RESEARCH NOTE

BABESIA MICROTI (PIROPLASMIDA: BABESIIDAE) IN NYMPHAL *IXODES RICINUS* (ACARI: IXODIDAE) IN THE CZECH REPUBLIC

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Abstract. A total of 350 nymphs of the common tick *Ixodes ricinus* (Linnaeus, 1758) were collected in an endemic focus of Lyme borreliosis (South Moravia, Czech Republic) and examined for the presence of the protozoan *Babesia microti* (França, 1909) by polymerase chain reaction (PCR), using primers specific for the *B. microti* gene encoding small subunit rRNA. The assay revealed five positive pools (out of 70 pools examined); the corresponding prevalence rate was about 1.5%. Sequence analysis of the PCR products confirmed their 100% homology with that of *B. microti*. The study represents the first evidence of *B. microti* in ixodid ticks in the Czech Republic.

Babesiosis is an emerging, tick-transmitted zoonotic disease caused by intraerythrocytic parasites of the genus *Babesia*. These piroplasmas are transmitted by ixodid ticks and are capable of infecting a wide variety of vertebrate hosts which are competent in maintaining the transmission cycle. Babesiae include at least three species pathogenic for humans: *Babesia bovis*, *B. divergens* and *B. microti* (Homer et al. 2000). Whereas the bovine parasite, *B. divergens*, is responsible for most European cases of human babesiosis, especially in splenectomized patients (Gorenflot et al. 1998), *B. microti* has not yet been implicated as a cause of autochthonous human illness in Europe (Foppa et al. 2002). Most human cases caused by *B. microti* have occurred in the north-eastern states of the USA and are transmitted by *Ixodes scapularis* (Spielman 1994).

However, *B. microti* is also present in European countries. First findings of *B. microti* in central Europe were reported in the blood from *Microtus arvalis*, *M. agrestis*, *Clethrionomys glareolus*, *Apodemus flavicollis* and *A. sylvaticus* rodents (Aeschlimann et al. 1975, Šebek 1975, Šebek et al. 1977). The occurrence of *B. microti* in rodents has been then reported from other European countries (Šebek et al. 1977, Šebek et al. 1980, Walter 1984, Telford et al. 2002). Thereafter, three species of the genus *Ixodes* have been found to carry and/or transmit *B. microti* in Europe: (1) *I. trianguliceps* in England (Hussein 1980) and Russia (Telford et al. 2002); (2) *I. ricinus* in Germany (Weber and Walter 1980, Walter 1981, Walter and Weber 1981), Slovenia (Duh et al. 2001), Switzerland (Foppa et al. 2002), England (Gray et al. 2002), Poland (Skotarczak and Cichocka 2001, Kuźna-Grygiel et al. 2002, Skotarczak et al. 2003) and Hungary (Kálmán et al. 2003); and (3) *I. persulcatus* in Lithuania (Alekseev and Dubinina 2003) and European Russia (Alekseev et al. 2003).

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The occurrence of *B. microti* in *I. ricinus* ticks has not yet been investigated in the Czech Republic. The purpose of the present study was to determine the prevalence of *B. microti* in *I. ricinus* ticks in one area of South Moravia (Czech Republic), where Lyme borreliosis is endemic. A total of 350 host-seeking nymphs of *I. ricinus* were collected in the surroundings of Valtice (South Moravia, Czech Republic) during 2003 by flagging the vegetation. The habitat was described in another paper (Hubálek et al. 1994). All tick specimens were frozen at –60°C until further processing. Immediately before DNA isolation, nymphs were pooled. Different pool sizes were used for two groups of ticks. We started with pools consisting of three nymphs, but after the first results obtained we shifted to pools of ten individuals (for technical reasons). All ticks were surface sterilized with 70% ethanol (PCR quality) and mechanically disrupted using a glass microblender. The total genomic DNA was extracted with QIAamp DNA Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. PCR detection of *B. microti* DNA was performed as described by Persing et al. (1992), including primers bab 1 and bab 4 specific for the *B. microti* gene encoding small subunit rRNA (ss-rDNA). Each reaction tube contained 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.001% Tween 20, 2.5 mM $MgCl_2$, 200 mM mixture of dNTPs, 2.5 U Taq purple DNA polymerase and 25 pmol of each primer. PCR reaction was performed in a PTC-200 Gradient Thermal Cycler (MJ Research, USA) under the following conditions: 1 min of denaturation at 94°C, 1 min of annealing at 55°C and 2 min of extension at 72°C consisting of 40 cycles. The PCR products were separated on 2% agarose gel, stained with ethidium bromide and visualised by UV light. DNA extraction and PCR handling were done in two separate rooms to avoid possible cross-contamination of the samples. Specific PCR products were further characterized by sequence analysis. DNA fragments were precisely excised from the gel and purified with the Gel Extraction Kit (Qiagen, Hilden, Germany). To ensure the specificity, the PCR products were sequenced twice in both directions using bab 1 and bab 4

