

## DETECTING DNAs OF *ANAPLASMA PHAGOCYTOPHILUM* AND *BABESIA* IN THE BLOOD OF PATIENTS SUSPECTED OF LYME DISEASE

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**Abstract:** Co-occurrence of granulocytic anaplasmosis, borreliosis and babesiosis in humans is a result of common vectors for the respective pathogens of these diseases, most commonly ticks from the genus *Ixodes*. Studies on ticks in Europe and also in Poland have shown that several pathogens may co-occur in individuals of *I. ricinus*.

A total of 96 hospitalised patients infected or suspected of being infected with borreliosis were screened for *A. phagocytophilum* and *Babesia* sp. DNA. Positive results of PCRs for *A. phagocytophilum* DNA were obtained for 10 patients, 8 of whom were diagnosed with borreliosis earlier, and 4 of whom were diagnosed with tick-borne encephalitis (on the basis of serological studies of serum and cerebrospinal fluid). None of the 10 patients had clinical or biochemical markers of anaplasmosis, corroborating the existence of asymptomatic anaplasmosis or self-limiting course, in Europe. Similarly, *Babesia* DNA was not found in the blood of any of the patients. The results of the studies show that in diagnosing tick-borne diseases, clinical examinations should consider infection by two or even three tick-borne pathogens.

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### INTRODUCTION

Tick-borne encephalitis (TBE) and borreliosis (Lyme disease) are the best known and most commonly diagnosed tick-borne diseases. Many studies have shown that infection by the spirochete *Borrelia burgdorferi* inducing borreliosis is often accompanied by coinfection with TBE (tick-borne encephalitis), *Babesia*, *Anaplasma* and *Ehrlichia*. Coexistence of granulocytic anaplasmosis, borreliosis and babesiosis in humans stem from the presence of common vectors for the respective pathogens

of these diseases, most commonly ticks from the genus *Ixodes*. In Europe, this is the common tick *Ixodes ricinus*. In vector ticks, infection with one of the agents frequently accompanies infection by the others.

In many European countries, including Poland [10], Lyme disease risk groups or those infected with the disease also possess antibodies against *Anaplasma phagocytophilum* (formerly *E. phagocytophilum*, the so-called HGE agent), a species of bacteria inducing granulocytic anaplasmosis. This corroborates the hypothesis that natural centres of *A. phagocytophilum* are

co-distributed with the former. The first clinical case of HGE in Europe was reported from Slovenia [17]. Studies by Lotrič-Furlan *et al.* [15] revealed the existence of further cases of HGE in this country. In all, 10 cases of HGE infection were recorded from Slovenia up to the year 2000 [16].

The first case of granulocytic anaplasmosis (HGE early) in Poland was described in 2001 by Tylewska-Wierzbanowska *et al.* [22]. Using the IFA method, antibodies against *A. phagocytophilum* were found in 2 out of 3 patients suspected of infection from northeastern Poland. This result was also supported by the PCR method. One of the patients was also shown to possess *B. burgdorferi* DNA. This suggests that areas in which Lyme disease occurs are often associated with the distribution of *A. phagocytophilum*.

The first diagnosed and described case of human babesiosis caused by *B. bovis* in Europe was reported from a Yugoslavian farmer in 1957 [4, 11]. In North America, the first incidence of babesiosis induced by *B. microti* was described in 1969 [9]. Thus far in Poland, only 1 case of imported human babesiosis has been described [12], although our studies of the vector *I. ricinus* [18-20] and the reservoir *Clethrionomys glareolus* [1] support the occurrence of *Babesia* in Poland.

In this study, patients hospitalized in the Clinic of Infectious Diseases and Neuroinfections, (Medical University of Białystok) infected or suspected of being infected with borreliosis or TBE [13].

