RISK OF TICK-BORNE BACTERIAL DISEASES AMONG WORKERS
OF ROZTOCZE NATIONAL PARK (SOUTH-EASTERN POLAND)

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Cisak E, Chmielewska-Badora J, Zwoliński J, Wójcik-Fatla A, Polak J, Dutkiewicz J: Risk of tick-borne bacterial diseases among workers of Roztocze National Park (south-eastern Poland). *Ann Agric Environ Med* 2005, **12**, 127–132.

Abstract: The objective of the present study was to assess the risk of borreliosis and anaplasmosis (ehrlichiosis) among the forestry workers of the Roztocze National Park (south-eastern Poland) by examination of *Ixodes ricinus* ticks living in park area with PCR method, and by the serological and clinical examination of the workers. In 406 examined ticks, the prevalence of infection with *Borrelia burgdorferi* sensu lato was 11.5%. The nested PCR reaction for determining the genospecies showed that the most common was *Borrelia burgdorferi* sensu stricto (55.3% of total positive) followed by *Borrelia afzelii* (38.3%). As many as 6.1% out of 115 examined ticks showed the presence of *Anaplasma phagocytophilum* DNA. The infection rate was high in males and females (14.3% and 11.1% respectively) and low in nymphs (1.5%). In 46 out 113 examined forestry workers (40.7%) the presence of specific IgG and/or IgM antibodies against *Borrelia burgdorferi* sensu lato was found in ELISA test, while only 4 out of 56 urban blood donors showed a positive response (p<0.0001).The prevalence of IgG antibodies against *Anaplasma phagocytophilum* determined with the use of indirect immunofluorescence test (IFA) was 17.7% in forestry workers compared to 5.4% in reference group of blood donors (p<0.05). No correlation was found between the presence of antibodies to *A. phagocytophilum* and to *B. burgdorferi* s. l. Clinical investigations of 113 forestry workers showed 3 cases of borreliosis (2.7%) and no cases of anaplasmosis (ehrlichiosis). In conclusion, forestry workers of the Roztocze National Park in south-eastern Poland are often exposed to *Ixodes ricinus* ticks infected with *Borrelia burgdorferi* and *Anaplasma phagocytophilum*, and show a high proportion of asymptomatic borreliosis and anaplasmosis (ehrlichiosis) manifested by a positive serologic response, while the number of clinical cases is relatively low.

INTRODUCTION

According to many reports, the risk of contracting diseases transmitted by ticks is greater in forestry wor-kers, farmers and others working in woody areas [3, 7, 8, 11, 12, 15, 19, 23, 26, 27, 28, 30, 31, 35, 44, 47, 54]. Of particular interest in this respect are two tick-borne bacterial diseases: borreliosis and anaplasmosis (ehrlichiosis)

[4, 5, 9, 14, 22, 29, 33, 40], which are transmitted in Europe by the common tick *Ixodes ricinus* and pose growing health problems [22, 30, 32, 34, 49].

Borreliosis (Lyme disease, Lyme borreliosis, Lb), caused by *Borrelia burgdorferi* spirochetes, is a polymorphous disease, which may cause skin, joints, as well as neurological, ocular and cardiac disorders. The species *Borrelia burgdorferi* sensu lato comprises at least three pathogenic genomic species: *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* and *Borrelia afzelii* [10, 20, 22, 42, 45, 46, 52, 53]. Clinical manifestation of Lb may vary depending on infective genospecies [22, 36, 42, 53]. The annual number of Lb cases in Poland shows a growing tendency - 3,817 in 2004 [25].

Human granulocytic ehrlichiosis (HGE) is caused by obligatory intracellular rickettsia *Anaplasma phagocytophilum* (*Ehrlichia phagocytophila*) [3, 5, 19, 30]. The disease is also called human anaplasmosis (HA). The clinical manifestation of the disease may vary from a subclinical to severe, or even fatal course. The common incidence of the infection in ticks and humans was evidenced in Europe with genetic and serologic methods [3, 13, 15, 16, 19, 30, 47], but so far the number of reported clinical cases of HA is low. The first clinical case of the disease in Europe was described in 1992 in Slovenia [34], and up to the year 2000 altogether 10 cases of HA infections were diagnosed in that country. The first cases of the disease in Poland were reported in 2001 [49].

