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# CO-INFECTION WITH *BORRELIA* SPECIES AND OTHER TICK-BORNE PATHOGENS IN HUMANS: TWO CASES FROM POLAND

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**Abstract:** Co-infection with *Borrelia* species and *Anaplasma phagocytophilum* or *Babesia* spp. was assessed in a retrospective study of tick-exposed individuals from southeastern Poland. The co-infection rate of these pathogens was found to be rather low (*Borrelia* spp./*Anaplasma phagocytophilum* – 4.2%, 1/24; *Borrelia* spp./*Babesia* spp. – 4.2%, 1/24). However, due to the increased prevalence of *Borrelia* spp. in *Ixodes ricinus* ticks in Poland and the recent emergence of new tick-borne infections, it is necessary to carefully evaluate the true risk of human infection with several pathogens using more sensitive and reliable diagnostic tools. This is the first report of human infection with *Babesia* spp. in Poland that has been confirmed by molecular techniques with homology of 98.9% to *B. divergens* or *Babesia* EU1.

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## INTRODUCTION

Tick-borne diseases (TBD) represent a significant and increasing threat to human health in the northern hemisphere [24]. In the last two decades, new infections transmitted mainly by *Ixodes ricinus* ticks have emerged across Europe, and the incidence of such infections in humans is rising steadily. It is well established that *I. ricinus* is an important vector of Lyme borreliae, but pathogens associated with tick-borne encephalitis, anaplasmosis, rickettsiosis and babesiosis can also be transmitted simultaneously [11].

Lyme borreliosis has become the most common vector-borne disease, not only in the United States, but also in Europe [41]. The former *Borrelia burgdorferi* sensu lato complex consists of at least 7 human pathogenic species: *B. burgdorferi*, *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. lusitaniae*, *B. spielmanii* and *B. bavariensis*. All are prevalent in Europe, and at least three of these bacterial genospecies (*B. burgdorferi*, *B. afzelii*, *B. garinii*) are responsible for Lyme borreliosis with associated multisystemic syndrome [10]. The number of diagnosed cases of borreliosis in Poland is increasing rapidly, especially in certified as the occupational disease groups, for instance forestry workers [5, 6]. More then 10,300 cases were reported by the Polish National Institute of Public Health in 2009, compared with 1,850 cases in 2000 (http://www.pzh.gov.pl). The reservoir hosts are primarily wild rodents, and the vectors are mainly nymphs of *I. ricinus*, the most widespread and abundant human biting ixodid tick in Poland [32]. Human granulocytic anaplasmosis (HGA), formerly known as human granulocytic ehrlichiosis (HGE), caused by *Anaplasma phagocytophilum* has recently been recognized as an emerging disease, occurring with increasing frequency all over the world. *A. phagocytophilum*, an obligate, intracellular bacterium that infects the granulocytes (primarily neutrophils) of mammals, has been detected in *Ixodes* ticks both in Europe and in the United States [1, 19, 29]. Human *A. phagocytophilum* infection was first described in the USA in 1994 [3] and in Europe from Slovenia in 1997 [30]. Since then, about 65 cases have been reported in Europe [8], including Poland [12, 17, 40].

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Babesiosis is caused by infection of erythrocytes with various species of protozoan parasites from the genus *Babesia*. Most cases of human babesiosis in the Unites States are caused by *Babesia microti* [26], although recently the WA1-type, MO1-type and CA1-type *Babesia* species have been shown to cause clinical symptoms [7]. More than 30 cases of human babesiosis have been recorded in Europe since 1956, all caused by the cattle species *B. divergens*. Three recently described human cases identified in Italy, Austria and Germany were caused by infection with a species named EU1, exhibiting molecular characteristics distinct from those of *B. divergens* [16, 18]. The rodent species *B. microti* is also present throughout Europe, although there has only been one verified case of human babesiosis due to infection with this species [20].

In recent years, co-infections of humans acquired from *Ixodes ricinus* ticks have been observed quite frequently in the United States [1, 4, 38] and in Europe [24, 44]. However, only three such cases have been reported so far in Poland [12, 17, 27]. In this retrospective study we examined *Borrelia*-seropositive individuals from southeastern Poland, where *I. ricinus* ticks are highly endemic, for co-infection with *A. phagocytophilum* and *Babesia* species.

