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PREVALENCE OF *ANAPLASMA PHAGOCYTOPHILUM* IN *IXODES RICINUS* TICKS DETERMINED BY POLYMERASE CHAIN REACTION WITH TWO PAIRS OF PRIMERS DETECTING 16S rRNA AND *ankA* GENES

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Abstract: A total of 684 *Ixodes ricinus* ticks (321 nymphs, 184 males, and 179 females) were collected by flagging lower vegetation in 6 forest districts located on the territory of Lublin province (eastern Poland). Ticks were examined by polymerase chain reaction (PCR) method for the presence of *Anaplasma phagocytophilum* DNA with two pairs of primers: EHR521/EHR747 for detecting 16S rRNA gene, and LA6/LA1 for detecting *ankA* gene. To study the relationship between infection in ticks and people occupationally exposed to tick bite, blood serum samples of 261 forestry workers employed in the same forest districts were examined by immunofluorescence method for the presence of specific antibodies against *A. phagocytophilum*. A total of 70 ticks out of 684 examined (10.2%) showed the presence of *A. phagocytophilum* 16S rRNA gene. The prevalence of infection was significantly dependent on tick's stage (χ^2=49.2, p<0.00001) and geographical locality (χ^2=34.4, p<0.00001). The percentage of *I. ricinus* females infected with *A. phagocytophilum* (24.6%) was significantly greater compared to males (6.5%) and nymphs (4.4%) (p<0.00001). Only 19 ticks out of 684 examined (2.8%) showed the presence of *A. phagocytophilum ankA* gene, significantly less compared to 16S rRNA gene (p<0.00001). The prevalence of infection demonstrated by the presence of *ankA* gene was also significantly dependent on tick's stage (χ^2=23.6, p<0.00001) but not on locality (χ^2=9.8, p=0.082). A significant correlation was found between the presence of *A. phagocytophilum* 16S rRNA gene in *I. ricinus* female ticks from the particular forest districts and the serologic response to *A. phagocytophilum* of forestry workers employed in the same districts (p<0.05). No significant correlation was found between the presence of *A. phagocytophilum ankA* gene in *I. ricinus* ticks and serologic response of exposed workers. In conclusion, detection of *A. phagocytophilum* infection in ticks by PCR with the use of EHR521/EHR747 primers detecting 16S rRNA gene is significantly more sensitive compared to LA6/LA1 primers detecting *ankA* gene.

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INTRODUCTION

Ixodes ricinus ticks transmit a range of human and animal pathogens, including bacteria *Anaplasma phagocytophilum* causing human granulocytic anaplasmosis [1, 2, 4, 21]. The pathogen can be detected by serological tests and by polymerase chain reaction (PCR) disclosing the presence of various DNA fragments. The EHR521/EHR747 primers' pair detecting 16S rRNA gene coding ribosomal RNA has been used most often for the detection

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of *A. phagocytophilum* DNA with PCR method [4, 6]. However, more recently, the use of LA6/LA1 primers' pair disclosing *ankA* gene coding AnkA protein has been recognised as a more sensitive PCR modification [19, 27].

The primary aim of the present work was to compare the usefulness of the aforementioned pairs of primers in the detection of *Anaplasma phagocytophilum* DNA in *Ixodes ricinus* ticks collected in the Lublin region (eastern Poland). The second aim was to study the relationship between the prevalence of *A. phagocytophilum* DNA in *Ixodes ricinus* ticks collected in various forest districts of the Lublin region, and the prevalence of seropositive reactions to *A. phagocytophilum* in the forestry workers from the same districts. Preliminary results of this study were reported elsewhere [28].

MATERIALS AND METHODS

Collection of ticks. Ticks were collected during spring/summer season in 2005 from the following forest districts located in the Lublin region (eastern Poland): • Puławy, situated in the northwestern part of the region, harbouring dry upland forests; • Lubartów, situated in central part of the region north of the city of Lublin, harbouring dry upland forests; • Lublin, suburban recreational area "Dąbrowa" in the central part of the region south to the city of Lublin, harbouring dry upland forests; • Sobibór, situated in the eastern part of the Lublin region in the area of the Włodawa-Łęczna Lakeland, harbouring wet lakeland forests; • Zwierzyniec, situated in southeastern part of the region, on the area of Roztocze Highlands, harbouring dry highland forests; • Gościeradów, situated in the southwestern part of the region, harbouring dry upland forests. Unfed *Ixodes ricinus* ticks (females, males and nymphs) were collected by dragging a woolen flag over lower vegetation and litter along the paths and edges of deciduous and mixed forests. Collected ticks were placed in glass tubes with 70% ethanol for further investigation.