The objective of the present study was to assess the risk of borreliosis and anaplasmosis among the forestry workers of the Roztocze National Park (RNP) located in Zwierzyniec (Lublin province, south-eastern Poland) by the examination of *Ixodes ricinus* ticks living in the forestry environment of RNP with PCR method, and by the serological and clinical examination of the workers.

MATERIALS AND METHODS

Study area. The Roztocze National Park (RNP) founded in 1974, is situated on scenic highlands in southeastern Poland (Lublin province). Currently, its area comprises 8,483 ha, including an 8,102 ha area of mixed forest composed of Scots pine (*Pinus sylvestris*), European beech (*Fagus sylvatica*), European hornbeam (*Carpinus betulus*), and other deciduous and coniferous trees.

Collection of ticks. Unfed *Ixodes ricinus* ticks (adults and nymphs) were collected during spring/summer season in 2003-2004 from the forestry area of RNP by dragging a light woolen flag (60 × 90 cm) over the lower vegetation, mostly on forest paths, in glades, and at the edge of the woods. Collected ticks were placed separately (adults), or in pools of 5-7 specimens (nymphs), in vials with 70% ethanol for further investigation.

Isolation of bacterial DNA from ticks. Bacterial DNA was isolated from collected ticks after removal from alcohol by boiling in 0.7 M ammonium hydroxide [37].

Detection of *Borrelia burgdorferi* s.l. DNA in ticks. Isolates were examined for the presence of DNA *Borrelia burgdorferi* s.l. by polymerase chain reaction (PCR) with oligonucleotide primer set FLA1/FLA2 [53] (Polgen, Łódź, Poland), specific for DNA *fla* gene sequence. All adult ticks were examined separately and nymphs in pools of 5, 6 or 7 specimens. In each PCR reaction were applied:

• matrix DNA,
• FLA1/FLA2 primers,
• Polish strain Bo-148c/2 as a positive control,
• redistilled water as a negative control,
• thermostable polymerase (DyNAzyme™ II DNA, Finnzymes Oy, Espoo, Finland),
• mixture of dTP nucleotides (DNA, Gdańsk, Poland).

The amplification was carried out in a thermal cycler (DNA, Gdańsk, Poland) according to Wodecka & Skotarczak [53]. The size of amplified DNA fragment was 482 base pairs (bp). Amplification products were identified in 1.5% agarose gel, after electrophoresis in standard conditions and staining with ethidium bromide.

Detection of *Borrelia burgdorferi* s.l. genospecies DNA in ticks. Species-specific primers BB1/BB2, BA1/BA2 and BG1/BG3 (Polgen, Łódź, Poland) designed for differentiation *B. burgdorferi* s.l. into three genospecies (*Borrelia burgdorferi* s.s., *Borrelia afzelii*, *Borrelia garinii*) were used in nested-PCR reaction of 482 bp fragment of the first PCR reaction product, obtained with FLA1/FLA2 primers [53]. The reagents applied in nested-PCR reaction were:

• each of the three above-mentioned pair primers,
• thermostable polymerase (DyNAzyme™ II DNA, Finnzymes Oy, Espoo, Finland),
• dNTPs (DNA, Gdańsk, Poland).

The nested-PCR reaction was carried out in a thermal cycler (DNA, Gdańsk, Poland), according to Stańczak *et al.* [42] and Wodecka & Skotarczak [53]. The sizes of amplified DNA fragments were: 76 bp for *B. burgdorferi* s.s., 103 bp for *B. afzelii* and 125 bp for *B. garinii*. Amplification products were identified in 1.5% agarose gel, after electrophoresis in standard conditions and staining with ethidium bromide.

Detection of *Anaplasma phagocytophilum* DNA in ticks was carried out with the use of PCR method. A set of primers, EHR 521 and EHR 747 (Polgen, Łódź, Poland), were used to amplify a 247 bp fragment of the 16S rDNA gene [43]. The conditions of PCR reaction, established by Grzeszczuk *et al.* [18] were applied. HGE-1-infected HL60 cells from the IFA kit (Focus Technologies, Cypress, CA, USA) constituted a positive control. As a negative control, redistilled water was used.

