

Natural Transmission of Zoonotic *Babesia* spp. by *Ixodes ricinus* Ticks

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To determine characteristics of natural transmission of *Babesia* sp. EU1 and *B. divergens* by adult *Ixodes ricinus* ticks, we examined tick salivary gland contents. We found that *I. ricinus* is a competent vector for EU1 and that their sporozoites directly invade erythrocytes. We conclude that EU1 is naturally transmitted by *I. ricinus*.

Ixodes ricinus is a ubiquitous triphasic tick found commonly in Europe. This arthropod feeds on a wide variety of vertebrate hosts, including small rodents and wild and domestic ungulates. It is therefore a potential vector of numerous pathogens, such as bacteria, viruses, and parasites, mainly apicomplexans. Among these pathogens, 2 zoonotic *Babesia* species have been described in Europe: the well-known cattle parasite *Babesia divergens* (1) and the more recently reported roe deer parasite *Babesia* sp. EU1 (2–4). Biological transmission of *B. divergens* by *I. ricinus* ticks has been proven by in vivo experimental infections (5); however, quantitative transmission studies that visualize and quantify sporozoites have never been conducted. For *Babesia* sp. EU1, biological evidence of natural transmission by *I. ricinus* ticks is still lacking; its presence has been assessed only by DNA amplification from whole ticks (4,6–8). Therefore, to analyze transmission of zoonotic *Babesia* spp. by *I. ricinus* ticks, we visualized, isolated, and identified infectious sporozoites from dissected tick salivary glands, the transmitting organs.

The Study

In 2008, ticks were collected from animals from 2 different biotopes where each *Babesia* species had been known

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to circulate: a farm on which a herd was infected with *B. divergens* and a reserve on which wild fauna were infected with *Babesia* sp. EU1. A dairy farm in La Verrie (Vendée, France) was selected as a favorable biotope for *B. divergens* transmission on the basis not only of the presence of numerous ticks on cows and in pastures in 2007 but also of the parasite circulation in the herd, attested by serologic testing (prevalence of 37.5% by immunofluorescence antibody test [IFAT]) and confirmed by its isolation from cattle erythrocytes (prevalence 25% by culture) (9). Of the cows tested by IFAT, 56% had positive results, which indicated that new infections from ticks were occurring within the herd. Because we assumed that sporozoite differentiation is stimulated by blood ingestion and because of experimental proof that female ticks can transmit *B. divergens* (10), we collected only adult ticks feeding on cows. The 324 collected ticks were morphologically identified as *I. ricinus* and weighed to estimate their repletion status (range 3–398 mg). Of these, 223 ticks (4.7–339 mg) were dissected under a stereomicroscope to isolate both salivary glands, which were subsequently crushed in 30 μ L phosphate-buffered saline in a 1.5-mL microtube with an adapted pestle. A droplet of this suspension was deposited on an 18-well slide, stained with May-Grünwald-Giemsa, and examined under a light microscope. When parasites were seen, and for 41 additional negative samples within the same weight range, 5 μ L of the infected suspension was added to the culture medium with bovine (*B. divergens* selective growth) or sheep (both species growth) erythrocytes, RPMI (Roswell Park Memorial Institute medium; Lonza, Basel, Switzerland), and 20% fetal calf serum (Lonza) in 96-well plates (11).

To identify the parasites, we directly sequenced the amplified 18S rDNA *Babesia* gene. PCR with Phusion High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) was performed on extracted DNA (Wizard Genomic DNA Purification Kit; Promega, Madison, WI, USA) from the remaining crushed salivary gland suspensions (Bab primers GF2 and GR2, 540 bases long, variable part of the gene) (4) and from resulting parasitized erythrocytes (primers CryptoF and CryptoR, 1,727 bases long, complete gene) (12).