## MATERIALS AND METHODS

A total of 30 blood samples were collected over a period of two years (2007–2008) from immunocompetent persons (mean age 35.5 years, males n=11, females n=19) living in southeastern Poland, where the risk of being bitten by an *I. ricinus* tick is very high. These individuals were either forestry workers or those partaking in outdoor activities, and all had reported tick infestation. Altogether, 24 out of the 30 subjects (80%) presented typical clinical signs of Lyme borreliosis (EUCALB, http://www.oeghmp.at/eucalb/), but they were not hospitalized. They were also confirmed as seropositive for *Borrelia* spp. based on conventional serological/molecular tests carried out in diagnostic laboratories (ELISA, Western Blot and/or PCR). Blood samples were collected in EDTA-containing tubes (1 mM) and frozen at -20°C until DNA extraction. DNA was isolated from whole blood using a MiniPrep Blood kit (AxyGen, USA).

**PCR analysis.** The DNA extracted from blood samples was used in a nested PCR with primers amplifying specific fragments of pathogen small-subunit ribosomal RNA genes: (1) the 16S rDNA of *A. phagocytophilum* [20], and (2) the 18S rDNA of *Babesia* spp. [2]. PCR amplification products were separated by 1.5% agarose gel electrophoresis, stained with 0.2 μg $ml^{-1}$ ethidium bromide and visualized under UV light. These DNA fragments were sequenced using an ABI-PRISM 377 automatic DNA sequencer (Applied Biosystems). The resulting sequences were assembled using the programme ABITM BigDye™ and compared with sequences deposited in the National Institute of Health genetic sequence database: GenBank.

Single *A. phagocytophilum*- and *B. divergens*-positive samples were verified at the Institute of Medical Microbiology, Friedrich-Schiller-University in Jena, Germany, using different PCR protocols [15, 19]. The amplified products were separated by electrophoresis on a 2% agarose gel, stained with SYBR-Green (Biozym Diagnostic, Germany) and visualized under UV light. Sequencing of these DNA fragments was performed using a DYEnamicTM ET Dye Terminator Cycle Sequencing Kit (GE Healthcare) and the reactions run on an ABI PRISM 310 genetic analyzer (PE Biosystems) according to the manufacturer's instructions.

**Sequence analysis.** Analyses of DNA sequences and phylogenetic relationships were carried out using the PHILIP software package, version 3.63. The sequences were aligned with the programme ClustalW. For distance analysis, a neighbour-joining tree was generated from a Kimura two-parameter distance matrix using the algorithms DNADIST and NEIGHBOR. Equivalent rDNA sequences from *Plasmodium falciparum* (GenBank accession No. M19172) and *Rickettsia helvetica* (L36212) were used as outgroups.

**Microscopic study.** Peripheral blood smears were prepared from fresh blood, air-dried, fixed in methanol and stained with Giemsa at pH 7.1. The smears were viewed at 1,000× magnification under oil immersion using an Olympus AX70 microscope. One *Babesia* spp.-positive smear was verified at the Institute of Medical Microbiology, Friedrich-Schiller-University in Jena, Germany, using a Zeiss Axioskop 40 microscope. Micrographs were recorded using a Canon PowerShot G6 digital camera and viewed with the programme Adobe Photo Shop Elements 4.0.

## RESULTS

*A. phagocytophilum* DNA was detected in one blood sample (3.3%, n=1/30 tested samples). A similar result was obtained for the DNA of *Babesia* spp., i.e. only one tested sample was positive (3.3%, n=1/30 tested samples). Thus, DNA of *A. phagocytophilum* or *Babesia* spp. was found in the blood of individuals suspected of Lyme borreliosis based on clinical observations and positive results of serological and/or molecular studies (both 4.2%, n=1/24).

