

HIGH PREVALENCE OF *ANAPLASMA PHAGOCYTOPHILUM* INFECTION IN TICKS REMOVED FROM HUMAN SKIN IN NORTH-EASTERN POLAND

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Abstract: In order to examine the risk of *Anaplasma phagocytophilum* infection in humans, 76 *Ixodes ricinus* ticks and 3 *Dermacentor reticulatus* removed from humans were examined for the presence of *Anaplasma phagocytophilum* by PCR targeting the 16S rRNA gene. The average infection rate was 23.7% (18/76) in *Ixodes ricinus* ticks. A very high infection rate reaching 36.8% (14/38) was detected in female *Ixodes ricinus*, followed by males – 16.6% and nymphs – 10.5%. No infection was noted in larvae. All *Dermacentor reticulatus* also tested negative. The positive PCR products were sequenced and deposited in the Gen Bank (Accession No. DQ006828).

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INTRODUCTION

Hard ticks (Ixodidae) are ubiquitous ectoparasites of vertebrates. Increasing abundance and activity of these arthropods in recent years constitute a significant medical problem [3, 17, 25] as they can transmit many pathogens that may cause severe diseases in wild and domestic animals and humans. *Ixodes (I.) ricinus*, the most prevalent tick species in Poland, which is responsible for the majority of tick bites in humans, is a known vector of tick-borne encephalitis [2] Lyme borreliosis [12, 20], babesiosis [19, 23] and newly diagnosed human granulocytic anaplasmosis, HGA (formerly human granulocytic ehrlichiosis - HGE) [18, 21, 22]. The latter is caused by the recently renamed *Anaplasma (A.) phagocytophilum* [3] which is also an etiologic agent of equine granulocytic ehrlichiosis and tick-borne fever of sheep and cattle in Europe. *Dermacentor (D.) reticulatus*, biting humans

accidentally, carries among others, *Francisella tularensis* [9], the etiologic agent of tularemia, and rickettsiae of the spotted fever group (SFG) [24].

HGA was described for the first time in 1994 in the USA [1]. Since then, many studies have focused on ecology, pathogenicity and clinical presentations of *A. phagocytophilum* infections. We aimed to evaluate the prevalence of *A. phagocytophilum* in ticks removed from humans in the region of the highest tick-borne diseases infection rates in Poland.

MATERIAL AND METHODS

Ticks were removed from the skin of adult patients who reported to the Department of Infectious Diseases Emergency Room, Medical University of Białystok from March 2004 to September 2005. The ticks were killed in hot water, placed in separate vials and fixed in 70%

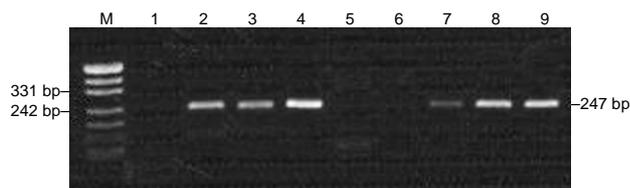


Figure 1. PCR amplification of *Anaplasma phagocytophilum* 16S rRNA gene fragment (247 bp) following DNA extraction from ticks removed from human skin; lane M - marker (PuC19 DNA/mspI Marker); lane 1 - negative control (double distilled water); lane 2 - positive control; lanes 3, 4, 7, 8, 9 - positive samples, 247 bp product; lanes 5, 6 - negative samples.