To confirm the identity of the infected ticks, we directly sequenced a variable part of the 16S rDNA mitochondrial gene of *Ixodes* ticks (310 bases long) (primers IrUp1 5'-TTGCTGTGGTATTTTGACTATAC-3' and IrDo2 5'-AATTATTACGCTGTTATCCCTGA-3'). We used DNA extracted from salivary glands.

Microscopic observation of crushed salivary gland suspensions identified small pear-shaped elements in only 3 ticks; weights were 11.7, 25.3, and 277 mg. These millions of pyriform parasites were considered to be sporozoites (13): they measured about 2 μ m in length and 1 μ m in diameter (Figure, panel A). Only a few parasites had unusual forms, which suggests binary fission (Figure, panel B).

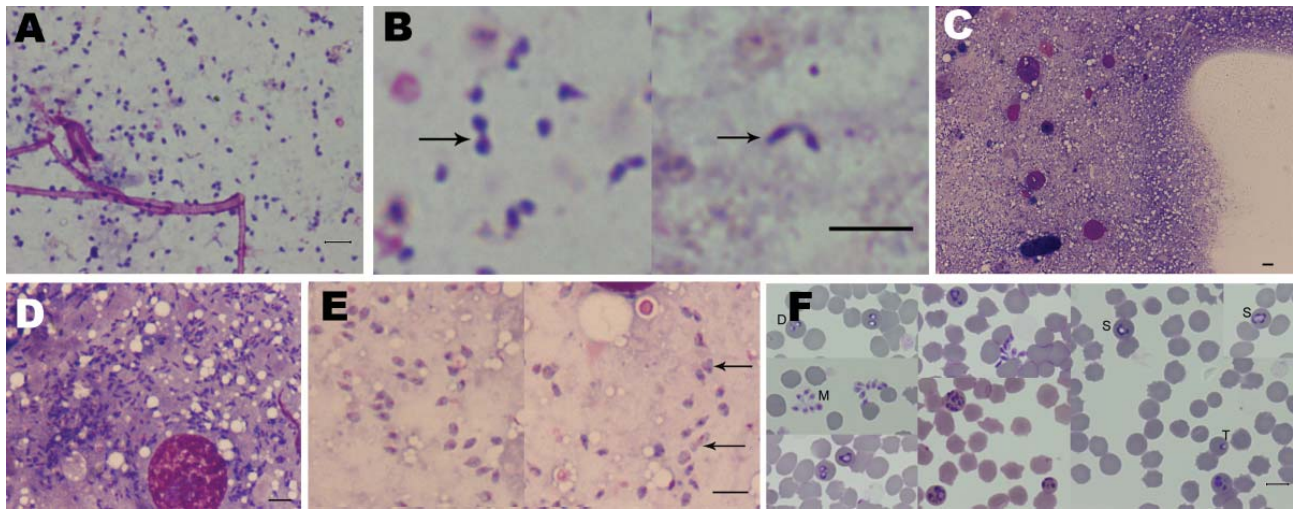


Figure. Microscopic appearance of *Babesia* sp. EU1 sporozoites isolated from tick salivary glands and of subsequent asexual development in erythrocytes. Sporozoites were stained with Giemsa and observed in the suspension of crushed salivary glands (A, B) and from salivary glands directly crushed between slides (C, D, E). Arrows indicate sporozoite dividing forms. A composite panel of asexual stages cultivated in sheep erythrocytes from these sporozoites is presented (F); developmental stages are indicated by letters (D, dividing stages; M, free merozoites; S, schizont-like form; T, trophozoite). Scale bars = 5 μ m.

Development of intraerythrocytic parasites was observed, which proved the parasites' capacity to directly infect erythrocytes. Of the 3 tick salivary glands containing pear-shaped elements, 3 days after inoculation onto a culture, $\approx 1/10,000$ erythrocytes was infected. Only sheep erythrocytes were invaded, which suggests infection with *Babesia* sp. EU1. From these 150- μ L starting wells, 10-mL amplified cultures (10% parasitized erythrocytes) could be established within 1 month (Figure, panel F). Typical *Babesiidae* developmental forms (trophozoite, dividing stages, and free merozoites) were observed, as were more atypical schizont-like parasites, which seemed to produce numerous merozoites. When sporozoites were not observed, parasites were never observed in the cultures of either bovine or sheep erythrocytes.