Sequence analysis of the amplicon produced using primers HER521/EHR747 revealed 99.9% homology with a recently described 16S rRNA gene sequence from *A. phagocytophilum* isolated from an individual in Poland [13]. Our isolate was also closely related to other strains of *A. phagocytophilum* originally isolated from *Microtus oeconomus* in Poland, *I. ricinus* in Spain and Germany, as well as from a human in Slovenia (Fig. 1).

The fragment amplified using primers Bab 18S for/Bab 18S rev showed 98.9% homology to the 18S rRNA gene sequence of a recently described *B. divergens* isolated from roe deer in Slovenia and *I. ricinus* in Poland, as well

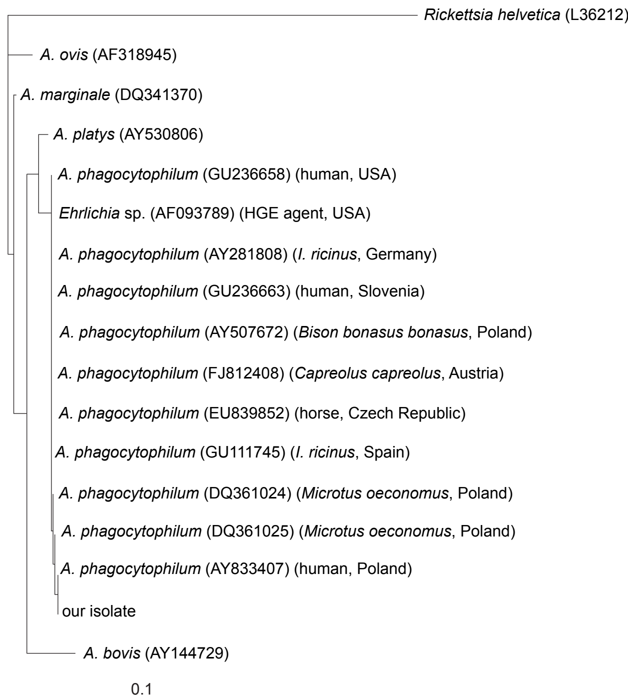


**Figure 1.** Phylogenetic tree of *Anaplasma* isolates from human blood and other *Anaplasma* species generated from 16S rRNA gene sequences by the neighbor joining method. The GenBank accession numbers of the 16S rDNA sequences used for phylogenetic analysis are given in parentheses. *Rickettsia helvetica* was used as an outgroup.

as to *Babesia* EU1 isolated from *I. ricinus* and roe deer elsewhere in Europe (Fig. 2). Examination of a smear of the *Babesia*-positive blood sample stained with Giemsa revealed *Babesia* spp. infection with a very low level parasitemia of 0.02% (Fig. 3).

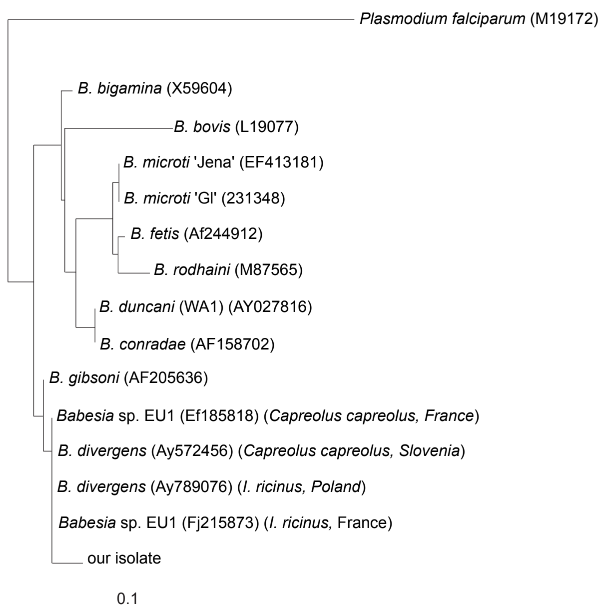


**Figure 2.** Phylogenetic tree of *Babesia* isolates from human blood and other *Babesia* species generated from 18S rRNA gene sequences by the neighbor joining method. The GenBank accession numbers of the 18S rDNA sequences used for phylogenetic analysis are given in parentheses. *Plasmodium falciparum* was used as an outgroup.

