Babesia capreoli* and very similar to *B. divergens* was found in the blood of a roe deer (*Capreolus capreolus*) found dead in central Italy. Sequences corresponding to the full coding region of the 18S ribosomal RNA (rRNA) gene were identical to a sequence reported for *Babesia divergens* from a reindeer (*Rangifer tarandus*) and 99.9% and 99.8% similar to those reported for *B. capreoli* and bovine origin *B. divergens*, respectively.

Key words: *Babesia capreoli*, *Babesia divergens*, *Capreolus capreolus*, Italy, molecular diagnostics, morphology, phylogeny.

Babesiosis is an emerging tick-borne disease caused by intraerythrocytic parasites of the genus *Babesia*. These protozoa affect domestic and wild animals, and some species can be transmitted to humans by ticks that have previously fed on these animals. Zoonotic species include *Babesia microti* (now belonging to the genus *Theileria*, as reported by Uilenberg, 2006) in North America and *Babesia divergens* in Europe (Zintl et al., 2003). Subclinical persistent infections of *B. microti* infections may also occur in Europe (Gray, 2006). *Babesia microti* normally infects rodents, while *B. divergens* is associated with bovines. Human cases also have been attributed to a newly recognized species, *Babesia duncani* (Conrad et al., 2006); to a *B. divergens*-like parasite in America; and to *Babesia* EU1 (closely related to *B. divergens*) and another *B. divergens*-like parasite in Europe (Centeno-Lima et al., 2003; Herwaldt et al., 2003; Gray, 2006).

Babesiosis can be an important cattle disease, and wildlife can represent a source of infection. Roe deer (*Capreolus capreo-*

lus) have been found to be infected with *Babesia capreoli* (Enigk and Friedhoff, 1962), *B. EU1* (Herwaldt et al., 2003), and other *Babesia* that are not well characterized. These include a species reported as *B. capreoli* but described only in GenBank® (Slemenda et al., in Gray, 2006), a species reported as nearly identical to *B. divergens* (Duh et al., 2005), and in Italy (Emilia-Romagna region), an unidentified species that morphologically overlaps with *B. capreoli* (Galuppi et al., 2005). To date, human Babesiosis in Italy has only been attributed to *B. EU1* (Herwaldt et al., 2003).

In the present study, we report the presence of a *B. capreoli/divergens*-like parasite in a roe deer from central Italy (Lazio region, Rieti Province) and assess the phylogenetic relationships of this isolate with species previously reported in this host or documented in Europe.

A young male roe deer, which was killed by a vehicle in the Lazio region, Rieti Province (42°31'N, 13°5'E) of central Italy, was delivered to the Istituto Zooprofilattico for examination during October 2005. During necropsy, blood was taken from the heart, and thin and thick blood smears were prepared. Blood smears were stained (10% Giemsa) and examined (40× and 100×) for blood parasites. Several coagula were stored at –20 C for molecular analyses.

Babesiosis was diagnosed on blood smears by noting parasitic inclusions in the erythrocytes; approximately 18% of erythrocytes were parasitized. Smears contained ring-shaped (diameter: 0.75–1.30 µm) and pyriform (0.9–1.4 × 0.4–

	111111	1111111111	1111111
	112223566	6666666777	7899000011 1111334566 6666677
	2381678702	46667779012	2259027711 2244094603 3334600
	5039660333	2269787040	7510976889 0103119532 3560406
<i>Babesia</i> roe deer	CTTTC TACTC	GGTTTTGTC	TGATCTTAAG CCAACTTTCT TTTATAG
<i>B.d.</i> reindeer Scotland
<i>B. capreoli</i> roe deer FranceT.....
<i>B.d.</i> bovine Ireland	A...A.....T.....C..
<i>B.d.</i> roe deer SloveniaG...	A.....TCC.....
<i>B.d.</i> gerbil Germany	T.....	A...A.....CA TG..T.....C..
<i>B.d.</i> humans PortugalC.....	.T..A..T..	..T.TCCG.. ..GT...T.GA
<i>B.d.</i> ? N. Ireland	.CGGG..TGG	A.GGAGA.CG	G..A..... .C.T..G.A AAAGC..