DNA isolation. A total of 684 *I. ricinus* ticks (321 nymphs, 184 males, and 179 females) were examined. Bacterial DNA was isolated from ticks after removal from ethanol and drying on filter paper by boiling in 0.7 M ammonium hydroxide, according to Rijpkema *et al.* [16], and stored at -20°C. Adult ticks were prepared separately while nymphs in pools of 5 specimens. Minimum infection rate in nymphs was calculated according to Kahl *et al.* [14].

Detection of *A. phagocytophilum* 16S rRNA gene by PCR. To amplify a fragment of 16S rRNA gene, the polymerase chain reaction (PCR) was carried out with the EHR521 (5'-TGT-AGG-CGG-TTC-GGT-AAG-TTA-AAG-3') and EHR747 (5'GCA-CTC-ATC-GTT-TAC-AGC-GTG-3') oligonucleotide pair of primers [8, 13, 15]. In each PCR reaction were applied: • matrix DNA, • EHR521/EHR747 primers (Eurogentec, Seraing, Belgium), • DNA of *A. phagocytophilum* retrieved from slides of the *Anaplasma phagocytophilum* IFA IgG kit (Focus Technologies, Cypress, California, USA) as a positive control, • redistilled water as a negative control, • thermostable polymerase (DyNAzyme™ II DNA, Finnzymes Oy, Espoo, Finland), • mixture of dNTP nucleotides (Fermentas, Vilnius, Lithuania). The amplification was carried out in a PTC-150 thermal cycler (MJ Research Inc., Waltham, MA, USA) in consecutive stages: • preliminary denaturation – 5 min at 94°C; • 40 cycles, each consisting of: 45 sec denaturation at 94°C, 45 sec annealing at 54°C, and 45 sec elongation at 72°C; • final elongation – 5 min at 72°C. The size of amplified DNA fragment was 247 base pairs (bp).

Detection of *A. phagocytophilum ankA* gene by PCR. To amplify fragment of *ankA* gene, the PCR was carried out with the LA6 (5'-GAG-AGA-TGC-TTA-TGG-TAA-GAC-3') and LA1 (5'-CGT-TCA-GCC-ATC-ATT-GTG-AC-3') oligonucleotide pair of primers (Eurogentec, Seraing, Belgium) [17, 27]. Other PCR reagents were the same as above. The amplification was carried out in a PTC-150 thermal cycler (MJ Research Inc., Waltham, MA, USA) in consecutive stages: • preliminary denaturation – 2 min at 94°C; • 8 cycles, each consisting of: 30 sec denaturation at 94°C, 30 sec annealing at decreasing temperatures (2 cycles at 62°C, 2 cycles at 60°C, 2 cycles at 58°C, and 2 cycles at 56°C), and 30 sec elongation at 72°C; • 28 cycles with the same parameters except for temperature of annealing which was steadily 54°C; • final elongation – 5 min at 72°C. The size of amplified DNA fragment was 444 bp.

Identification of PCR products. Amplification products were identified in 2% agarose gel (BASICA, LE, Prona, EEC), after electrophoresis in standard conditions and staining with ethidium bromide solution (2 μg/ml). Amplified fragments were visualised in a transilluminator under UV light (UV-953, JW Electronic, Warsaw, Poland).

Serological examination of forestry workers. To study the relationship between infection in ticks and people occupationally exposed to tick bite, a group of 261 forestry workers, 45 women and 216 men, mean age 42.2 ± 9.5 years, were examined in the years 2003-2004. The workers were employed in the same forest districts from which ticks were collected: Puławy (46 persons), Lubartów (39 persons), Dąbrowa (30 persons), Sobibór (47 persons), Zwierzyniec (55 persons), and Gościeradów (44 persons). As a control group, 56 male blood donors living in the city of Lublin, mean age 35.5 ± 9.6 years, were examined. The samples of peripheral blood were drawn by puncture of the elbow vein and serum was separated by centrifugation. Serum samples were examined for the presence of IgG antibodies against *Anaplasma phagocytophilum* with the immunofluorescence test kit *Anaplasma phagocytophilum* IFA IgG (Focus Technologies, Cypress, California, USA), according to manufacturer's instructions. Sera showing

fluorescence in the dilution 1:64 were considered positive [28, 29].

Statistical analysis. Statistical analysis was performed with the use of χ^2 test, Student's t-test and Pearson's linear test for correlation. The $p \leq 0.05$ level was considered significant. The statistical analysis was carried out with the use of the StatisticaTM ver. 5.0 package (Statsoft©, Inc., Tulsa, Oklahoma, USA).