PCR amplification, sequencing, and comparison with *Babesia* spp. 18S rDNA genes (BLAST [www.ncbi.nlm.nih.gov/blast/Blast.cgi] search in GenBank) showed the sequences to be 100% identical to the *Babesia* sp. EU1 sequence (AY046575) for the 3 infected ticks (sporozoites and culture). The partial (sporozoites) and complete (culture) 18S rDNA sequences obtained have been deposited in GenBank, accession nos. FJ215872 and FJ215873. Identity of the ticks was confirmed by sequence analysis and comparison with the 16S rDNA *I. ricinus* gene (U14154).

For the wild fauna reserve, we used the same approach. At the reserve of Chizé (Deux-Sèvres, France), where high prevalence *Babesia* sp. EU1 has been described (4), we captured 18 roe deer, then collected and analyzed blood samples from them. Presence of *Babesia* sp. EU1 was attested by culture of samples from 4 of the deer. For

31 female ticks, half of the ticks were processed as previously described, and the salivary glands of the other half were simply crushed between 2 slides so parasites could be better seen and quantified. With the latter method, a huge number of sporozoites, $\approx 10^7$ to 10^8 , were observed (Figure, panels C, D). The inner structures were well preserved, nuclei were clearly visible, and we could observe apparent dividing forms (Figure, panel E). From the ticks collected from roe deer, only 2 tick salivary glands contained parasites; PCR products using Bab primers showed 100% identity with *Babesia* sp. EU1 (AY046575).

Conclusions

Our study shows that *I. ricinus* ticks are competent vectors for *Babesia* sp. EU1. Not only can these ticks carry *Babesia* sp. EU1 DNA, but more importantly, they enable these parasites to complete their life cycle up to the production of infectious sporozoites. Direct invasion of erythrocytes by *Babesia* sp. EU1 undoubtedly classifies this species in the genus *Babesia*, a feature generally not proven for most *Babesia* spp.

The proportions of *Babesia* sp. EU1-infective ticks found in our study (3/223 from cattle farm and 2/31 from wild fauna reserve, not statistically different) are comparable to published prevalence of infected ticks (1%–2%) collected either from animals or vegetation (6–8,14,15). Whatever the biotope, *Babesia* sp. EU1 is always present, threatening also in anthropized zones (farming areas). Millions of parasites inside salivary glands were observed and could be injected to the vertebrate host, from the early stage of the tick feeding (11.7 mg) until repletion (277 mg),

which represents a massive infection. These 2 epidemiologic features, combined with the increasing number of immunocompromised persons, should lead to more awareness of the risk related to this zoonotic pathogen.

B. divergens sporozoites were never seen in the salivary glands of adult *I. ricinus* ticks, even when ticks were collected from cattle. This finding is despite the large number of ticks examined (223), the prevalence of nymphs carrying *B. divergens* DNA collected from the farm pastures (87% in 2007 on 113 nymphs analyzed, data not shown), and the infectious status of the herd (serologic prevalence 56%). We therefore raise questions about the main transmitting stage (larvae, nymph, or adult?) and about the quantitative transmission of *B. divergens* by *I. ricinus* ticks (low number of produced and infectious sporozoites?). In Europe, human babesiosis could be caused by these 2 *Babesia* spp., each of which is transmitted by *I. ricinus* ticks but probably with different sporozoite-production features.

