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PRESENCE OF POTENTIALLY PATHOGENIC *BABESIA* SP. FOR HUMAN IN *IXODES RICINUS* IN SWITZERLAND

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Abstract: We have designed and performed a new PCR method based on the 18S rRNA in order to individuate the presence and the identity of *Babesia* parasites. Out of 1159 *Ixodes ricinus* (Acari: Ixodidae) ticks collected in four areas of Switzerland, nine were found to contain *Babesia* DNA. Sequencing of the short amplicon obtained (411-452 bp) allowed the identification of three human pathogenic species: *Babesia microti*, *B. divergens*, for the first time in Switzerland, *Babesia* sp. EU1. We also report coinfections with *B.* sp. EU1-*Borrelia burgdorferi* sensu stricto and *Babesia* sp. EU1-*B. afzelii*.

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INTRODUCTION

The tick *Ixodes ricinus* may carry various pathogens with the potential of generating serious human and animal diseases such as borreliosis, babesiosis and tick-borne encephalitis. Babesiosis is caused by the infection of erythrocytes by a protozoan belonging to the genus *Babesia*. The symptoms are broad, ranging from an apparently silent infection to a fulminant malaria-like disease that can result occasionally in human death [15]. The *Babesia* species represent one of the major tick-borne pathogens that infect human and animal red blood cells all over the world [16].

In North America, the predominant etiological agent of human babesiosis is *Babesia microti*, a parasite of small mammals. In addition, a *Babesia* species, designated WA1, was also shown to cause disease in humans [17]. *B. microti* is also present in Europe [6, 8, 22], where a few human cases, not well documented, have been reported [12]. Recently, Meer-Scherrer *et al.* [20] described the first *B. microti* infection in a Swiss patient.

Since the late 1950s, European human infection have been attributed to *B. divergens*, usually a cattle parasite [12, 24]. However, in the absence of a molecular-based confirmation, the identification of the species causing human cases is uncertain. A new candidate species pathogenic in humans, designated EU1 (*Babesia* sp. EU1), has been recently documented [14]. Phylogenetic analysis has shown that EU1 and *B. odocoilei* form a sister group to *B. divergens*. The serological assays presently available are not able to discriminate between EU1 and *B. divergens* infections [14], hence all the cases reported to date have been ascribed to *B. divergens*. European human babesiosis is infrequent and concerns mostly splenectomised patients. Infections with *B. divergens* are likely to be more severe than those caused by *B. microti* [12].

Since 1997, DNA sequences from 2 cases of human babesiosis due to *B. divergens* are available on GenBank. Both concerned splenectomised patients: 1 was related to a fatal case in a 66-year-old patient in Portugal [5], the other to a 34-year-old patient in the Canary Islands [21].

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Molecular analysis have confirmed that the protozoans involved are *B. divergens*.

Other cases of human babesiosis have been described by molecular techniques in Italy and in Austria [14]. Herwaldt *et al.* [14] documented how 2 asplenic patients were not infected by *B. divergens*, but by the new candidate organism EU1. In addition, in Slovenia the presence of a *Babesia* sp. in *I. ricinus* ticks has also been reported [6]. The partial sequence of the gene 18S rRNA has been shown to be identical to the corresponding gene of EU1. In conclusion, these fragmentary pieces of information do not allow us to have a representative picture of the distribution in Europe of the different *Babesia* sp.

The aim of this study is to determine the prevalence of *Babesia* sp. in *I. ricinus* in Switzerland. For this purpose, we designed and performed a new PCR method, which we applied to more than 1,000 *I. ricinus* ticks collected from 4 different regions of the country.

MATERIALS AND METHODS

Tick sampling. In spring and autumn of the years 2002 and 2003, *I. ricinus* ticks were collected in 4 regions of Switzerland (Neuchâtel, Ticino, Zürich and Valais). In Canton Neuchâtel and in Canton Zürich, an area of 1049 m^2 and a more extended surface were flagged, respectively. In Canton Valais, 3 areas were chosen as sampling locations: Mt. d'Orge (Sion), Gueroz (Val Trient) and Finges. In Ticino (South Switzerland), ticks were collected from the animal hosts (dogs, cows, cats, goats and asses) and humans. Ticks were immediately introduced in 100% ethanol and conserved at 4ºC until taxonomic identification (based on morphological characteristics).