FIGURE 1. Variable sites of the 18S ribosomal RNA (rRNA) gene from the roe deer isolated in this study, the European *Babesia divergens* (B.d.), and *B. capreoli* sequences deposited in GenBank®.

0.7 μm) protozoa; observed parasites had a dark-stained nucleus and a lightly stained cytoplasm with vacuoles in the center. Pear-shaped trophozoites occurred either singly or in pairs assembled at their pointed extremities. Several parasites were located at the periphery of the erythrocyte. Morphologic features were very similar to *B. divergens*, which in the natural host is reported as pyriform and ring-shaped stages sized $1.5\text{--}1.9 \times 0.4\text{--}1 \mu\text{m}$ and $1.48\text{--}1.8 \mu\text{m}$, respectively, and in unusual hosts, $1.9\text{--}2.6 \times 0.8\text{--}1.18 \mu\text{m}$ and $1\text{--}2 \mu\text{m}$, respectively (Zintl et al., 2003).

The DNA was extracted from coagula, and the complete 18S ribosomal RNA (rRNA) gene (approximately 1,700 base pairs [bp]) was amplified by PCR with the specific primers CRYPTO F (5'-AACCTGGTTGATCCTGCCAGT-3') and CRYPTO R (5'-GCTTGATCCTTCTGCAGGTTACCTAC-3') (Herwaldt et al., 2003). Amplicons were purified and sequenced. Sequences were assembled and subjected to a GenBank® Blast homology search (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov).

nlm.nih.gov). The PCR amplification yielded a specific product of 1,724 base pairs. Sequence analysis and Blast search indicated an identity of 100% with the strain isolated in a reindeer from Scotland (*Rangifer tarandus*) and ascribed to *B. divergens* (Langton et al., 2003), 99.9% with *B. capreoli* found in roe deer in France, and 99.8% with a bovine *B. divergens* isolate from Ireland. Differences were detected at only four positions (Fig. 1). Sequence comparison with other European isolates also indicated high homology (99.7%) to a *Babesia* isolate from roe deer from Slovenia, whereas the other *B. divergens* isolates from Portugal (humans), Germany (gerbil), and Northern Ireland (unknown host) differed from 9 to 26 bp.

The complete sequences of the 18S rRNA genes for the bovine *B. divergens* for *B. divergens*-like sequences reported for European cervids, humans, and gerbils, for *B. EU1* reported in humans in Italy and Austria, for *B. capreoli*, and for additional *Babesia* species that were retrieved from the GenBank® database (see Fig. 2 for the accession numbers) were