Table 1. Prevalence of *Babesia microti* in *Ixodes ricinus*, South Moravia, 2003.

Sample	No. of nymphs	Pool size	No. of pools	No. of pools positive	MIR[1]	MLE[2]
I	150[3]	3	50	1	0.67%	0.67%
II	200[4]	10	20	4[5]	2.00%	2.21%
Total	350		70	5	1.43%[6]	1.55%[6]

[1]minimum infection rate; [2]maximum likelihood infection rate; [3]coll. September; [4]coll. April (100 specimens) and September (100 specimens); [5]three of the September collection; [6]weighted average

Table 2. Prevalence of *Babesia microti* in host-seeking *Ixodes ricinus* in Europe.

Country/Reference	Larvae	Nymphs	Adults	Total[1]
GERMANY				
Walter 1981	–	2/375 (0.5)[2]	–	2/375 (0.5)
SLOVENIA				
Duh et al. 2001	–	9/69 (13.0)	4/70 (5.7)	13/139 (9.4)
POLAND				
Skotarczak and Cichocka 2001	12/385 (3.1)	49/1160 (4.2)	69/550 (12.5)	118/1710 (6.9)
Kuźna-Grygiel et al. 2002	–	8/412 (1.9)	0/49 (0.0)	8/461(1.7)
Skotarczak et al. 2003	4/19 (21.1)	26/234 (11.1)	41/280 (14.6)	67/514 (13.0)
SWITZERLAND				
Foppa et al. 2002	–	14/408 (3.4)	–	14/408 (3.4)
HUNGARY				
Kálmán et al. 2003	–	–	–	4/452 (0.9)

[1]nymphs and adults, total; [2]no. positive / no. examined (% positive) individuals

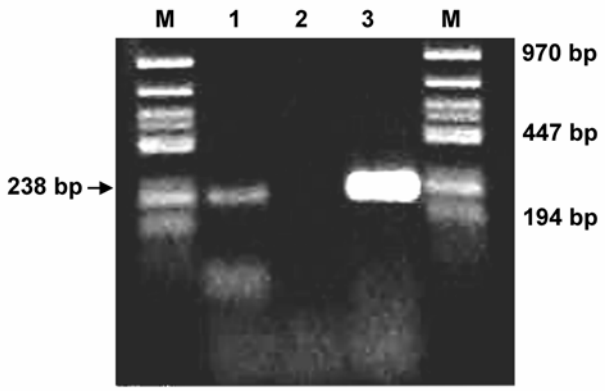

Fig. 1. PCR product of *Babesia microti* DNA from nymphal *Ixodes ricinus*. M – ladder; lanes 1, 2 and 3 – positive sample, negative control, and positive control (*B. microti* DNA), respectively.

primers. CEQ 2000 Dye terminator Cycle sequencing Kit was used, sequences were analysed on an ABI Prism 877 ITC automated DNA sequencer (Beckman Coulter, USA) using DNASTAR software (DNASTAR, London, UK), and compared with those in the GenBank. BLAST programs of the National Center for Biotechnology Information (Bethesda, MD, USA) were used for database searches. Minimum infection rate (MIR) was estimated as the ratio of the number of positive pools to the total number of individual ticks tested (in per cent). The other method of estimating the proportion of infected individuals in pooled samples was the maximum likelihood estimation (MLE), which gives results approaching the real situation more precisely than MIR (Gu et al. 2003). The corresponding software program MIR-IR, obtained from the authors, was used for the latter estimation.