DNA extraction of the collected ticks. Total DNA was extracted from each tick by using a Dneasy Tissue kit (Qiagen AG, Basel, Switzerland) according to the manufacturer's instructions. Each sample was minced with a disposable sterile scalpel within a microtube. After digestion with proteinase K (20 µg/ml) samples were applied to the columns for absorption and washing of DNA. Finally, DNA was eluted in 200 µl of buffer available from the kit and stored at 4ºC. In order to show that negative results were not due to inhibition of the PCR reaction, the efficiency of the DNA extraction was validated on 100 negatively samples amplifying a 338–339 bp DNA fragment corresponding to the 12S rDNA gene of the ticks [2].

DNA extraction of blood samples. The DNAs of the *Babesia* strains used to validate the method were extracted from blood samples of infected animals (Tab. 1), provided by the Institute of Parasitology in Bern, with the QIAmp DNA Blood Mini kit (Qiagen AG, Basel, Switzerland) according to the manufacturer's instructions.

Table 1. Blood samples[a] containing different *Babesia* strains.

Strains	Source	Geographic origin	GenBank accession No.
B. equi	Horse blood	Switzerland (Bern)	AY648885
B. canis	Dog blood	Switzerland	AY648872
B. divergens	Cattle blood	Austria	AY648871
B. major	Cattle blood	Austria	AY648886
B. bigemina	Cattle blood	Austria	AY648884
B. canis canis	Dog blood	Switzerland (Aargau)	AY648874
B. divergens	Cattle blood smear	Switzerland (Fribourg)	AY648873

[a]The samples were provided by the Parasitology Institute of the University of Bern, Switzerland.

Table 2. *Babesia* sequences downloaded from GenBank and used as references in the phylogenetic tree of Figure 2.

Reference strains	Source	GenBank accession number
Babesia sp. EU1	GenBank	AY046575[a]
B. divergens	GenBank	AJ439713[a]
B. odocolei	GenBank	U16369[a]
B. canis	GenBank	AY072926
B. caballi	GenBank	AY309955[a]
B. bigemina	GenBank	X59604[a]
B. rodhaini	GenBank	M87565[a]
B. microti (North America)	GenBank	AF231348[a]
B. microti (Switzerland)	GenBank	AY144692
B. equi	GenBank	AY150064[a]
B. canis canis	GenBank	AY527063/AY072926[a]
B. canis vogeli	GenBank	AY150061/AY072925[a]

[a] complete sequence of the 18S rRNA gene (1'700 bp) available in GenBank

Detection of *Babesia* DNA by PCR. The PCR reactions for the detection of *Babesia* from blood samples and *Ixodes* ticks were performed with 5 µl of the extracted DNAs as template, 0.5 µM of each primer (see below), 1 Unit Taq Polymerase (Qiagen AG, Basel, Switzerland) in a total volume of 50 µl (buffer provided by the manufacturer). The reaction mixtures were subjected to an initial denaturation step of 10 min at 94ºC, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min, and elongation at 72ºC for 2 min. Amplification was completed by a further 5 min step at 72ºC. The reactions were performed in a DNA Thermal Cycler (Perkin-Elmer Applied Biosystems, Rotkreuz, Switzerland). We designed the primers used both for PCR amplification and sequencing on the basis of the common sequence of the 18S rRNA gene of the genus *Babesia* (primer BJ1: 5'-GTC TTG TAA TTG GAA TGA TGG-3';

primer BN2: 5'-TAG TTT ATG GTT AGG ACT ACG-3'). According to the sequence of the 18S rRNA gene of *B. divergens* AJ439713 (Tab. 2), the position of the amplified fragment is 488-912.

