RESEARCH NOTE

DETECTION OF *ANAPLASMA* DNA IN *IXODES RICINUS* TICKS: PITFALLS

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Abstract. A total of 150 nymphal *Ixodes ricinus* (L., 1758) (Acari: Ixodidae) from the Czech Republic were examined for *Anaplasma phagocytophilum* (Foggie, 1949) Dumler et al., 2001 by PCR using EHR521/747 primers: 22 of 50 pools were positive (minimum prevalence, 14.7%). However, sequencing of the PCR products did not show complete homology with *A. phagocytophilum* (91%) while the closest relationship (95%) was found to "*Candidatus* Ehrlichia walkerii". The results indicate a need for care in interpretation of *Anaplasma* PCR results and for PCR optimization for detecting *A. phagocytophilum* in ticks.

The causative agent of human granulocytic anaplasmosis (HGA) (formerly called human granulocytic ehrlichiosis, HGE) is *Anaplasma phagocytophilum* (Foggie, 1949) Dumler et al., 2001 (formerly called *Ehrlichia phagocytophila*) (Anaplasmataceae, Rickettsiales), a gramnegative obligate intracellular bacterium with tropism to leukocytes in the vertebrate host. This is an emerging zoonotic disease transmitted by ixodid ticks and first described in the USA, where several hundred cases have been reported since 1994 (Bakken and Dumler 2006). A limited number of laboratory-confirmed cases of human anaplasmosis due to *A. phagocytophilum* have been reported from countries in Europe, including Austria, Italy, Latvia, the Netherlands, Norway, Poland, Czech Republic, Slovenia, Spain, and Sweden (Bakken and Dumler 2006), and the common tick *Ixodes ricinus* (L.) has been identified as the principal vector of this rickettsial agent in Europe (Parola and Raoult 2001). In Europe, prevalence of *A. phagocytophilum* in *I. ricinus* differs considerably according to various authors (Table 1).

The purpose of this study was to assess prevalence of *A. phagocytophilum* in nymphal *I. ricinus* ticks in an area of South Moravia (Czech Republic) where Lyme borreliosis is endemic (Hubálek et al. 2003). Host-seeking nymphal *I. ricinus* were collected by flagging low vegetation during September 2003. All tick specimens were frozen at –60°C until examination. Immediately before DNA isolation, nymphs were surface-sterilized with 70% ethanol (PCR quality), then pooled (3 nymphs per pool) and mechanically disrupted using a sterile glass microblender. The total genomic DNA was extracted with QIAamp DNA Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. PCR detection of *A. phagocytophilum* was performed as described previously including primers EHR521 (5'-TGT AGG GGG TTC GGT AAG TTA AAG-3') and EHR747 (5'-GCA CTC ATC GTT TAC AGC GTG-3') which amplify a 247 bp partial sequence of *A. phagocytophilum* 16S rRNA gene (Pancholi et al. 1995). 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 technique was performed in a PTC-200 Gradient Thermal Cycler (MJ Research, USA) under the following conditions: 30 sec of denaturation at 94°C, 30 sec of annealing at 55°C and 1 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 under UV light. DNA extraction and PCR handling were done separately in two 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). The nucleotide sequences were determined by direct sequencing of PCR products. To ensure the specificity, the PCR products were sequenced twice in both directions using EHR521 and EHR747 primers. CEQ 2000 Dye terminator Cycle sequencing Kit was used, sequences were analysed on the 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.

A total of 150 nymphal *I. ricinus* in 50 pools were screened. Specific products of *A. phagocytophilum* were detected in 22 pools, which gives a minimum prevalence of 14.7%. Randomly selected PCR products from positive specimens were subjected to sequence analysis for confirmation and compared with sequences deposited in the GenBank database. Surprisingly, all sequences demonstrated only 91% nucleotide identity with the *A. phagocytophilum* AF481855.1, which was detected in cervids in Slovenia (Petrovec et al. 2002). The highest homology (95% nucleotide identity) was shown to a new "*Candidatus* Ehrlichia walkerii" (AY098730.1), which was detected in *I. ricinus* removed from asymptomatic patients in Belluno, Italy (Brouqui et al. 2003, Sanogo et al. 2003), followed by *Ehrlichia*-like sp. "Schotti variant" (AF104680; 95% nucleotide identity) and "*Candidatus* Neoehrlichia mikurensis" (AB074460.1; 95% nucleotide identity). Furthermore, another sequencing of 16S rRNA gene has confirmed the first results.

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Table 1. Minimum prevalence of rickettsiae declared as *Anaplasma phagocytophilum* in *Ixodes ricinus* in Europe according to various authors.