In total, 5 of 70 pools (350 nymphal *I. ricinus*) were positive, giving MIR 1.43% (0.14 infected tick per 1,000 ticks). The alternative estimation by MLE approach yielded a very similar value, 1.55% (Table 1). Fig. 1 shows an example of one positive PCR specimen. The PCR products, subjected to sequence analysis, showed a 100% nucleotide homology with other *B. microti* strains deposited in GenBank: M 93660 (USA), AF 373331 (Slovenia), AF 231348 (Germany), AY 056017 (Switzerland) and AB0 83375 (Japan).

The prevalence of *B. microti* in *I. ricinus* found in this study (about 1.5% with both MIR and MLE calculations) is close to the infection rates reported in other European countries like Germany, Switzerland and Hungary, while considerably higher values were occasionally found in Slovenia or Poland (Table 2). European data show that *B. microti* occurs in all stages of the *I. ricinus* vector. This study has confirmed the presence of *B. microti* in the Czech Republic, where it had been earlier microscopically observed in rodents (Šebek 1975, Šebek et al. 1977). The lack of recognized human pathology associated with European strains of *B. microti*, despite exposure to infectious tick bites, may be a consequence of a lower virulence of European strains compared to the North American babesiae. Disease episodes due to *B. microti*, on the other hand, may be overlooked by general practitioners because of the relatively nonspecific symptoms (at least in mild cases) and a common presumption that this agent rarely, if at all, infects *I. ricinus* (Foppa et al. 2002).

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References

AESCHLIMANN A., BROSSARD M., QUENET G. 1975: Contribution a la connaissance des piroplasmes de Suisse. Acta Trop. 32: 281–289.

ALEKSEEV A.N., DUBININA H.V. 2003: Multiple infections of tick-borne pathogens in *Ixodes* spp. (Acarina, Ixodidae). Acta Zool. Lithuan. 13: 311–321.

ALEKSEEV A.N., SEMENOV A.V., DUBININA H.V. 2003: Evidence of *Babesia microti* infection in multi-infected *Ixodes persulcatus* ticks in Russia. Exp. Appl. Acarol. 29: 345–353.

DUH D., PETROVEC M., AVSIC-ZUPANC T. 2001: Diversity of *Babesia* infecting European sheep ticks (*Ixodes ricinus*). J. Clin. Microbiol. 39: 3395–3397.

FOPPA I.M., KRAUSE P.J., SPIELMAN A., GOETHERT H., GERN L., BRAND B., TELFORD S.R. 2002: Entomologic and serologic evidence of zoonotic transmission of *Babesia microti*, eastern Switzerland. Emerg. Infect. Dis. 8: 722–726.

GORENFLOT A., MOUBRI K., PRECIGOUT E., CARCY B., SCHETTERS T.P.M. 1998: Human babesiosis. Ann. Trop. Med. Parasitol. 92: 489–501.

GRAY J., von STEDINGK L.V., GURTELSCHMID M., GRANSTRÖM M. 2002: Transmission studies of *Babesia microti* in *Ixodes ricinus* ticks and gerbils. J. Clin. Microbiol. 40: 1259–1263.

GU W., LAMPMAN R., NOVAK R.J. 2003: Problems in estimating mosquito infection rates using minimum infection rate. J. Med. Entomol. 40: 595–596.

HOMER M.J., AGUILAR-DELFIN I., TELFORD S.R., KRAUSE P.J., PERSING D.H. 2000: Babesiosis. Clin. Microbiol. Rev. 13: 451–469.