Sequencing of PCR products. PCR products (411-452 bp) were purified by using an Amicon Microcon Millipore Kit (Millipore, Milano, Italy), eluted in 20 µl of buffer and stored at 4ºC. Cycle sequencing reactions were performed in total volumes of 15 µl using an ABI Prism Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer Applied Biosystems, Rotkreuz, Switzerland) on an ABI Prism 310 GENETIC Analyser (Perkin-Elmer Applied Biosystems), according to the manufacturer's instructions. DNA sequencing was performed in both directions.

Limit of detection. To assess the analytical sensitivity of the PCR assays for the multicopy target 18S rRNA gene, the DNA concentration of an extract originating from a *B. divergens* strain from Austria (Tab. 1) was determined by fluorimetry (Fluorimeter TD-700, Witec AG, Switzerland). The extract was then serially diluted 10-fold in sterile water. Five µl of each dilution were used as template for the PCR reaction.

DNA sequence analysis. To verify the taxonomic status of *Babesia* sp. detected in our collection of ticks, we performed a phylogenetic analysis with the amplicons obtained from the positive samples and the sequences downloaded from GenBank (Tab. 2). The 18S rRNA sequences were handled and stored with the Lasergene program Editseq (DNAstar Inc., Madison, WI USA) and aligned with Megalign (DNAstar Inc.). Phylogenetic analyses of the 18S rRNA sequences were performed by the neighbour-joining (NJ) method, with Kimura-2-parameters distances (performed using MEGA Molecular Evolutionary Genetics Analysis version 2.1, [19]). The reliability of internal branches was assessed by boots-trapping with 1,000 (NJ) pseudoreplicates [7].

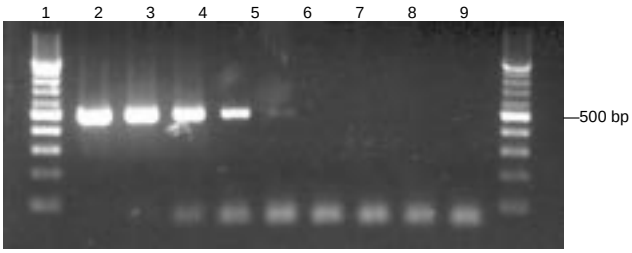

Figure 1. PCR assay on a serial dilution of purified genomic DNA from *Babesia divergens* (10-fold dilutions starting from 55 ng/ml in lane 1). The 425 bp amplicon has a detection limit of 550 fg/ml which corresponds to 27.5 fg/reaction (lane 5). The bands at the bottom are due to residual primers from the reaction. DNA size markers, 100 bp ladder, have been loaded on each side.

Nucleotide sequence accession number. The 18S rRNA sequences of *Babesia* used to validate the method and the *Babesia* detected in the *I. ricinus* ticks have been deposited in GenBank: the accession numbers are in Table 1 and Figure 2, respectively.

RESULTS

PCR of the collected ticks. A total of 1'159 ticks (294 from Ticino, 294 from Neuchâtel, 285 from Zürich and 286 from Valais) were tested for the presence of *Babesia* by amplification of the 18S rRNA gene (Tab. 3). A total of 9 samples (0.8%) (5 from Ticino, 2 from Neuchâtel and 2 from Zürich) showed single bands of 411-452 bp, which were confirmed to be *Babesia* DNA by sequencing (Fig. 2). The five animals (2 dogs, 1 cat and 2 cows) carrying the infected ticks in Ticino did not show any clinical signs of *Babesia* infection, such as fever, anorexia, anaemia, or jaundice. The peripheral blood of these animals could not be analysed in this study.

Table 3. *Babesia* found in *I. ricinus* ticks collected in four regions of Switzerland.