Country	Nymphs	Adults	Total[a]
Finland (Makinen et al. 2003)	0/111 (**0.0**)[b]	0/343 (**0.0**)	0/454 (**0.0**)
Norway (Jenkins et al. 2001)	1/185 (**0.5**)	2/156 (**1.3**)	3/341 (**0.9**)
Denmark (Skarphédinsson et al. 2007)	10/69 **(14.5)**	15/37 **(14.6)**	25/106 **(23.6)**
Estonia (Makinen et al. 2003)	–	3/100 (**3.0**)	3/100 (**3.0**)
Poland (Grzeszczuk et al. 2002)	1/74 (**1.4**)	59/302 (**19.5**)	60/376 (**16.0**)
Austria (Sixl et al. 2003)	–	12/235 (**5.1**)	12/235 (**5.1**)
Czech Republic (Hulínská et al. 2002)	–	–	2/90 (**2.2**)
Slovakia (Derdáková et al. 2003)	0/20 **(0.0)**	5/40 (**12.5**)	5/60 (**8.3**)
Hungary (Srétér et al. 2004)	–	6/452 (**1.3**)	6/452 (**1.3**)
Slovenia (Petrovec et al. 1999)	–	3/93 (**3.2**)	3/93 (**3.2**)
Republic of Moldova (Koči et al. 2007)	–	–	18/198 **(9.1)**
Italy (Mantelli et al. 2006)	100/1014 (**9.9**)	–	100/1014 (**9.9**)
United Kingdom (Ogden et al. 1998)	5/135 (**3.7**)	5/114 (**4.4**)	10/249 (**4.0**)
The Netherlands (Wielinga et al. 2006)	–	–	4/1580 (**0.3**)
France (Ferquel et al. 2006)	4/1065 **(0.4)**	2/171 (**1.2**)	6/1236 (**0.5**)
Switzerland (Pusterla et al. 1999)	3/575 (**0.5**)	18/1092 (**1.6**)	21/1667 (**1.3**)
Germany (Baumgarten et al. 1999)	–	6/275 (**2.2**)	6/275 (**2.2**)
Baltic Region (Russia) (Alekseev et al. 2001)	–	–	3/295 (**1.0**)
Bulgaria (Christova et al. 2003)	10/42 (**23.8**)	56/185 (**30.3**)	66/227 (**29.1**)
Portugal (Santos et al. 2004)	6/142 (**4.2**)	–	6/142 (**4.2**)
Spain (Oteo et al. 2000)	?/? **(24.1)**	–	?/? **(24.1)**

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

These findings indicate potential difficulties in molecular detection of the HGA agent in ixodid ticks, when the primer pair EHR521 and EHR747 is used. According to a comparative study (Massung and Slater 2003), the primers EHR521 and EHR747 were found to be highly sensitive, but with a poor specificity, since they detected in addition to *A. phagocytophilum* also *Rickettsia rickettsii*, *Bartonella henselae*, *Ehrlichia chaffeensis*, and probably other rickettsial endosymbionts of ticks. Moreover, Massung et al. (2003) found a non-pathogenic (in mouse model) variant "Ap-1" of *A. phagocytophilum* occurring more often (about 10 times) than the pathogenic variant "Ap-ha"; at the same time, genetic difference between both variants was found to be negligible (only two nucleotides) in 16S rRNA gene sequence. Furthermore, sequence of a non-pathogenic variant of *A. phagocytophilum* was amplified from *I. ricinus* ticks collected in Spain (Portillo et al. 2005). Recent data suggest that Ap-1 is restricted to ruminant species and represents a lineage distinct from Ap-ha, which infects humans and numerous other mammals (Massung et al. 2006).

Our results, in accord with those of Shukla et al. (2003), emphasize the importance to sequence rickettsial PCR products for confirmation of their specificity. A very high prevalence of *A. phagocytophilum* in *I. ricinus* ticks in Europe was reported in, e.g., Bulgaria (Christova et al. 2003), Denmark (Skarphédinsson et al. 2007), Poland (Grzeszczuk et al. 2002), Slovakia (Derdáková et al. 2003) or Spain (Oteo et al. 2000). Some of these figures might have been over-estimated due to missing confirmation of PCR products by sequencing (Shukla et al. 2003). The relatively very low incidence of reported clinical cases of HGA (much lower than that of Lyme borreliosis) in Europe should be a reflection of the lower actual prevalence of the human pathogenic variant of *A. phagocytophilum* (compared to *Borrelia burgdorferi* sensu lato) in *Ixodes ricinus* ticks, but this contrasts with some data reported (Table 1). Our findings as well as reports of other authors (Massung et al. 2003, Shukla et al. 2003) indicate clearly that a high caution is necessary for correct interpretation of the PCR-based results of *A. phagocytophilum* presence in ixodid ticks.

In conclusion, standardisation of molecular detection of the HGA agent seems to be desirable. Most importantly, a novel, more specific primer pair, which would differentiate the human-pathogenic variant of *A. phagocytophilum* from the non-pathogenic one by PCR, is highly needed.

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