RESULTS

Infection of *I. ricinus* ticks with *A. phagocytophilum* demonstrated by the presence of 16S rRNA gene. A total of 70 ticks out of 684 examined (10.2%) showed the presence of *A. phagocytophilum* 16S rRNA gene (Tab. 1). The prevalence of infection was significantly dependent on tick's stage (χ^2=49.2, p<0.00001) and locality (χ^2=34.4, p<0.00001). The percentage of *I. ricinus* females infected with *A. phagocytophilum* (24.6%) was significantly greater compared to males (6.5%) and nymphs (4.4%) (p<0.00001). No significant difference was found to exist between the prevalence of infection in males and nymphs (p>0.1). The greatest prevalence of infection was found in ticks collected in forest districts located in the central part of Lublin region, close to city of Lublin: Lubartów (28.1%) and Lublin (25.0%) (Tab. 1).

Infection of *I. ricinus* ticks with *A. phagocytophilum* demonstrated by the presence of *ankA* gene. A total of 19 ticks out of 684 examined (2.8%) showed the presence of *A. phagocytophilum ankA* gene (Tab. 2). The prevalence of infection was significantly dependent on tick's stage (χ^2=23.6, p<0.00001), being significantly greater in females than in other stages (p<0.01). The prevalence was not dependent on locality (χ^2=9.8, p=0.082). Similar the case of 16S rRNA gene, the greatest prevalence of infection was found in ticks collected in the forest districts of Lubartów and Lublin (Tab. 2).

Relationship between the presence of *A. phagocytophilum* 16S rRNA and *ankA* genes in *I. ricinus* ticks. A total prevalence of infection demonstrated by the presence of 16S rRNA gene was nearly 4 times greater compared to that demonstrated by the presence of *ankA* gene, and the difference was statistically significant (p<0.00001). 78 out of 684 examined ticks (11.4%) contained *A. phagocytophilum* DNA demonstrated by the presence of either gene (16S rRNA or *ankA*). 11 (1.6%) showed the presence of both genes, 59 (8.6%) – only of 16S rRNA, 8 (1.2%) – only

Table 1. Infection of different stages of *Ixodes ricinus* ticks from various forest districts of Lublin province with *Anaplasma phagocytophilum* demonstrated by the presence of 16S rRNA gene.

Forest district	Nymphs*			Males			Females			Total		
	N	Pos.	%	N	Pos.	%	N	Pos.	%	N	Pos.	%
Puławy	54	1	1.9	50	1	2.0	39	12	30.8	143	14	9.8
Lubartów	10	2	20.0	12	1	8.3	10	6	60.0	32	9	28.1
Lublin	37	2	5.4	18	5	27.8	25	13	52.0	80	20	25.0
Sobibór	20	1	5.0	10	0	0.0	10	3	30.0	40	4	10.0
Zwierzyniec	140	5	3.6	24	3	12.5	18	5	27.8	182	13	7.1
Goscieradów	60	3	5.0	70	2	2.9	77	5	6.5	207	10	4.8
Total	321	14	4.4	184	12	6.5	179	44	24.6	684	70	10.2

*minimum infection rate (nymphs examined in pools); N = number of examined ticks; Pos. = number of positive ticks; % = percent of positive ticks.

Table 2. Infection of different stages of *Ixodes ricinus* ticks from various forest districts of Lublin province with *Anaplasma phagocytophilum* demonstrated by the presence of *ankA* gene.

Forest district	Nymphs*			Males			Females			Total		
	N	Pos.	%	N	Pos.	%	N	Pos.	%	N	Pos.	%
Puławy	54	0	0.0	50	1	2.0	39	2	5.1	143	3	2.1
Lubartów	10	0	0.0	12	0	0.0	10	2	20.0	32	2	6.3
Lublin	37	0	0.0	18	1	5.6	25	5	20.0	80	6	7.5
Sobibór	20	0	0.0	10	0	0.0	10	1	10.0	40	1	2.5
Zwierzyniec	140	2	1.4	24	0	0.0	18	2	11.1	182	4	2.2
Gościeradów	60	0	0.0	70	1	1.4	77	2	2.6	207	3	1.4
Total	321	2	0.6	184	3	1.7	179	14	7.8	684	19	2.8

*minimum infection rate (nymphs examined in pools); N = number of examined ticks; Pos. = number of positive ticks; % = percent of positive ticks.

Table 3. Serological response of forestry workers employed in various forest districts of Lublin province to *Anaplasma phagocytophilum*, and its correlation with the infection of *Ixodes ricinus* ticks in these districts assessed by PCR with two pairs of *A. phagocytophilum* primers.