Areas	Total number of ticks examined	Collected from	Infection rate[a] (%)			*Babesia* sp. detected by PCR (stage of the infected tick/Accession No.)		
			Total	Nymph stage	Adult stage	*Babesia* sp. EU1	*B. divergens*	*B. microti*
Ticino	294 (1L[b], 9N, 284A)	Animals	5/294 (1.7)	1/9 (11.1)	4/284 (1.4)	Sample 3 (F/AY648881) Sample 4 (F/AY648879) Sample 5 (F/AY648880)	Sample 6 (F/AY648875) Sample 7 (N/AY648876)	–
Neuchâtel	294 (155N, 139A)	Vegetation	2/294 (0.7)	2/155 (1.3)	0/139 (0)	Sample 1 (N/AY648877) Sample 2 (N/AY648878)	–	–
Valais	286 (82N, 204A)	Vegetation	0/286 (0)	0/82 (0)	0/204 (0)	–	–	–
Zürich	285 (246N, 39A)	Vegetation	2/285 (0.7)	2/246 (0.8)	0/39 (0)	–	–	Sample 8 (N/AY648882) Sample 9 (N/AY648883)

A, Adults; N, Nymphs; L, Larvae and F, Adult Female; [a]Number of ticks infected/number of ticks examined (%); [b]tested negative for *Babesia*.