HUBÁLEK Z., HALOUZKA J., JUŘICOVÁ Z., SVOBODOVÁ Š. 1994: Seasonal distribution of borreliae in *Ixodes ricinus* ticks. Zentralbl. Bakteriol. 280: 423–431.

HUSSEIN S.H. 1980: *Ixodes trianguliceps*: seasonal abundance and role in the epidemiology of *Babesia microti* infection in north-western England. Ann. Trop. Med. Parasitol. 74: 531–539.

KÁLMÁN D., SRÉTÉR T., SZÉLL Z., EGYED L. 2003: *Babesia microti* infection of anthropophilic ticks (*Ixodes ricinus*) in Hungary. Ann. Trop. Med. Parasitol. 97: 317–319.

KUŹNA-GRYGIEL W., BUKOWSKA K., CICHOCKA A., KOSIK-BOGACKA D., SKOTARCZAK B. 2002: The prevalence of piroplasms in a population of *Ixodes ricinus* (Acari: Ixodidae) from north-western Poland. Ann. Agric. Environ. Med. 9: 175–178.

PERSING D.H., MATHIESEN D., MARSHALL W.F., TELFORD S.R., SPIELMAN A., THOMFORD J.W., CONRAD P.A. 1992: Detection of *Babesia microti* by polymerase chain-reaction. J. Clin. Microbiol. 30: 2097–2103.

SKOTARCZAK B., CICHOCKA A. 2001: The occurrence of DNA of *Babesia microti* in ticks *Ixodes ricinus* in the forest areas of Szczecin. Folia Biol. (Krakow) 49: 247–250.

SKOTARCZAK B., RYMASZEWSKA A., WODECKA B., SAWCZUK M. 2003: Molecular evidence of coinfection of *Borrelia burgdorferi* sensu lato, human granulocytic ehrlichiosis agent, and *Babesia microti* in ticks from northwestern Poland. J. Parasitol. 89: 194–196.

SPIELMAN A. 1994: The emergence of Lyme disease and human babesiosis in a changing environment. Ann. N. Y. Acad. Sci. 740: 146–156.

ŠEBEK Z. 1975: Blutparasiten der wildlebenden Kleinsäuger in der Tschechoslowakei. Folia Parasitol. 22: 11–20.

ŠEBEK Z., ROSICKÝ B., SIXL W. 1977: The occurrence of babesias affecting small terrestrial mammals and the importance of this zoonosis in Europe. Folia Parasitol. 24: 221–228.

ŠEBEK Z., SIXL W., STÜNZNER D., VALOVÁ M., HUBÁLEK Z., TROGER H. 1980: Zur Kenntnis der Blutparasiten wildlebender Kleinsäuger in der Steiermark und im Burgenland. Folia Parasitol. 27: 295–301.

TELFORD S.R. III, KORENBERG E.I., GOETHERT H.K., KOVALEVSKI Y.V., GORELOVA N.B., SPIELMAN A. 2002: [Detection of natural foci of babesiosis and granulocytic ehrlichiosis in Russia.] Zh. Mikrobiol. Epidemiol. Immunobiol. 6: 21–25. (In Russian.)

WALTER G. 1981: Isolierung von *Babesia microti* (França 1912) aus freilebenden Nymphen von *Ixodes ricinus* (Linnaeus 1758). Acta Trop. 38: 187–188.

WALTER G. 1984: Zur Übertragung und zum Parasitämieverlauf von *Babesia microti* (Stamm "Hannover I") bei Rötelmaus (*Clethrionomys glareolus*) und Erdmaus (*Microtus agrestis*). Acta Trop. 41: 259–264.

WALTER G., WEBER G. 1981: Untersuchung zur Übertragung (transstadial, transovarial) von *Babesia microti*, Stamm "Hannover I", in *Ixodes ricinus*. Tropenmed. Parasitol. 32: 228–230.

WEBER G., WALTER G. 1980: *Babesia microti* (Apicomplexa: Piroplasmida): electron microscope detection in salivary glands of the tick vector *Ixodes ricinus* (Ixodidea: Ixodidae). Z. Parasitenkd. 64: 113–115.

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