ORIGINAL ARTICLES AAEM

Ann Agric Environ Med 2004, 11, 261–264

THE RISK OF EXPOSURE TO *ANAPLASMA PHAGOCYTOPHILUM* INFECTION IN MID-EASTERN POLAND

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Tomasiewicz K, Modrzewska R, Buczek A, Stańczak J, Maciukajć J: The risk of exposure to *Anaplasma phagocytophilum* infection in Mid-Eastern Poland. *Ann Agric Environ Med* 2004, **11**, 261–264.

Abstract: Both the presence of *Anaplasma phagocytophilum* in ticks and the seroprevalence of human granulocytic anaplasmosis have been reported in different parts of Europe. There are few reports concerning this problem in Poland. The aim of the study was to assess the prevalence of *Anaplasma phagocytophilum* in ticks, and to detect antibodies against the HGE agent in serum of forest workers in the region of Mid-Eastern Poland. In our opinion, this should reflect the real probability of infection of people exposed to *Ixodes* tick bites. Seroactivity against *Anaplasma phagocytophilum* was detected in 20.6% of persons in the study group. Coexistence of anti-*Borrelia burgdorferi* was present in 84.6% of individuals seropositive to *A. phagocytophilum*. The PCR test identifying *Anaplasma phagocytophilum* was positive in 13.1% of overall tick samples. The highest prevalence of infection (45.7%) was found in female ticks. Anaplasmal DNA was detected in 4.5% of male ticks and only in 0.9% of nymphs. The results of our study confirmed the existence of *A. phagocytophilum* in the natural environment of Mid-Eastern Poland. As the risk for infection exists, it should call the attention of public health services to the possibility of an increasing number of patients with this disease.

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Key words: ehrlichioses, Human Granulocytic Anaplasmosis, borreliosis, tick borne diseases.

INTRODUCTION

Infection by intracellular bacteria belonging to the genus *Ehrlichia* is an emerging public health problem in many areas of the world. Ehrlichioses were primarily described as infectious diseases of animals and therefore a veterinary problem. Nevertheless, in 1953, the first case of infection with the agent later named *Ehrlichia sennetsu* was described in Japan. In 1987, the first documented case of human monocytic ehrlichiosis (HME) was reported. Since that time, hundreds of well-documented *E. chaffeensis* infections were diagnosed in the United States. Bakken *et al.* in 1994 described the first case of human granulocytic ehrlichiosis (HGE) [1, 6, 11]. In 2001, Dumler *et al.* proposed reclassification of *E. phagocytophila* genogroup and creating a new species *Anaplasma phagocytophilum* [9].

The identification of new infectious agents prompted the development of research into their existence in different regions of the world, primarily in Europe and the

Received: 30 April 2004
Accepted: 24 November 2004

United States. In Europe, the first *E. chaffensis* infection was described in Portugal in 1991, and in Slovenia in 1997 the first molecularly confirmed clinical case of HGE was reported [21, 26]. Serologic evidence of granulocytic ehrlichiosis in humans has been also described in several European countries, including Germany, Sweden, Norway, Great Britain, Switzerland, France, Belgium and Italy [2, 3, 7, 10, 14, 16, 22, 24, 27, 31]. Poland officially "joined" this group of countries in 2000, when Grzeszczuk *et al.* published a preliminary report confirming the presence of human anaplasmosis in our country [12].

Anaplasma phagocytophilum was detected in ticks in several European countries, using molecular techniques. It is highly interesting that studies conducted in Great Britain, Slovenia and France revealed the genetic diversity of *A. phagocytophilum* [4, 14, 24, 25]. On the other hand, there was no genetic diversity among isolates in Switzerland and Italy [4, 8]. As many authors have noticed, the fact of using different primers in the polymerase chain reaction (PCR) should be considered while one interprets these results [4, 24].

Assessment of the scope *A. phagocytophilum* of the presence of in ticks in a particular region, and of the risk for exposure in a population is a great challenge for scientists. Reports confirming *A. phagocytophilum* infection in ticks in different European countries should call the attention of public health services to the possibility of an increasing number of patients with this little-known disease. Taking this problem into consideration, we started this study to verify whether *A. phagocytophilum* is present in ticks in the region of Mid-Eastern Poland. At the same time, we tested seroactivity against *A. phagocytophilum* in people exposed to *Ixodes* tick bites in this region, to assess the real probability for acquiring the infection.