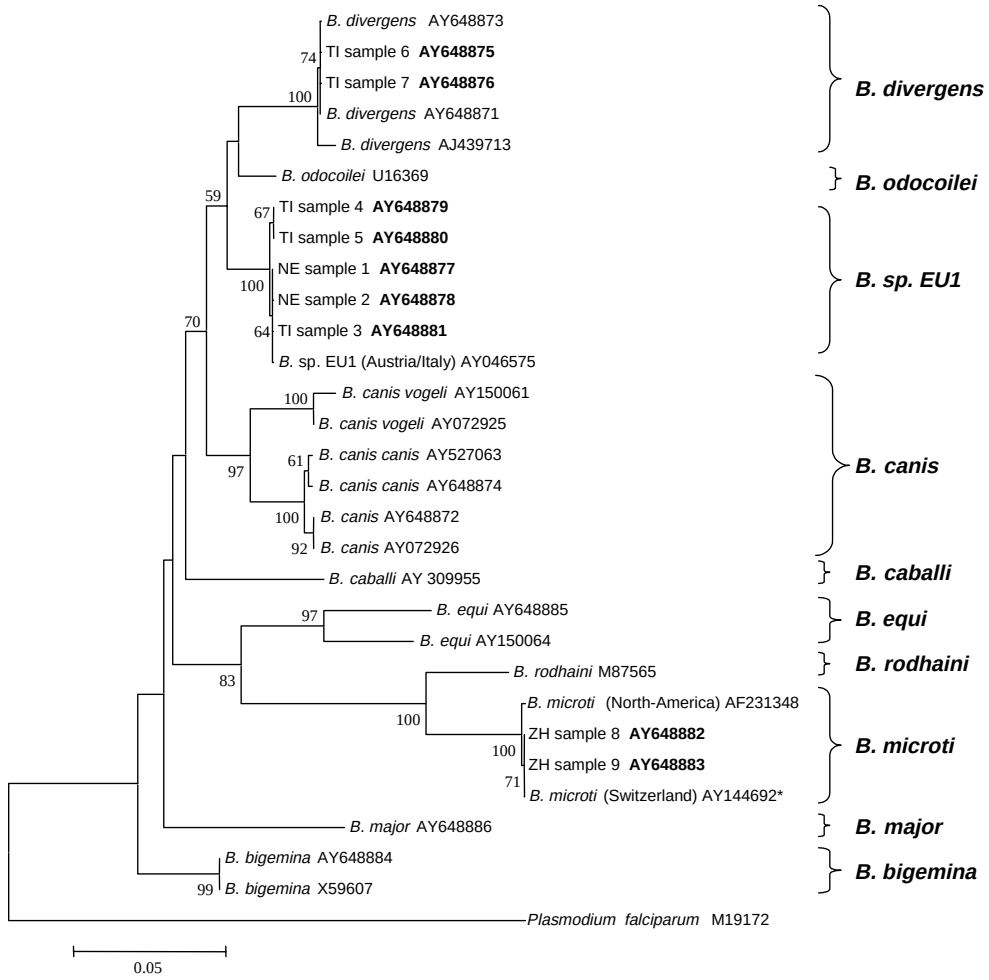

Figure 2. Neighbour-joining tree of 1'000 bootstrap pseudo-replicates with Kimura-2-parameters distances from 18S rRNA (411-452 bp) sequences. Bootstrap proportions are indicated when greater than 50%. The tree illustrates the phylogenetic relationship of the *Babesia* species detected in Switzerland. The codes refer to the geographic origin of the collected ticks (NE = Neuchâtel, TI = Ticino and ZH = Zürich). Sample 3 originates from an *I. ricinus* tick collected from a cow, whereas the ticks of samples 4 and 5 originated from a dog and a cow, those of 6 and 7 from a dog and a cat, respectively. *Plasmodium falciparum* has been used as the outgroup. The GenBank accession numbers of the *Babesia* sequences detected in *I. ricinus* are in bold. The sequence of *B. microti* marked by * is identical to that from Berlin (AF231349), which is not reported in the tree. The scale bar represents 5% of divergence.

Validation of the PCR method. All 7 infected blood samples (Tab. 1) used to validate the method were successfully amplified, which supports its reliability. The PCR experiments on serial dilutions of purified genomic DNA from *B. divergens* showed that the limit of detection under ideal sample condition was 27.5 fg/reaction (Fig. 1). The PCR tests did not produce amplicons when DNA extracted from *T. gondii* and *L. infantum* were used as template (data not shown).

All 100 *Babesia*-negative ticks, tested to validate the DNA extraction by amplifying a fragment of the 12S rDNA gene, generated positive results (data not shown). This confirms that our *Babesia*-negative results are not due to inhibition of the PCR reaction.

Sequence analysis. In order to verify whether the size of the amplified short fragment is sufficient to discriminate the different species, we downloaded from Genbank the sequences of the 18S rRNA gene of 10 strains (Tab. 2) and compared the nucleotide variability of the short fragments (411-452 bp) to that of the whole sequences (1'700 bp). The values obtained were 25.3% and 20.3%, respectively, which can be considered similar.

We then determined the species of the *Babesia* detected in our ticks by comparing the sequences of the 18S rRNA short amplicons with the corresponding fragments of the 12 *Babesia* sequences used as references (Tab. 2) and of the 7 *Babesia* sequences used to validate our method (Tab. 1). A phylogenetic tree was constructed by the NJ method with Kimura-2-parameters (Fig. 2).

Three positive samples (samples 1 and 2, two nymphs from Neuchâtel, and sample 3, a female from Ticino) were identified as EU1 organisms (Tab. 2, GenBank accession no. AY046575). Two other samples from Ticino (samples 4 and 5, two females) were found to be closely related to EU1 with an identity of 99.8% (the sequences differed by only 1 base). The last two positive amplicons from Ticino (sample 6 and 7, a female and a nymph, respectively) perfectly overlapped with *B. divergens* sequences obtained from a blood cattle sample (Tab. 1,

GenBank accession no. AY648871) and from a blood smear cattle (Tab. 1, AY648873). Finally, samples 8 and 9 (two nymphs) from Zürich were identical to the GenBank sequence of *B. microti* found in a tick from Switzerland (Tab. 2, GenBank accession No. AY144692) and differed by 1 bp from the North American reference strain *B. microti* (Tab. 2, GenBank accession No. AF231348).

DISCUSSION

We have described a new PCR method based on the 18S rRNA gene that allows the detection of *Babesia* in *I. ricinus* ticks and in blood samples. The amplified fragments show an important number of mutations. In addition, the variability of the target fragments (25.3%) is similar to the variability of the complete gene sequences (20.3%). Because of this variability, the short amplicon of 411-452 bp of the 18S rRNA gene may be used for accurate species resolution. Indeed, we could differentiate all the *Babesia* species we considered (Fig. 2). Moreover, in the *B. canis* cluster, we clearly could separate the two subspecies *B. canis vogeli* and *B. canis canis* (Fig. 2).

The analytical sensitivity of the PCR assays has been shown to be 27.5 fg/reaction of *B. divergens* DNA. However, the majority of the preview reports describing PCR for the detection of *Babesia* in ticks did not report the sensitivity, with the exception of a study which showed a limit of detection of 50 organisms/ml of canine whole blood [3].

