

## HUMAN ANAPLASMOSIS IN NORTH-EASTERN POLAND: SEROPREVALENCE IN HUMANS AND PREVALENCE IN *IXODES RICINUS* TICKS

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**Abstract:** Sera of 500 inhabitants of north-eastern Poland, 450 suspected for Lyme borreliosis and 50 blood donors (control group), were analysed for the presence of IgG antibodies against *Anaplasma phagocytophilum*, the causative agent of human anaplasmosis (HA), known so far as human granulocytic ehrlichiosis (HGE). Forty one (9.1%) sera of the study group and one serum (2%) of the control group were positive using indirect fluorescence assay (IFA). The seropositivity tended to be more frequent among males (10.3%) than females (7.6%) and among the rural (10.3%) than urban population (7.5%); however, differences were of no statistical significance ( $p = 0.4$ ). No age difference was found between the seropositive and the seronegative individuals ( $p = 0.77$ ). The only factor increasing the risk of HA seropositivity found was forestry employment ( $p < 0.05$ ). Additionally, a total of 559 *Ixodes ricinus* ticks, collected in the same area as sera, were investigated for the presence of *A. phagocytophilum* by the polymerase chain reaction (PCR) and 41 (8.7%) of them were found to be positive. The infection level ranged from 2.3–13.7%, depending on the area studied. Bacteria were significantly less frequently detected in nymphs - 2.1% (5/235) than in adult ticks - 13.6% (44/324) and in males - 4.2% (74/165) than in females - 23.3% (37/159) ( $p \leq 0.05$ ). The obtained results confirm both the occurrence of HA foci in north-eastern Poland with *I. ricinus* as the principal vector of the *A. phagocytophilum* infection, and forestry workers as the main group at risk.

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### INTRODUCTION

Human anaplasmosis (HA), previously known as human granulocytic ehrlichiosis (HGE), is an emerging tick-borne infectious zoonosis characterised by headache, fever, myalgia, malaise, thrombocytopenia, and elevated liver enzymes. Occasionally, gastrointestinal and respiratory

symptoms are involved. The disease can vary from subclinical or mild to sporadically severe or even fatal. Between 17–54% patients are hospitalised [2, 8].

The precise taxonomic status of its causative agent has been definitively established in a recent reclassification. The formerly so-called “HGE agent”, along with the closely related veterinary pathogens: *Ehrlichia phagocytophila*

and *Ehrlichia equi*, have been considered one species, designated to the genus *Anaplasma* and renamed *A. phagocytophilum* corrig. (Foggie 1949) [7, 18].

The first clinical case of molecularly confirmed human anaplasmosis was described in Slovenia in 1997 [21] and in Poland in 2001 [31]. The epidemiology and ecology of the disease is under intensive evaluation. Several seroepidemiologic studies in different European countries have demonstrated prevalence of antibodies to *A. phagocytophilum* in approx. 10% of tick-exposed humans [2, 4, 8, 9, 13, 30]. Concerning Poland, the Białowieża Primeval Forest focus has recently been serologically and molecularly identified [10].

The objectives of this study were to estimate the prevalence of antibodies against *A. phagocytophilum* among north-eastern Poland inhabitants at different risks of exposure to ticks, and to evaluate the infection level of *Ixodes ricinus* ticks with the HA agent in the same area studied.

## MATERIALS AND METHODS

**Serum samples and indirect immunofluorescence assay (IFA).** A total of 450 serum samples, including 365 sera of adults (mean age 42.6 years, SD = 12.5, male/female ratio 1.2:1) and 85 sera of children (mean age 10.8 years, SD = 4.1) were enrolled in the study. All of them were collected between September 1999–December 2000 and originally submitted to the Department of Infectious Diseases, Medical University of Białystok for serological diagnostics of Lyme borreliosis. Antibodies to *Borrelia burgdorferi* were assayed by the commercial enzyme-linked immunosorbent assay (ELISA) test kit (Biomedica), according to the manufacturer's instruction. Positive or equivocal results were confirmed by Western Blott (DPC Bierman, Germany).