## MATERIAL AND METHODS

The study involved 96 patients hospitalised in 2002 at the Clinic of Infectious Diseases and Neuroinfections (Medical University of Białystok) infected or suspected of being infected with borreliosis and TBE [16]. The group, consisted of 43 women and 53 men at age  $x = 48$  years (from 17 to 71 years) were assessed for the presence of *A. phagocytophilum* and *Babesia* sp. DNA. The patients were divided into 5 groups: group I with erythema migrans (9 individuals), group II with neuroborreliosis (11 individuals), group III with borreliosis induced inflammation of joints (arthralgia) (36 individuals) and group IV with early disseminated Lyme borreliosis (18 individuals). Borreliosis was ruled out in 22 patients (group V). Diagnosis of clinical symptoms was based on clinical observation and positive results of serological studies (ELISA) using Recombinant-Borrelia IgM and IgG tests (Biomedica), corroborated by Western-blot assays. Additionally, patients suffering from neuroborreliosis were subjected to serological tests of cerebrospinal fluid. Blood samples in EDTA for PCRs were sampled during the first few days of hospitalisation before antibiotic treatment and preceding diagnosis. Positive PCR results asserting the presence of *Borrelia burgdorferi s.l.* DNA were obtained for 24 of 96 patients (25%) [13]. DNA was isolated from the 96 blood samples using the QIAamp® DNA Mini Kit (Qiagen, Germany).

**Amplification of *A. phagocytophilum* DNA.** PCR was performed using the oligonucleotide primers LA1 and LA6 to amplify a fragment of the *epank1* gene specific-species for *E. egui*, *E. phagocytophila* and the HGE agent and yielding a product of 444bp [23]. As the standard sample, we used HGE agent DNA that the Molecular Laboratory of the Department of Genetics, University of Szczecin, received by courtesy of Professor Yasuko Rikihisa from the Department of Veterinary Biosciences, Ohio State University, USA. The PCR was always carried out together with a negative control, i.e. without DNA.

The thermal profile was as follows: initial denaturation at 94°C, 2 min, 8 cycles; denaturation 92°C, 30 s, annealing 62-56°C, 30 s (temperature was decreased by 2°C every two cycles), elongation 72°C, 30 s and 28 cycles: denaturation 94°C-30s, annealing 54°C-30 s, elongation 72°C-30 s. The amplification products were visualized by electrophoresis on 2% agarose gels stained with ethidium bromide.

**DNA amplification of *Babesia*.** A fragment of the  $\beta$ -tubuline gene was amplified. PCR was performed using primers F34 and R323. The thermal profile of Caccio *et al.* [5] was applied. PCR products were visualized on 2% agarose gels. Reactions were carried out in a Biometra thermal cycler (Austria). As positive amplification controls, 1 ng of *B. microti* merozoite DNA from the University of Warsaw, Poland and 10 ng of *B. divergens* merozoite DNA from the Precigout Laboratoire de Biologie Cellulaire et Moléculaire Montpellier, France were used.

## RESULTS

DNA of *A. phagocytophilum* was detected in 10 blood samples (10.42%) of 96 infected or potentially infected patients (Tab. 1). *B. burgdorferi s.l.* DNA was previously detected in 3 of these 10 individuals, 8 had been clinical diagnosed with borreliosis beforehand [13] and 4 were diagnosed with tick-borne encephalitis (on the basis of serological tests of serum and cerebrospinal fluid). Two of the latter were also diagnosed with borreliosis on the basis of serological results and clinical symptoms.

DNA of *A. phagocytophilum* was not found in the blood of patients from group I with erythema migrans. *A. phagocytophilum* DNA was detected in 2 individuals from group II (with neuroborreliosis). One of these patients was hospitalised because of tick-borne encephalitis and neuroborreliosis. The course of the disease was very severe and complicated with lapses in consciousness and limb paralysis. Biochemical markers were not found in this patient, i.e. thrombocytopenia, the presence of morule in blood cells and increased aminotransferase activity. Hepato-splenomegalia was not recorded either. In group III (inflammation in joints), PCR amplification of *A. phagocytophilum* DNA was successful in 4 cases (2 women and 2 men). One of these patients was also positive for *B. burgdorferi s.l.* DNA. Similar to

**Table 1.** Groups of patients hospitalized in the Clinic of Infectious Diseases and Neuroinfections in Białystok during 2002 and assessed for DNA of tick-borne pathogens *A. phagocytophilum* and *Babesia* sp.