We have detected *B. microti*, *B. divergens* and *Babesia* sp. EU1 in *I. ricinus* ticks collected in four areas of Switzerland. The comparison of the tick infection rate between the studied areas is difficult to make because of the different origins of the samples: indeed, ticks were collected from animal hosts in Ticino and from vegetation in the other three areas. However, the infection rate values were low, whatever the collection method (0.7–1.7%). A similar infection rate (1%) was reported from a study on ticks collected in 4 different areas of Southern Germany [13]. In contrast, ticks tested in Slovenia and in Poland had a positivity rate for *Babesia* spp. of 9.6% and 16.3%, respectively [6, 22]. The reasons for such infection rate differences remain to be clarified.

This is the first study reporting the presence of *Babesia* sp. EU1 in Switzerland. This organism was detected in ticks collected in two areas located North (Neuchâtel) and South of the Alps (Ticino). The presence in Switzerland of *B. microti* and *B. divergens* was also confirmed. Although *B. divergens* has been described in various Swiss cantons (Valais, Ticino and Jura) [1, 10], until now there have been no reports of clinical human case due to indigenous strains of this species. Concerning *B. microti*, a study reported the presence in rodents of specific antibodies in two sites of the Canton Bern [9]. In addition, the presence of antibodies directed against *B. microti* was evidenced in inhabitants of an eastern Swiss area [8]. Recently, Meer-Scherrer *et al*. [20] described the first human case due to *B. microti* in a Swiss patient. However, the behaviour and the virulence of the European *B. microti* strains might be different from those of North America. Interestingly, in another study, it has been suggested that *B. microti* in the United States may be more virulent for humans than the *B. microti*-like isolated in Japan [25]. It is important to consider that *B. microti* actually consists of a genetical species complex based on the 18S rRNA gene: *Babesia* isolated in rodents divide in two clades, one zoonotic, including our samples, and the other maintained only in rodents [11].

Recently, two cases of human babesiosis, in Italy and Austria, have been attributed to EU1 [14]. Moreover, in Slovenia ticks have been found infected by this species [6]. The EU1 sequences obtained in these different countries, including Switzerland, are identical or very close (99.8% of similarity) and thus no specific geographic association may be identified. An important issue concerning *Babesia* sp. EU1 is to determine whether it is an emergent species. Only the 4 bordering European countries mentioned above have been found to host this pathogen. However, the serological tests used in the past are not able to distinguish this species and hence, it is not possible to know its real prevalence. Further surveys similar to ours should be implemented in the European countries to track this new *Babesia* species.

Another issue which has to be clarified is related to the *Babesia* reservoirs. Zintl *et al*. suggested that *B. divergens* is maintained in cattle which have recovered from previous infections or present mild subclinical infections [26]. Thus, cattle movement across Europe may influence the distribution of this pathogen. Moreover, the reservoir host for EU1 is presently unknown and for this reason its distribution throughout European countries is not predictable.

A serological study in Western France indicated that asymptomatic infection with *B. divergens* may co-exist with *Borrelia burgdorferi* sensu lato [12]. The ticks we have analysed in this work have been also examined for infection by *B. burgdorferi* s.l. [4]. We report two coinfections, detected molecularly, the first in a tick from the Neuchâtel forest (sample 2) which was found to host both EU1 and *B. burgdorferi* s.s., and the second in a tick from a cow in Ticino (sample 5) which contained *Babesia* sp. EU1 and *B. afzelii*. Coinfections with *B. microti* and *B. burgdorferi* s.l. are well documented in North America as well as in Europe [18, 20, 23].

To conclude, we would like to stress that the association of *I. ricinus* with *Babesia* sp. EU1, *B. divergens* and *B. microti* is important because of their zoonotic relevance. Indeed, ticks may be considered as epidemiological markers for the distribution of different infectious agents such as *Borrelia burgdorferi* sensu lato, *Erlichia*, *Rickettsia* and TBEV.

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