All sera derived from inhabitants of the 3 areas of north-eastern Poland, known as endemic for tick-borne encephalitis and Lyme borreliosis: the Białowieża Primeval Forest Region (Puszcza Białowieska), the city of Białystok and surrounding areas, and the eastern part of the Mazurian Lake District. The control group consisted of 50 age-matched blood donors, denying tick bites.

Samples were stored at -20°C until the time of analysis for the presence of anti-*A. phagocytophilum* antibodies that was performed within 6 months from the time of collection. Two serum samples from Polish patients with acute HA, acquired and confirmed during their stay in Connecticut (USA) two months prior to the present study, were tested as additional positive controls.

The commercial IFA kit (HGE-IFA IgG Test Kit, MRL Diagnostics, USA) was used to detect IgG antibodies against *A. phagocytophilum* antibodies. The serum screening dilution was 1:64 according to the manufacturer and titres  $\geq$  1:64 were considered positive. Positive samples were titrated to 1:512 dilution.

A questionnaire concerning age, employment (in the forest or not), area of residence, rural or urban environment and tick bites was answered by every participant.

**Ticks sampling, DNA extraction and polymerase chain reaction (PCR).** Questing adults and nymphs of *Ixodes ricinus* were collected in 16 different sites in the Mazurian Lake District (June–September 1999), the city of Białystok and its vicinity (May 2001), and in the Białowieża Primeval Forest (April 2001). Collections were performed by flag dragging over vegetation alongside deer trails, along forest paths and at the edge of the forest. All specimens were transported alive to the laboratory where they were fixed in 70% ethanol for further investigation by PCR.

The DNA was extracted from each individual tick by lysis in ammonium hydroxide (NH<sub>4</sub>OH) [23]. Templates were stored at -20°C. The primer set EHR 521 and EHR 747, designated to amplify a fragment of 16S rDNA [19], was used to detect the presence of DNA of *A. phagocytophilum*. Tick lysates from positive reactions obtained in our previous investigations, authenticated by DNA sequencing [10], served as positive controls while negative controls used double distilled water (DDW).

All reactions were carried out in Perkin Elmer GeneAmp PCR System 2400 thermal cyclers according to published protocol [19]. The resulting amplification products were visualised by ethidium bromide staining and 2% agarose gel electrophoresis with Tris-Borate-EDTA (pH 8.2) as running buffer. Samples were considered positive if the expected 247 bp fragment was seen.

**Statistical analysis.** Comparison of categorical variables was performed with two-sided Fisher exact test or Pearson's  $\chi^2$  test, as appropriate, using Statistica PL software. Log-linear analysis was applied to confirm the correlations between HA seropositivity and selected variables.

Odds ratio and 95% confidence intervals were calculated with Epi Info software (Centres for Disease Control and Prevention). Mean age between the groups was compared by the Mann-Whitney test. For all statistical analyses,  $p \leq 0.05$  was considered statistically significant.

## RESULTS

**Serologic analyses.** We encountered seroreactivity for *A. phagocytophilum* in 9.1% (41/450) individuals tested and in one out of 50 healthy blood donors (2%). Although the IgG antibody titres ranged from 1:64–1:512, in the majority they were low. Of 41 positive sera, 28 had titres 1:64; six had titres 1:128, four had titres 1:256 and three had titres 1:512.

The highest seropositivity was detected among the inhabitants of the Mazurian Lake District - 11.2% (14/125) followed by the Białystok area - 10% (19/190) and the Białowieża Primeval Forest region - 5.9% (8/135), but the differences were not considered significant ( $p = 0.287$ ).

There was no age difference between seronegative and seropositive individuals; mean age 36.3 vs. 36.9 years, respectively, ( $p = 0.77$ ). Seropositivity was observed more