Examined population. A total of 100 male and 13 female forestry workers employed in Roztocze National Park and in collaborating forest inspectorate in Zwierzyniec were examined. The mean age of the

examined workers amounted to 41.5 ± 9.3 years. The forestry workers were interviewed regarding exposure to ticks. All of them claimed frequent tick bites during the entire period of employment.

The reference group consisted of 56 healthy male blood donors living in the city of Lublin, at mean age 35.5 ± 9.6 years.

Samples of peripheral blood for serological studies were collected from all workers and members of the reference group.

Serological examination for the presence of anti-Borrelia antibodies. Sera of forestry workers and referents were examined for the presence of anti-*Borrelia burgdorferi* s.l. antibodies with the use of ELISA test, determining the level of specific IgM and IgG immunoglobulins (Bellco Biomedica, Vienna, Austria). The test was carried out according to producer's instructions.

Serological examination for the presence of anti-Anaplasma antibodies. The presence of IgG antibodies against *Anaplasma phagocytophilum* was detected with the use of indirect immunofluorescence test (IFA) (Focus Technologies, Cypress, CA, USA).

Clinical examinations. All the seropositive workers were subjected to clinical examinations in the Outpatient Department of the Institute of Agricultural Medicine in Lublin. They comprised physical, dermatological, and neurological examinations.

Statistical analysis was carried out using chi-square test.

RESULTS

Infection of *Ixodes ricinus* ticks with *Borrelia burgdorferi*. Altogether 47 (11.6%) out of 406 collected *Ixodes ricinus* ticks (individual and pooled samples) were considered positive for DNA *B. burgdorferi* s.l. (Tab. 1). Adult females were infected in 12.1%, males in 6.7%, and the minimum infection rate in nymphs amounted to 12.2%.

Further study of 47 tick samples positive for *B. burgdorferi* s.l. DNA showed that *B. burgdorferi* s.s. DNA was present in 26 samples (55.3% of total positive), *B. afzelii* DNA in 18 (38.3%), and mixed infections (with *B. burgdorferi* s.s. and *B. afzelii*) in 1 (2.1%) (Tab. 2). *B. garinii* DNA was never found and 2 (4.3%) of total positive samples remained unidentified.

Infection of *Ixodes ricinus* ticks with *Anaplasma phagocytophilum*. In 7 out of 115 examined ticks, the DNA fragment specific for *Anaplasma* was found (6.1%). The infection rate was high in males and females (14.3% and 11.1% respectively), and low in nymphs (1.5%) (Tab. 3).

Table 1. *Borrelia burgdorferi* sensu lato DNA in ticks collected from vegetation in the area of Roztocze National Park.

Tick stage	Positive/examined	Percent infected
Females	7/58	12.1%
Males	3/45	6.7%
Nymphs*	37/303	12.2%**
Total	47/406	11.6%**

*examined in pools of 5 or 6 specimens; ** minimum infection rate

Table 2. Genospecies of *Borrelia burgdorferi* found in ticks from the Roztocze National Park.

Genospecies	*B. burgdorferi* s.s.	*B. afzelii*	*B. garinii*
B. burgdorferi s.s.	26	1	0
B. afzelii		18	0
B. garinii		0	

Table 3. *Anaplasma phagocytophilum* DNA in ticks collected from vegatation in the area of Roztocze National Park.

Tick stage	Positive/examined	Percent infected
Females	3/27	11.1%
Males	3/21	14.3%
Nymphs*	1/67	1.5%**
Total	7/115	6.1%**

*examined in pools of 5 or 7 specimens; ** minimum infection rate

Table 4. Occurrence of antibodies to *Borrelia burgdorferi* sensu lato and *Anaplasma phagocytophilum* in forestry workers of the Roztocze National Park and reference group of urban blood donors.