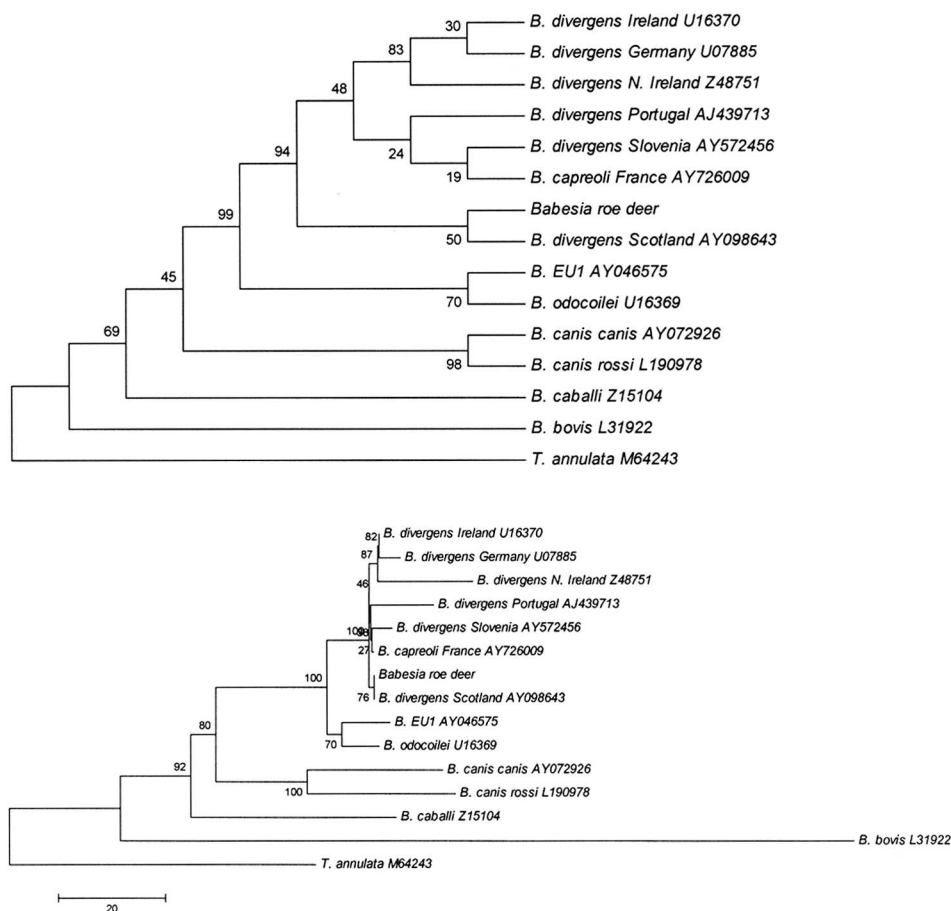


FIGURE 2. Phylogenetic relationships using maximum parsimony (top) and neighbor-joining (bottom) methods between the Italian *Babesia* isolate from roe deer and other related *Babesia* species, based on aligned 1,722 nucleotides of the 18S rRNA gene. Numbers at nodes indicate the bootstrap values; the scale bar represents the number of different nucleotides.

aligned with our roe deer isolate by using the program CLUSTAL W. The 18S rRNA sequence for *Theileria annulata* was included as an outgroup. Phylogenetic analysis was performed using the program MEGA version 3.1, and phylogenetic trees were constructed using parsimony and neighbor-joining methods. The robustness of the trees was evaluated by 3,000 bootstrap replications. The topologies (Fig. 2) revealed total homology with *B. divergens* found in Scotland, and a very close relationship with *B. capreoli*, which clusters together with other *B. divergens* described in Europe (association strongly supported by high bootstrap values), and

a lower affinity to species found in Italy (*B. EU1*) and in the USA (*B. odocoilei*), and those reported from humans.

Studies on Italian populations of *C. capreolus*, to date, have focused mainly on ectoparasites, intestinal and tissue nematodes, and, only recently, on blood parasites (Galuppi et al., 2005). The presence of *Babesia*-like parasites has been described, but the lack of genetic characterization precludes comparisons with *Babesia* detected in these studies.

The parasite that we found in the roe deer in this study was not morphologically consistent with *B. capreoli*. Instead, the morphology of the parasite was similar to

bovine *B. divergens* M'Fadyean and Stockman (1911). Morphologic variation can be dependent on host, which for *B. divergens* is atypical in this case (Canning et al., 1976; Gray et al., 1991).

In support of the morphologic data, the 18S rRNA gene sequence from the roe deer parasite was identical to the *B. divergens*-like isolate from reindeer in Scotland. This isolate also was reported as morphologically indistinguishable from *B. divergens* (Langton et al., 2003). It is possible that our roe deer and reindeer *Babesia* are closely related to each other but are not *B. divergens*. It also is possible that the inclusion of GenBank sequences attributed to *B. capreoli* in our genetic analysis masked the genetic relatedness of these *Babesia* with *B. divergens*. In either case, in the absence of confirmation based on additional parasitized animals and the absence of biologic data on the infectivity of this parasite for gerbils or cattle, we cannot definitively identify this *Babesia* and prefer to refer to it as *B. capreoli*/*divergens*-like. Additional work is needed to better identify *Babesia* spp. present and to understand the importance of roe deer as hosts for these parasites in Italy.

The authors are grateful to P. Mollo for her excellent technical assistance and to E. Bighi for reviewing the English. This study was partially supported by grants from the Ministry of University and Researches (PRIN 2004) and Italian Ministry of Health (Ricerca corrente, 2005).

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Received for publication 12 December 2006.