Group	Symptom	No of patients	No. of DNA <i>A. phagocytophilum</i> positive	No. of DNA <i>Babesia</i> sp. positive
I	EM	9	0	0
II	Neuroborreliosis	11	2	0
III	Arthralgia	36	4	0
IV	Disseminated LB	18	2	0
V	No borreliosis symptoms/or TBE	22	2	0
Total		96	10	0

group II, biochemical and clinical symptoms of *A. phagocytophilum* infection were not detected. In group IV (disseminated Lyme borreliosis) 2 men were positive for *A. phagocytophilum* DNA, in 1 of them *B. burgdorferi s.l.* DNA was also amplified. The other patient was hospitalized with tick-borne encephalitis and clinically and serologically determined borreliosis. The course of TBE in this patient was severe and chronic. In group V (patients suspected of borreliosis), 2 individuals were positive for *A. phagocytophilum* DNA. Coexistence of the latter pathogen with *B. burgdorferi s.l.* was revealed in 1 of the patients. Significantly, both patients had been diagnosed with tick-borne encephalitis, proved by the presence of IgM antibodies against the TBE virus in their cerebrospinal fluid and serum. The course of the disease was more severe in all patients in which coinfection with the TBE virus or *B. burgdorferi* occurred. All blood samples screened for *Babesia* DNA were negative.

## DISCUSSION

Our studies on the vector *I. ricinus* for the presence of tick-borne pathogens [19, 20] in northwestern Poland revealed double (*B. burgdorferi s.l.* and *Babesia microti*) and triple (*B. burgdorferi s.l.*, *B. microti* and HGE agent) coinfection of ticks. Positive PCR results for *B. burgdorferi s.l.* DNA were shown for 16,7% of the *I. ricinus* population, 13,3% for *B. microti* DNA and 4,5% for *A. phagocytophilum* DNA. A similar assessment for human granulocytic anaplasmosis and the etiological factor of Lyme borreliosis in *I. ricinus* in northern Poland was carried out by Stańczak *et al.* [21]. *A. phagocytophilum* DNA was recorded in 14,85% of the studied ticks, *B. burgdorferi s.l.* in 6,6%, and coinfection by *B. burgdorferi s.l.* and *A. phagocytophilum* detected in 5% of the population of *I. ricinus*. In the study by Cisak *et al.* [7], TBEV and *B. burgdorferi s.l.* was isolated from a pool of 24 *I. ricinus* ticks collected from eastern Poland.

*Anaplasma phagocytophilum* (= *E. phagocytophila*), formerly known as the HGE agent, induces a tick-borne zoonosis - ehrlichiosis (HGE - human granulocytic ehrlichiosis), now named human anaplasmosis (HGA).

Clinical symptoms appear several days (often 7) after a tick bite. After penetrating the skin, anaplasmae disperse through lymph and blood vessels attacking granulocytes. Human anaplasmosis is a disease affecting many bodily systems and usually has a mild course. Its due course normally runs from between 2-3 weeks to 2-3 months. Flu-like symptoms dominate in the early phase of the disease: fever, headache and muscle ache. After a few days, nausea, vomiting, diarrhoea, stomach ache, coughing and shortness of breath appear. Enlargement of lymph nodes, rash, and periods of unconsciousness may also occur. The symptoms listed above are not specific. The most severe complications in anaplasmosis include breathing failure, kidney failure, damage to the central nervous system, and alimentary canal haemorrhage.