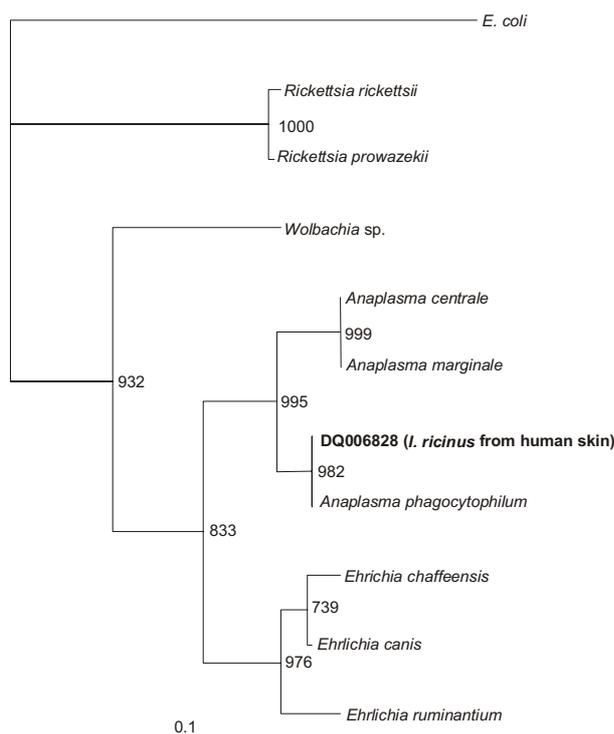


Figure 2. Dendrogram showing the relationship of the 216 bp fragment of 16S rRNA (rrs) gene of *Anaplasma phagocytophilum* from *Ixodes ricinus* ticks removed from human skin (Acc. No. DQ006828) and other related bacteria using ClustrX algorithm.

ethanol for further investigation for the presence of *A. phagocytophilum* by PCR. Each tick was individually evaluated prior to DNA extraction by a qualified entomologist with regard of species according to Siuda [17], gender and engorgement. The latter feature was estimated in comparison with our collection of ticks during different stages of feeding.

DNA extraction. DNA was extracted by the ammonium hydroxide lysis (NH_4OH) according to Rijpkema *et al.* [15]. All ticks were examined individually. Lysates were stored at -20°C until examining.

Polymerase Chain Reaction. PCR was performed according to Pancholi *et al.* [14]. The primers EHR 521 (5'- TGT AGG CGG TTC GGT AAG TTA AAG-3') and EHR 747 (5'- GCA CTC ATC GTT TAC AGC GTG -

3') were used to amplify the 16S rRNA gene fragment of *A. phagocytophilum*. The tick lysates from positive reactions obtained in our previous investigation [23] served as a positive control and the double distilled water as a negative control.

All PCR reactions were carried out in Perkin Elmer GeneAmp PCR System 9700 thermal cyclers. Amplification products were analysed after electrophoresis in a 2% agarose gel stained with ethidium bromide. DNA bands of 247 base pairs (bp) were considered as positive results (Fig. 1).

DNA Sequencing and data analysing. The PCR products of positive samples were purified and sequenced with an ABI Prism 3100 Genetic Analyser (Applied Biosystem, USA). Sequences were edited, assembled with the Vector NTI and/or BioEdit programme and compared with the gene sequences obtained from the GenBank database using the BLAST programme. Alignments and dendrograms were constructed using the ClustalX programme and neighbour - joining method [26]. Dendrograms were visualized using Tree View v. 1.6.1 [13]. The following reference bacteria were included in phylogenetic analysis: *Rickettsia rickettsii* (acc. No. M7322), *Rickettsia prowazekii* (Acc. No. M21789), *Wolbachia sp.* (Acc. No. DQ305292), *Anaplasma centrale* (Acc. No. AF283007), *Anaplasma marginale* (Acc. No. AF311303), *Anaplasma phagocytophilum* reference strain (Acc. No. U02521), *Ehrlichia chaffeensis* (Acc. No. M73222), *Ehrlichia canis* (Acc. No. M73226). *Escherichia coli* (Acc. No. DQ305292) was used as an out-group for reference comparison (Fig. 2).

Statistical analysis. Comparison of categorical variables was performed by chi-square test, $p \leq 0.05$ was considered statistically significant.

RESULTS

A total of 79 ticks were collected and investigated. They were identified as *D. reticulatus* - 3 males specimens and *I. ricinus* - 76 specimens. The majority of *I. ricinus* consisted of females ($n=38$), followed by nymphs ($n=19$), males ($n=12$) and larvae ($n=7$). Ticks were at different stages of engorgement (Tab. 1).