	Positive/examined (%)		p
	Forestry workers	Reference group	
Borrelia burgdorferi s.l. IgG and/or IgM	46/113 (40.7%)	4/56 (7.1%)	<0.0001
Anaplasma phagocytophilum IgG	20/113 (17.7%)	3/56 (5.4%)	<0.05

Prevalence of serologic reactions to *Borrelia burgdorferi*. In 46 out of 113 examined forestry workers (40.7%) the presence of specific IgG and/or IgM antibodies against *Borrelia burgdorferi* s.l. was found. People from the reference group showed seropositive reactions to *B. burgdorferi* s.l. only in 4 cases (7.1%). The difference between prevalence of specific anti-*Borrelia burgdorferi* antibodies in both groups was highly significant (p<0.0001) (Tab. 4). The antibody response showed some dependence on age, being highest in the oldest group of workers, and lowest in the young subjects (Fig. 1).

Prevalence of serologic reactions to *Anaplasma phagocytophilum*. Specific antibodies against *Anaplasma phagocytophilum* were found statistically more frequently
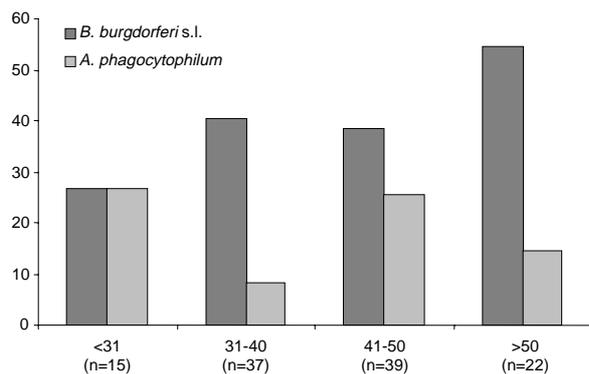
Figure 1. Seropositive reactions to *Borrelia burgdorferi* sensu lato and *Anaplasma phagocytophilum* in forestry workers, depending on age (*B. b.* s.l.: $\chi^2=5.10$, p=0.16; *A. ph.*: $\chi^2=3.05$, p=0.38).

Table 5. Coexistence of antibodies to *Borrelia burgdorferi* and *Anaplasma phagocytophilum* in forestry workers of the Roztocze National Park.

		Anaplasma phagocytophilum IgG		
		+	-	Total
Borrelia burgdorferi	+	6	40	46
IgM and/or IgG	-	14	53	67
Total		20	93	113

$\chi^2=1.15$; p=0.28

in forestry workers compared to the reference group (17.7% *vs.* 5.4%, p<0.05) (Tab. 4). Dependence on age was not observed, as the highest antibody response was found in the youngest (up to 30 years) and upper middle-aged workers (41-50 years) (Fig. 1).

No correlation was found between the presence of antibodies to *A. phagocytophilum* and to *B. burgdorferi* s.l. (Tab. 5).

Clinical manifestations. Out of 46 seropositive workers, in 3 clinical borreliosis was diagnosed. The main clinical manifestations included: borreliosis *erythema*, periodical strong arthralgic pain, dysfunction of extremities, sleepiness, headaches and dizziness.

No clinical cases of anaplasmosis (ehrlichiosis) were found among the examined forestry workers.

DISCUSSION

The total infection rate of *Ixodes ricinus* ticks with *Borrelia burgdorferi* s.l. found in the present study (11.6%) was similar to rates reported by Skotarczak & Wodecka [39], Wodecka & Skotarczak [53] and Bukowska *et al.* [6] for north-western Poland, by Petko *et al.* [33] for southern Poland and by Stańczak *et al.* for northern Poland [43] and for the total territory of the country [41]. This was also similar to the infection rates reported from the Czech Republic [46], Slovakia [44], Norway [24] and Spain [2], and lower compared to the rates reported from southern Germany [30] and eastern Slovakia [13].

The results of this study indicating that *B. burgdorferi* s.s. was the most prevalent genospecies on the examined territory are consistent with those reported by Wodecka & Skotarczak [53] from north-western Poland, but not with the results of Stańczak *et al.* [42] who reported the prevalence of *B. afzelii* in *I. ricinus* ticks collected in various localities in Poland. Nevertheless, some other European authors have also reported the prevalence of *B. burgdorferi* s.s. genospecies in ticks collected in central Hesse in Germany [52], in north-eastern Italy [10], in the Eindhoven area in the Netherlands [20], in southern

Bohemia [46], and in eastern Slovakia [45]. By contrast, Oehme *et al.* [30] found a prevalence of *B. afzelii* in ticks collected in south-western Germany.