**Table 1.** Prevalence of antibodies against *Anaplasma phagocytophilum* among 450 residents of north-eastern Poland.

Group	n	No. (%) of seropositive <sup>a</sup>	Univariable analysis			Multivariable log-linear analysis
			p <sup>b</sup>	OR <sup>c</sup>	95% CI <sup>d</sup>	p
Males	253	26 (10.3)	0.409	1.09	0.71-2.7	–
Females	197	15 (7.6)				
Rural inhabitants	263	27 (10.3)	0.406	1.41	0.72-2.78	–
Urban inhabitants	187	14 (7.5)				
Tick bites reported	371	36 (9.7)	0.096	1.31	0.49-3.47	–
Tick bites denied	66	5 (7.6)				
No data	13	–				
Forestry workers	168	28 (16.7)	0.0002	4.14	2.08-8.24	0.00003
Others	282	13 (4.6)				
EM reported	58	4 (6.9)	0.806	0.71	0.24-2.07	0.821
EM denied	392	37 (9.4)				
Total	450	41 (9.1%)				

n - number of participants; <sup>a</sup>IFA titer  $\geq 1:64$ ; <sup>b</sup>p  $\leq 0.05$  was considered statistically significant; <sup>c</sup>OR - odds ratio; <sup>d</sup>CI - confidence intervals.

frequently among males 26/227 (10.2%) than females 15/182 (7.6%) and among rural 27/263 (10%) than urban inhabitants 14/187 (7.5%), but the differences were not significant (p = 0.41) (Tab. 1).

Among 41 seropositive individuals, 36 (87.8%) recalled tick bites during the 12 months prior to the study while the rest of them denied attacks of ticks. However, prevalence of antibodies against *A. phagocytophilum* among those who reported and denied contact with *I. ricinus*, 9.7% (36/371) and 7.6% (5/66), respectively, did not differ significantly (p = 0.096) (Tab. 1).

Of 168 forestry workers, 28 (16.7%) were HA seropositive, while among the other serosurvey subjects 13/282 (4.6%), and this difference was statistically significant (p = 0.002) (Tab. 1).

No difference in anti-*A. phagocytophilum* antibodies seroreactivity was found in 58 participants reporting erythema migrans during the 12 months prior to the study and the remaining subjects (p = 0.81) (Tab. 1).

Among the analysed group, the positive IgM and/or IgG antibody seroreactivities for Lyme disease spirochete antigen were previously noted in 59 individuals (13.1%),

of whom 7 (11.9%) were also seroreactive against *A. phagocytophilum*. Detection of antibodies against *B. burgdorferi* did not influenced the prevalence of HA seropositivity (p = 0.464). For the purpose of statistical analysis, equivocal results (IgM in 6 cases and IgG in 4) were considered negative.

**Prevalence of infection in ticks.** A total of 559 individual *I. ricinus*, collected from 16 sites in 3 areas of north-eastern Poland, was screened for the presence of DNA of *A. phagocytophilum* by PCR. Positive results were obtained in 49 (8.7%) of them (Tab. 2). Prevalence of infection ranged from 2.3% in the Mazurian Lake District to 13.7% in the Białowieża Primeval Forest but did not differ statistically (p = 0.32). In the particular collection sites the percentages of infected specimens varied between 0–22.5%. Nymphs showed a significantly lower positivity rate - 2.1% (5/235), compared to adult ticks - 13.6% (44/324). The percentage of infected females - 23.3% (37/159) was 5.5 times higher than in the males - 4.2% (7/165). These differences were statistically significant (p < 0.05) (Tab. 3).

**Table 2.** Prevalence of *Anaplasma phagocytophilum* among questing *Ixodes ricinus* in the 3 different areas of north-eastern Poland (p = 0.32).

Study area (year)	Ticks		
	tested	infected	
	n	n	% $\pm$ SE
Mazurian Lake District (1999)	175	4	(2.3 $\pm$ 0.97)
Białystok and vicinity (2001)	165	15	(9.1 $\pm$ 0.64)
Białowieża Primeval Forest (2001)	219	30	(13.7 $\pm$ 2.4)
Total	559	49	(8.7 $\pm$ 1.4)

**Table 3.** Prevalence of *Anaplasma phagocytophilum* among nymphal and adult *Ixodes ricinus* in north-eastern Poland (p < 0.05).

Stage	tested		infected	
	n		n	% $\pm$ SE
Males	165		7	(4.2 $\pm$ 1.1)
Females	159		37	(23.3 $\pm$ 3.5)
Adults total	324		44	(13.6 $\pm$ 2.0)
Nymphs	235		5	(2.1 $\pm$ 0.7)

## DISCUSSION

Although serological studies cannot definitely determine whether the detected antibodies were stimulated by the specific infectious agent being assayed or whether the activity is the result of infection by a related organism, the indirect immunofluorescent testing for antibodies against *A. phagocytophilum* proved to be a sensible and sensitive method for diagnosing HA infection [12, 14] and was used in the present study.