MATERIALS AND METHODS

The serological studies of IgG against *A. phagocytophilum* were performed in 63 individuals (59 males and 4 females; mean age 48 ± 7.2 years), with occupational exposure to tick bites. The study group consisted of forest workers from Lublin province and the eastern part of Świętokrzyskie province (Starachowice district). All of study participants had a history of tick bites, usually multiple and during the preceding 12 months. Each participant was informed of the aim of the study and informed consent was given. All serum samples were frozen at -20ºC immediately after centrifugation. Special attention was paid to the history of Lyme borreliosis symptoms (particularly erythema migrans) and/or symptoms of anaplasmosis.

The control group consisted of 30 healthy blood donors (all males; mean age 35 ± 3.2), all of whom denied tick bites.

The presence of anti-*A. phagocytophilum* IgG was examined using an indirect immunofluorescnence antibody assay (IFA) test kit HE IFA IgG: Focus Technologies, USA. The laboratory assay was used according to the manufacturer' instructions. Results with titer ≥ 1:64 were considered as positive; and as negative, titers < 1:64. Simultaneously the serum was screened for anti-*Borrelia burgdorferi* antibodies IgG and IgM using immunoenzyme assay (ELISA) kit Borrelia IgM, IgG (Biomedica, Austria).

Detection of *Anaplasma* phagocytophillum in ticks was performed only in adult organisms and nymphs thought most commonly to attack humans. Ticks were collected in the same regions in which the screened persons resided. Samples were kept in 70% ethanol. Prepared ticks were investigated with PCR according to Rijpkema *et al.* method. The primers EHR 521 and EHR 747 were used to amplify a fragment of 16S rDNA specific for *Anaplasma phagocytophilum* [19, 20, 23].

DNA amplification was carried out in 25 µl of reaction mixture consisting of 0.65 U of Taq polymerase, 2.5 µl reaction buffer for polymerase, 2.5 ml 50 mM $MgCl_2$, 2.5 µl of 2.5 mM dNTPs mixture, 0.5 µl EHR 521 and 0.5 µl EHR 747 primers in concentration 10 µM each, 13.35 µl of sterile double-distilled water (DDW) and 2.5 µl of DNA sample.

In each PCR reaction, *Anaplasma phagocytophilum* infected HL60 cells served as the positive control, and DDW does as negative control. All reactions were carried out in Perkin Elmer Gene Amp PCR system 2000 thermal cyclers (Perkin Elmer, USA).

Detection of 247 bp specific amplification product was carried out by 2% agarose gel electrophoresis. The electrophoresis was accomplished with a current intensity of 500 mA and voltage of 150V.

Statistical analysis. Statistics of variables was completed using Pearson's χ^2 test, and $p \leq 0.05$ was considered statistically significant.

RESULTS

In the study group of forest workers seroactivity against *Anaplasma phagocytophilum* was detected in 13 of 63 individuals (20.6%) (Tab. 1). Coexistence of anti-*Borrelia burgdorferi* was present in 11 of 13 (84.6%) individuals seropositive to *A. phagocytophilum*; this was obviously statistically significant ($p < 0.05$).

None of blood donors (control group) had antibodies against *A. phagocytophilum*.

Simultaneously, we analyzed the study participants according to incidence of symptoms and serological evidences of *Borrelia burgdorferi* infection. Seropositivity was found in 60.3%. Surprisingly, 1 of 30 healthy blood donors (3.3%) tested positive for *Borrelia burgdorferi* IgG assay.

Of the 694 tick samples prepared, 91 (13.1%) were positive in the PCR test identifying *A. phagocytophilum*. The highest prevalence of infection was found in female ticks (79 of 173, = 45.7%). Anaplasmal DNA was detected in 9 of 202 male ticks (4.5%) and only in 3 of 319 nymphs (0.9%) (Tab. 2). The rate of infection was significantly higher in adult ticks than in nymphs ($p<0.05$).

Table 1. Prevalence of IgG against *A. phagocytophilum* and IgM/IgG against *B. burgdorferi* in the study population of forest workers.

Anti -*B. burgdorferi* IgM/IgG		Anti-*A. phagocytophilum* IgG	
		positive	negative
Positive	38 (60.3%)	11 (17.5%)	27 (42.9%)
Negative	25 (39.7%)	2 (3.2%)	23 (36.5%)
Total	63	13 (20.6%)	50 (79.4%)

Table 2. Prevalence of *Anaplasma phagocytophilum* in ticks collected during the study.

Tick developmental stage	Total number of examined ticks	No. of ticks infected with *Anaplasma phagocytophilum*
Female ticks	173	79 (45.7%)
Male ticks	202	9 (4.5%)
Nymphs	319	3 (0.9%)
Total	694	91 (13.1%)

DISCUSSION

In recent years, only a few reports have been published which confirm the existence of human granulocytic ehrlichiosis agent in ticks, and seropositivity for *Anaplasma phagocytophilum* in populations exposed to tick bites in Poland [12, 13, 29, 30].