Laboratory studies reveal leucopenia, thrombocytopenia, increased activity of aminotransferases, lactic dehydrogenase, and an increased concentration of urea and creatinine. Cerebrospinal fluid is characterized by a small increase of lymphocytosis and larger concentrations of proteins. In Rtg chest examinations, in almost half of infected patients, lung inflammation can be observed [8, 14, 17].

Walls *et al.* [23] examined 31 blood samples for human anaplasmosis (HGA) from patients in New York, Minnesota and Wisconsin. An immunofluorescent antibody assay (IFA) revealed 21 positive samples. Results for the control group were negative. The same group of samples was subjected to PCRs with primers for 2 different genetic markers specific for *A. phagocytophilum*: a conservative fragment of the 16S rRNA (primers GE9 and GE10) gene and a fragment of the *epank1* gene specific for the *A. phagocytophilum* genogroup (primers LA6 and LA1). In 21 patients in which antibodies against *A. phagocytophilum* were detected, only 10 were amplified with primers GE9 and GE10; however, 20 were amplified by primers LA6 and LA1. Studies by Walls *et al.* [23] suggest higher specificity of the *epank1* gene in detecting the HGA agent and its important role in diagnostics of the disease. This is why we used primers complementary to a fragment of the *epank1* gene in PCRs for *A. phagocytophilum* DNA.

Anaplasmosis detection can be corroborated by cultures, blood smear examinations (Wright, Giemsa-stained smears), serology, or PCR on an acute-phase blood sample. In Europe, HGE verification is based on PCR and the immunofluorescent test (IFA). In the USA, the Center for Disease Control (CDC) established the case definition of HGE in 1997 (CDC, 70 u Blanco), but this is being revised. Anaplasmosis is described as an acute illness, accompanied by headache, myalgias, rigors and/or malaise for which 1 of 3 laboratory criteria must be present: 1) seroconversion; 2) a positive PCR; or 3) the presence of morule in blood, bone marrow, or CSF leucocytes, and an IFA antibody titer <64. Recently, Blanco and Oteo [3] have suggested that because a positive PCR result in the absence of either serological evidence or the isolation of anaplasmae could be a false

positive, the confirmation of a case would require a positive PCR and detection of morule.

However, morule were absent in the described cases of human anaplasmosis in Europe, although European HGA cases have a less severe course [3]. In European countries, infection by *A. phagocytophilum* is usually referred to as asymptomatic [24], asymptomatic and self-limiting course [2] or asymptomatic or mild [6]. Our studies have shown similarly that PCR+ cases do not always have the clinical symptoms or biochemical markers characteristic for *A. phagocytophilum* infection, i.e. thrombocytopenia, the presence of morule in blood cells and increased aminotransferase activity. Hepato-splenomegalia was not recorded either. Our PCR based study of 96 patients (serum, cerebrospinal fluid and knee joint fluid) from the Clinic of Infectious Diseases and Neuroinfections (Medical University of Białystok) revealed 24 (25%) PCR positive cases for *B. burgdorferi* s.l. DNA [12]. PCRs for *Babesia* and *A. phagocytophilum* DNA in the same group did not reveal the presence of the former pathogen; the latter, however, occurred in 10 (10.42%) patients, with 3 of these also positive for *B. burgdorferi* s.l. DNA. Additionally, ehrlichiae DNA was present in the blood of 2 men from the group of patients in which borreliosis was ruled out. All other cases positive for *A. phagocytophilum* DNA had been diagnosed earlier with different forms of Lyme borreliosis, and *B. burgdorferi* s.l. DNA was detected in 2 of these patients. Among the 10 patients with *A. phagocytophilum* DNA, 4 were diagnosed with tick-borne encephalitis (on the basis of serological tests of serum and cerebrospinal fluid). Two of the latter were also diagnosed with borreliosis on the basis of serological results and clinical symptoms. The course of the disease was more severe in all patients in which coinfection with the TBE virus or *B. burgdorferi* occurred. It seems that the severity of the course of the disease may be influenced by two or three pathogens, i.e. the TBE virus, *B. burgdorferi* and *A. phagocytophilum*.

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