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References

- Zintl A, Mulcahy G, Skerrett HE, Taylor SM, Gray JS. *Babesia divergens*, a bovine blood parasite of veterinary and zoonotic importance. *Clin Microbiol Rev*. 2003;16:622–36. DOI: 10.1128/CMR.16.4.622-636.2003
- Herwaldt BL, Caccio S, Gherlinzoni F, Aspöck H, Slemenda SB, Piccaluga P, et al. Molecular characterization of a non-*Babesia divergens* organism causing zoonotic babesiosis in Europe. *Emerg Infect Dis*. 2003;9:942–8.
- Haselbarth K, Tenter AM, Brade V, Krieger G, Hunfeld KP. First case of human babesiosis in Germany—clinical presentation and molecular characterisation of the pathogen. *Int J Med Microbiol*. 2007;297:197–204. DOI: 10.1016/j.ijmm.2007.01.002
- Bonnet S, Jouglin M, L’Hostis M, Chauvin A. *Babesia* sp. EU1 from roe deer and transmission within *Ixodes ricinus*. *Emerg Infect Dis*. 2007;13:1208–10.
- Joyner LP, Davies SF, Kendall SB. The experimental transmission of *Babesia divergens* by *Ixodes ricinus*. *Exp Parasitol*. 1963;14:367–73. DOI: 10.1016/0014-4894(63)90044-4
- Duh D, Petrovec M, Avsic-Zupanc T. Molecular characterization of human pathogen *Babesia* EU1 in *Ixodes ricinus* ticks from Slovenia. *J Parasitol*. 2005;91:463–5. DOI: 10.1645/GE-394R
- Casati S, Sager H, Gern L, Piffaretti JC. Presence of potentially pathogenic *Babesia* sp. for human in *Ixodes ricinus* in Switzerland. *Ann Agric Environ Med*. 2006;13:65–70.
- Hilpertshauer H, Deplazes P, Schnyder M, Gern L, Mathis A. *Babesia* spp. identified by PCR in ticks collected from domestic and wild ruminants in southern Switzerland. *Appl Environ Microbiol*. 2006;72:6503–7. DOI: 10.1128/AEM.00823-06
- Malandrin L, L’Hostis M, Chauvin A. Isolation of *Babesia divergens* from carrier cattle blood using in vitro culture. *Vet Res*. 2004;35:131–9. DOI: 10.1051/vetres:2003047
- Donnelly J, Peirce MA. Experiments on the transmission of *Babesia divergens* to cattle by the tick *Ixodes ricinus*. *Int J Parasitol*. 1975;5:363–7. DOI: 10.1016/0020-7519(75)90085-5
- Chauvin A, Valentin A, Malandrin L, L’Hostis M. Sheep as a new experimental host for *Babesia divergens*. *Vet Res*. 2002;33:429–33. DOI: 10.1051/vetres:2002029
- Duh D, Petrovec M, Bidovec A, Avsic-Zupanc T. Cervids as *Babesia* hosts, Slovenia. *Emerg Infect Dis*. 2005;11:1121–3.
- Mehlhorn H, Shein E. The piroplasms: life cycle and sexual stages. *Adv Parasitol*. 1984;23:37–103. DOI: 10.1016/S0065-308-X(08)60285-7
- Nijhof AM, Bodaan C, Postigo M, Nieuwenhuijs H, Opsteegh M, Franssen L, et al. Ticks and associated pathogens collected from domestic animals in the Netherlands. *Vector Borne Zoonotic Dis*. 2007;7:585–95. DOI: 10.1089/vbz.2007.0130
- Schmid N, Deplazes P, Hoby S, Ryser-Degiorgis MP, Edelhofer R, Mathis A. *Babesia divergens*-like organisms from free-ranging chamois (*Rupicapra r. rupicapra*) and roe deer (*Capreolus c. capreolus*) are distinct from *B. divergens* of cattle origin—an epidemiological and molecular genetic investigation. *Vet Parasitol*. 2008;154:14–20. DOI: 10.1016/j.vetpar.2008.02.028

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