The seroepidemiological studies conducted in many countries showed a high occupational risk of infection with *Borrelia burgdorferi* among forestry workers [23, 27, 28, 31, 35, 38, 44, 47], manifested by greater prevalence of anti-*Borrelia* antibodies. The prevalence of antibodies to *B. burgdorferi* s.l. found in the present study was high (40.7%) and significantly greater compared to reference group (p<0.0001). It was similar to the seroprevalence rates in forestry workers reported by Pancewicz *et al.* [32] from Podlasie province, an endemic area of Lyme borreliosis in Poland (43.6%), by Oehme *et al.* [30] from south-western Germany (18.0-52.0%), by Oteo *et al.* [31] from Spain (38%), by Nadal *et al.* [27] from Switzerland (35%), and by Hristea *et al.* [21] from western part of Romania (31.7%). A higher seropositivity of foresters (61.5%) was reported by Niścigorska *et al.* [29] from north-western Poland and by Tomasiewicz *et al.* [47] from mid-eastern Poland (60.3%). A lower prevalence of seropositive forestry workers (15.2%) was reported by Zhioua *et al.* [54] from France, by Angelov *et al.* [1] from Bulgaria (15.3%), and by Štefančiková *et al.* [44] from Slovakia (16.8%). Santino *et al.* [38] found a low seroprevalence of *B. burgdorferi* among occupationally exposed Italian workers (7.5%) which, however, was significantly greater compared to unexposed controls (1.2%). By contrast, Werner *et al.* [51] in south-western Sweden has not found a significant difference between seroprevalence of *B. burgdorferi* in an exposed group of forestry workers and farmers and an unexposed group of clerks (7.6 *vs.* 5.3%). Munchhoff *et al.* [26] observed the dependence of antibody response on age (rate of seropositivity increasing with age), similar to the present study.

In 3 forestry workers (2.7% of the total and 6.5% of seropositive individuals), a clinical borreliosis was diagnosed. This observation seems to confirm the views expressed by some earlier authors [27, 29, 35, 54] that clinical signs of the disease are rare compared to the overall infection rate evidenced by seropositivity. On the other hand, the morbidity index stated in the examined group is much greater compared to the overall index in Poland (2,700:10/100,000) [25], which indicates a notable risk of occupational borreliosis among forestry workers.

The percentage of *I. ricinus* ticks infected with *Anaplasma phagocytophilum* found in this study was 6.1%, higher compared to that obtained by Skotarczak *et al.* [40] in north-western Poland (4.5%) and lower compared to those reported by Grzeszczuk *et al.* [19] from north-eastern Poland (8.7%), and by Stańczak *et al.* [43] from northern Poland (14.0%). The infection rate found in this study was higher compared to those reported from Scotland (1.5-3.3%) [50] and south-western Germany (1.6%-3.1%) [16, 30] and lower compared to that reported from Norway (6-11.5%) [24] and eastern Slovakia (13.3%) [13].

Forestry workers examined in this study showed a significantly higher rate of seropositive reactions to *Anaplasma phagocytophilum* compared to unexposed reference group (p<0.05). This is in accord with the majority of reports from other European countries, proving that people occupationally exposed to tick bite show significantly more frequently the presence of specific anti-*Anaplasma* antibodies than urban dwellers [3, 15, 19, 38, 47]. The seroprevalence of *Anaplasma phagocytophilum* found in forestry workers examined in this study (17.7%) corresponds well with the results reported for this occupational group by other researchers from Poland [19, 47] and Germany [3, 15, 16, 30]. A lower seropositivity was found in forestry workers from the Netherlands [17] and Italy [38], and in English farmworkers [48].

CONCLUSION

Workers of the Roztocze National Park in south-eastern Poland are often exposed to *Ixodes ricinus* ticks infected with *Borrelia burgdorferi* and *Anaplasma phagocytophilum*, and show a high proportion of asymptomatic borreliosis and anaplasmosis (ehrlichiosis) manifested by a positive serologic response, while the number of clinical cases is relatively low.

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