The 5.9–11.2% prevalence of anti-HA agent antibodies in the inhabitants from north-eastern Poland revealed in our study correlates well with that noted in other European countries (7.3–15%) [6, 8, 9, 11, 19]. Local variations observed by us were of no statistical significance ( $p = 0.278$ ) which could be explained by lack of visible differences in environmental and climatic conditions in the 3 areas studied, followed by the statistically comparable infection levels of ticks with *A. phagocytophilum* noted there.

In our study, neither sex nor place of residence had any significant bearing on the HA seroprevalence. Although a positive correlation between the number of tick bites and seropositivity for *A. phagocytophilum* would be expected, we did not find any such correlation, just as previously by Bakken *et al.* [1] and Wittesjö *et al.* [32]. Correspondingly, Thomas *et al.* [30] and Dumer *et al.* [8] did not identify any relationship between self-reported tick bite and being positive for HA, HME or Lyme borreliosis. This can partially be explained by the small size of nymphs readily attacking humans that may not have been detected and therefore not reported. This results in difficulties in defining the groups according to the tick bite history.

Forestry employment was identified as the only risk factor of HA seropositivity in our study ( $p = 0.0002$ , OR = 4.14, CI = 2.08–8.24), which was confirmed with the log-linear analysis ( $p = 0.00003$ ). We encountered a similar dependency in the study concerning tick-borne encephalitis, also transmitted by *I. ricinus* in Poland [22]. On the contrary, results of our previous investigation from the local community of the Białowieża village revealed a lack of association between the HA seropositivity and the type of employment [12]. This could probably be explained by the fact that this particular village is located in the middle of the Białowieża Primeval Forest and its residents are permanently exposed to tick bites, even in their backyards or gardens, and spend a lot of their leisure time in the forest (e.g. picking wild mushrooms or berries).

Although other authors [2, 23] have shown that the Lyme borreliosis history and/or seropositivity had some bearing on the anti-HGE frequency, we did not find any such association.

Results of our seroepidemiological survey are supported by the results of tick investigations. By detecting *A. phagocytophilum* in *I. ricinus* collected in north-eastern Poland we provide further evidence of the occurrence of HA foci in this region. Although the

infected specimens were not distributed evenly among the collection sites and all 3 areas investigated, the differences were not statistically significant ( $p > 0.05$ ). The overall prevalence of *A. phagocytophilum* in ticks of 8.7% noted here differs from those reported from northern (19.2%) [28] and north-western Poland (1.4%) [25]. However, all three values are situated within the range of tick infection rates showed in the other European countries, for instance in Germany - 1.6% [9], in Sweden - 6.6% [29] and in Italy - 24% [5].

Most of the tick collection sites were located in popular tourist areas and/or in the National Park where there is an increased risk of exposure, not only to forestry workers but also to hikers, mushroom gatherers and berry-pickers whose knowledge about the HA is nil. There is therefore a need for awareness by tourists and inhabitants of the risk and symptoms of the disease. Besides, as an emerging zoonosis the disease is also little or almost unknown among physicians. Moreover, the known phenomenon of co-existence of antibodies against *A. phagocytophilum* and *B. burgdorferi* in humans [16, 17] and mixed infection with both pathogens in ticks [3, 28] suggests that one may acquire concurrent infections as a consequence of a single tick bite. Therefore there is the necessity of conducting simultaneous diagnostic test in persons suspected for HA or Lyme borreliosis. Recent studies supply the data on further threats represented by tick-bite, as *Babesia* spp. infections [15, 26, 27].

In conclusion, the obtained results confirm the perpetuation of *A. phagocytophilum* in north-eastern Poland, identify *I. ricinus* as a vector of the infection, and forestry employment as a risk factor for HA seropositivity. Further intensive surveillance should be undertaken, including its reservoir hosts, vector and tick-exposed populations, to clarify the epidemiology of human anaplasmosis in Poland.

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