Ticks were mostly attached to the lower extremities ($n=34$; 43.0%) and groins ($n=8$; 10.1%), then to the trunk ($n=23$; 29.1%), neck and head ($n=14$; 17.7%) of the patients.

Altogether, DNA of *A. phagocytophilum* was detected in 18 out of 76 *I. ricinus* ticks (23.7%). The percentage of infected female ticks was very high, reaching (36.8%) while only 16.6% of males tested positive, differences not statistically significant. The lowest infection rate was detected in nymphs - 10.5%, which was significantly lower than in females. *I. ricinus* larvae were negative as were all 3 *D. reticulatus* examined (Tab. 1).

Table 1. Prevalence of *Anaplasma phagocytophilum* in *Ixodes ricinus* and *Dermacentor reticulatus* ticks removed from patients in Department of Infectious Diseases and/or Emergency Room, Medical University of Białystok.

Tick species	Number of positive/number of tested (% positive)				
	Larvae	Nymphs	Females	Males	Total
<i>Ixodes ricinus</i>	0/7**	0/7 3/12**	4/15* 9/18** 1/5***	2/12	
Total	0/7	2/19 (10.5) ^P	14/38 (36.8) ^P	2/12 (16.6)	18/76 (23.7)
<i>Dermacentor reticulatus</i>				0/3	
Total				0/3	0/3

* - slightly engorged, ** - partially engorged, *** - fully engorged, ^P - $p \leq 0.05$.

All PCR products amplified were identical to each other. Sequence homology searches revealed that they were 100% identical with the prototype sequence of *A. phagocytophilum* from a first described human patient from Wisconsin, USA (Acc. No U02521). The sequences were deposited in the GenBank under Accession No. DQ006828.

DISCUSSION

The 23.7% of *I. ricinus* ticks infected with *A. phagocytophilum* detected in our study is unexpectedly high. A north-eastern Italian study revealed only 1 (0.3%) *I. ricinus* positive for *A. phagocytophilum* of 357 ticks removed from 353 asymptomatic individuals [16].

None of the patients from our study who were infested with ticks reported back for medical consultation. This fact reflects either lack of transmission to humans, for instance due to a short duration of tick feeding, or either mild or asymptomatic *A. phagocytophilum* infection. The latter was observed in our earlier study among forestry workers who were highly exposed to ticks [4].

A. phagocytophilum has been of medical concern for a relatively short period of time, since its first description in human patients from Minnesota and Wisconsin, USA [1]. Several studies address tick infection rates in Poland [5, 6, 18, 21, 22, 27], but all focus on host-seeking ticks. *A. phagocytophilum* prevalence in ticks ranges from 0% in Głębokie Public Bath and Landscape Park of Ińsko [18] up to 13.1% in Lublin province and Starachowice district [27], 14% in the Tricity forests [23] and 16% in the Białowieża Primeval Forest [6]. However, all these studies corroborate that the highest infection rate is among females *I. ricinus* ticks, reaching 47.6% [23]. These Polish data are in contrast with German results, showing an equal *A. phagocytophilum* prevalence in males and females *I. ricinus* ticks [10].

Such a high infection rate with *A. phagocytophilum* may partly be a result of the blood meal triggering bacterial "reactivation" in infected ticks, the phenomenon observed with *Rickettsia rickettsii* in *D. andersoni* ticks

owing to bacterial replication in the tick. This in effect might lead to a higher yield of PCR products [8].

The small number of human patients described so far in Poland [7, 28] as well as in other European countries [11, 25] is in contrast with such a high prevalence of the pathogen in ticks. In our prospective clinical study only 2 confirmed and 2 probable cases of human granulocytic anaplasmosis were diagnosed out of 68 febrile patients after tick bite evaluated [7].

The existing disparity between high *A. phagocytophilum* infection rate in ticks, the seroprevalence in humans comparable to those in the USA and a low number of human cases in Europe, indicate the necessity for further studies on comparative *A. phagocytophilum* pathogenicity in Europe and North America.

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