For the first time, we report here the results of a study conducted in Mid-Eastern Poland. The area is rich in forests, wild animals, and is well known as a reservoir of ticks infected with *Borrelia burgdorferi*. According to official reports of Polish Institute of Hygiene, in the study area about 200 new cases of borreliosis are reported yearly.

The prevalence of antibody against *Borrelia burgdorferi* in our study group was 60.3%, much higher than that observed in a comparable study in northeastern Poland (24.6%) [13]. This result confirmed the frequent exposure to tick bites, including occupational risk of infection.

In detecting antibodies against *A. phagocytophilum* in 13 of 63 individuals (20.6%) we have proved the presence of *A. phagocytophilum* in our region in a quite high frequency. It is interesting to compare this result with another studies concerning the occurrence of *A. phagocytophilum* in other region of Poland – the Białowieża Primeval Forest [13]. The rate of seroprevalence in our study was even 3 times higher than that found by Grzeszczuk *et al.* in Białowieża Primeval Forest (6.2%). The reason for such a huge difference may be the choice of tested individuals, although both studies included mostly individuals with a history of tick bites (in the latter, only 4 of 130 denied tick bites) [13]. The frequency of anti-*A. phagocytophilum* in our study resembles that found in Germany (14%) and Switzerland (17%) [10, 28], and surprisingly was much higher than observed in Russia [32].

It is worth mentioning that in 11 individuals both antibodies against *A. phagocytophilum* and *Borrelia burgdorferi* were present. The coexistence of these markers of dual infection can be easily explained by similar a route of infection and epidemiological relationships. On the other hand, 2 of 13 individuals seropositive to *A. phagocytophilum* had no markers of borrelial infection. The results of our study are in agreement with those of other authors, that patients with Lyme borreliosis are far more likely to have the anti-HGE agent than seronegative controls [2, 13].

Investigating of *A. phagocytophilum* prevalence in vectors may help to assess the risk for infection in humans. In Poland, *Ixodes ricinus* is thought to be a principal vector in both Lyme borreliosis as well as human anaplasmosis. Our study included a huge, representative number of ticks, and the overall rate of infection in ticks was 13.1% (91 of 694). This percentage is higher, compared to results of similar studies in France, Sweden, and in China, Korea and Russia [5, 15, 24, 31, 32].

Few studies have been carried out in Poland concerning the presence of *A. phagocytophilum* in ticks that country. Stańczak *et al.* have isolated *A. phagocytophilum* in 19,1% *Ixodes ricinus* ticks in the Pomerania Region (North Poland) [30], and Grzeszczuk *et al*. have found the agent in 16% of ticks collected in the Białowieża Primeval Forest [13]. The prevalence of *A. phagocytophilum* infection in ticks studied by Skotarczak *et al.* in north-western Poland was much lower (4.5%) [29].

The rate of *A. phagocytophilum* infection varies in particular developmental stages of *Ixodes* ticks. We have observed the highest value in female adult ticks – 45.6% (79 of 173). Male ticks were infected significantly less frequently – 4.45% (9 of 202), and the lowest value was observed in nymphs – 0.94% (3 of 319). A similar percentage of infected nymphs has been noted by Polish authors [13], as well as by several others in Western Europe and the United States [17]. It should be mentioned here that there are reports with controversial results. British, Italian and Swedish studies have shown the infection rates in nymphs were greater in adult ticks [8, 14, 31]. The difference in *A. phagocytophilum* prevalence in adult and nymphal stages are probably connected to tick behavioral preferences and host dependence. Although all stages of *Ixodes ricinus* parasitize a large variety of animal hosts, larvae prefer small rodents, nymphs to birds and larger animals, and adults ticks-large mammals [4].

Because of the highly non-specific clinical manifestation of human anaplasmosis, appropriate diagnosis cannot be carried out practically based on the history of tick bites and symptoms. It should be confirmed with sensitive and specific laboratory tests; although an indirect immunofluorescence antibody assay, which is used for seroepidemiological surveys, cannot always confirm active disease and differentiate between disease and previous long-ago exposure to *A. phagocytophilum*. The

history of seropositive individuals in the study group revealed symptoms which could be caused by *Anaplasma* or other infectious agents, particularly *B. burgdorferi*. In the opinion of many authors, reliable diagnosis of human anaplasmosis can be achieved with PCR technique [14, 19, 20].

The results of our study confirm the existence of *A. phagocytophilum* in the natural environment of Mid-Eastern Poland and the risk for infection, particularly in some groups of the population. This has led us to the conclusion, that physicians, including primary care professionals, should become more aware of the clinical and epidemiological features of the infection.

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