Forest district	Serological response			Correlation of serological response with infection rate in *I. ricinus* ticks demonstrated by the presence of 16S rRNA gene	
	N	Pos.	%		
Puławy	46	12	26.1	Nymphs	r = 0.707, p = 0.116
Lubartów	39	14	35.9	Males	r = 0.221, p = 0.674
Lublin	30	7	23.3	Females	r = 0.824, p = 0.044
				Total ticks	r = 0.727, p =0.100
				Correlation of serological response with infection rate in *I. ricinus* ticks demonstrated by the presence of *ankA* gene	
Sobibór	47	8	17.0	Nymphs	r = 0.022, p = 0.967
Zwierzyniec	55	13	23.6	Males	r = -0.089, p = 0.867
Gościeradów	44	6	13.6	Females	r = 0.653, p = 0.160
Total	261	60	23.0	Total ticks	r = 0.582, p = 0.226

N = number of examined forestry workers; Pos. = number of seropositive workers; % = percent of seropositive workers; r = correlation coefficient; p = probability of correlation coefficient.

of *ankA* gene, and 606 (88.6%) – of neither gene. A significant relationship between the presences of both genes was found (χ^2=48.3, p<0.00001).

Relationship between the frequency of positive serological reactions to *A. phagocytophilum* in forestry workers and prevalence of *A. phagocytophilum* DNA in *I. ricinus* ticks. A total of 60 out of 261 forestry workers (23.0%) showed the presence of antibodies to *Anaplasma phagocytophilum*. The response rate in the control group was low (5.4%), being significantly smaller than in forestry workers (p<0.01). A significant correlation was found between the *A. phagocytophilum* infection of *I. ricinus* female ticks from the particular forest districts, demonstrated by the presence of 16S rRNA gene and the serologic response to *A. phagocytophilum* of forestry workers employed in the same districts (p<0.05) (Tab. 3). The correlation between the presence of *A. phagocytophilum* 16S rRNA gene in other tick stages (nymphs, males) and serologic response of exposed workers was positive, but not significant. No significant correlation was found between the presence of *A. phagocytophilum ankA* gene in *I. ricinus* ticks and serologic response of exposed workers (Tab. 3).

DISCUSSION

The results of the present work indicate that the prevalence of *Anaplasma phagocytophilum* infection in ticks determined with PCR method may show a marked variation depending on the primers used. The prevalence determined with the use of EHR521/EHR747 primers' pair detecting 16S rRNA gene was on average 10.2%. It was within the range of 1.4-38.5% reported for ticks from different localities in northern and eastern Poland by earlier authors using this set of primers [8, 9, 10, 12, 17, 18, 20, 22, 23, 24, 25, 26] and close to the prevalence reported by Derdáková *et al.* [5] from eastern Slovakia which amounted to 8.3%.

The mean prevalence of *Anaplasma phagocytophilum* infection in ticks determined with the use of LA6/LA1 primers' pair detecting *ankA* gene was nearly 4 times lower and amounted to 2.8%. This result is not consistent with the views that the application of primers detecting *ankA* gene increases the sensitivity of PCR reaction for detection of *A. phagocytophilum* [11, 17, 19, 27], and with the results of Rymaszewska [17] who recorded in Poland a higher prevalence of *A. phagocytophilum* infection in ticks with the use of primers detecting the *ankA* gene compared to those detecting 16S rRNA gene (11.5% vs. 5.8%), and of Christova *et al.* [3] who found in Bulgaria 19.8% ticks infected with *A. phagocytophilum* with the use of these primers. By contrast, our results indicate a low sensitivity of primers detecting *ankA* gene and conform to those reported by Fingerle *et al.* [7] who found in Germany 24.8% ticks infected with *A. phagocytophilum* with the use of primers detecting 16S rRNA gene, while only 1.6% with the use of primers detecting *ankA* gene. Summarising, divergent results obtained with different primers indicate the need for standardisation of PCR method for detection of *Anaplasma phagocytophilum*, with the use of sequencing.

The prevalence of *Anaplasma phagocytophilum* infection in *Ixodes ricinus* ticks collected in eastern Poland showed a large variability depending on the stage and geographical location. Tick females were infected significantly more often than males and nymphs. The potential role of *I. ricinus* females in the transmission of *A. phagocytophilum* infection to occupationally exposed people has been confirmed by finding a significant correlation between the presence of *A. phagocytophilum* 16S rRNA gene in tick females from particular forest areas, and the serologic response to *A. phagocytophilum* of forestry workers employed in these areas.

CONCLUSIONS

1. Detection of *Anaplasma phagocytophilum* infection in ticks by PCR with the use of EHR521/EHR747 primers detecting 16S rRNA gene is significantly more sensitive compared to LA6/LA1 primers detecting *ankA* gene.

2. *Ixodes ricinus* females are infected with *A. phagocytophilum* significantly more often than males and nymphs, which indicates the key role of tick females in the pathogen transmission to humans and animals.

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