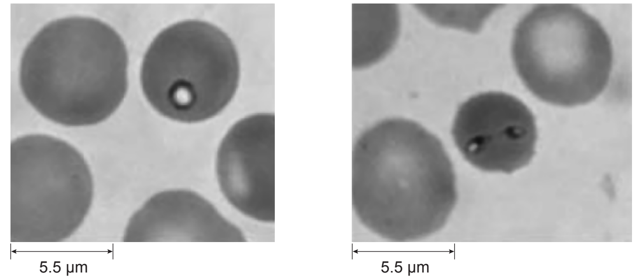


**Figure 3.** Photomicrographs of *Babesia* spp. in Giemsa-stained peripheral blood smears showing the characteristic ring forms in infected and double-infected erythrocytes.

## DISCUSSION

The purpose of this study was to retrospectively investigate the risk of co-infection with *Borrelia* spp., *A, phagocytophilum* and *Babesia* spp. in tick-exposed individuals from southeastern Poland. Co-infection of the vector *I. ricinus* with different pathogens appears to be quite common in Europe [14, 28, 31]. In Poland, the prevalence of co-infected *I. ricinus* ticks harbouring two pathogens in combination (*Borrelia* spp./*A. phagocytophilum*; *Borrelia* spp./*Babesia microti*; *A. phagocytophilum*/*Babesia microti*) varies from 0.12%–8.30%, depending on the site of tick sampling [34, 35, 36, 42, 45]. However, the combined presence of all three pathogens is rare in adult ticks, occurring in only 0.06%–1.1% of cases. Since all three pathogens share a common tick vector and can occur in the same endemic zoonotic reservoir, their acquisition, co-infection, and transmission to humans is quite possible. Such co-infections may have medical significance as symptom severity is frequently increased [4]. However, the mechanisms by which, for example, Lyme borreliosis and human babesiosis and/or anaplasmosis could potentiate the severity of one another remain unknown. These tick-borne diseases also share many of the same quite non-specific symptoms, making it difficult to differentiate between the infections in their early stages [37]. Human co-infections with the pathogens *Borrelia* spp. and *A. phagocytophilum* with clinically (erythema migrans) and serologically (seroconversion) confirmed Lyme borreliosis as well as asymptomatic anaplasmosis (positive serology or PCR for *A. phagocytophilum*) have been described in Poland [12, 17] and other countries [23, 25]. Nevertheless, the few previous reports of such co-infections indicate that the resulting disease is more severe and prolonged [39]. Since the late 1950s, two species of *Babesia*, *B. divergens* from cattle in Europe and *B. microti* from rodents in the northeastern and upper midwestern parts of the USA, have been shown to infect humans [26]. The species *B. microti*, *B. divergens*, *B. odocoilei*-like, and *Babesia* EU1 are known to be prevalent in *I. ricinus* ticks across Europe, including Poland [9, 15, 33, 43]. In the present study, molecular techniques were used to verify the first human infection with *Babesia* spp. recorded in Poland. Disappointingly, however, despite the relatively

high prevalence of ticks infected with *B. microti* (1%–4%) [33], there have so far been no reports of ticks infected with *B. divergens* or *Babesia* EU1. In the present study, the separate individuals infected with *A. phagocytophilum* and *Babesia* spp. were both co-infected with *Borrelia* species. Unfortunately, their clinical details are not known, but the fact that they were not hospitalized suggests that they presented with mild symptoms. However, the frequency of co-infection was low (4.2%, 1 out of 24 individuals seropositive for *Borrelia* spp.) and the small number of co-infected subjects makes statistical comparisons meaningless.

The identification of the first human case of *Babesia* spp. infection in Poland with homology of 98.9% to *B. divergens* or *Babesia* EU1 indicates that human babesiosis does occur in Poland and also in other European countries where *Babesia* spp. have been detected in free-living ticks. The lack of reported human cases may be due to low medical awareness of this infection and the absence of well-evaluated diagnostic tools such as serological tests or molecular biological assays for routine detection in diagnostic laboratories [21].

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