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Molecular and serological evidence of spotted fever group rickettsiae and *Borrelia* co-infection in *Ixodes ricinus* and humans in north-eastern Poland

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Tick-borne spotted fever group (SFG) rickettsioses are considered emerging vector-borne infectious diseases with potentially severe consequences for public health, particularly in the temperate regions of Europe. The aim of the study was to determine the prevalence and the diversity of *Rickettsia* spp. in questing *Ixodes ricinus* ticks in north-eastern Poland, evaluate the serological evidence of exposure to SFG rickettsiae among adult residents of this region, as well as to determine the level of SFG rickettsiae co-infection with *Borrelia* spirochaetes in ticks and humans. In a total of 4090 ticks, the presence of *Rickettsia* spp. was detected by PCR and confirmed by sequencing. IgG antibodies against SFG *Rickettsia* and *Borrelia* were detected using ELISA. Rickettsial DNA was revealed in 11.3% of females, 11.8% of males and nymphs of 6.5% (MIR, minimum infection rate). Based on DNA sequencing, *R. helvetica* and *R. monacensis* were identified. IgG antibodies against SFG rickettsiae were confirmed in 38.7% of adult residents of north-eastern Poland. The co-infection rate of SFG rickettsiae with *Borrelia* spirochaetes in questing adult *I. ricinus* ticks was low (2.6%), compared to the co-infection rate (16.1%) observed based on the presence of immunological responses in the study group of residents from northeastern Poland. These findings provide important evidence filling the gap between the environmental detection of SFG rickettsiae in vector populations and the limited number of diagnosed clinical cases in humans.

Keywords *Ixodes ricinus*, Forest workers, City residents, Seroprevalence, Coinfection

The rickettsiae are obligate, intracellular Gram-negative bacteria, transmitted via hematophagous ectoparasitic arthropod vectors such as ticks, fleas, lice or mites from wild or domestic vertebrate hosts[1]. Within the genus *Rickettsia*, one of the most numerous groups classified based on phenotypic characteristics, vector hosts and phylogenetic organization is the spotted fever group (SFG), which is almost exclusively tick-borne[2]. In recent decades, the medical importance of the *Rickettsia* species transmitted by ticks has increased considerably[3,4]. Among those, species such as *R. rickettsii* and *R. conorii* are causative agents of the oldest known tick-borne

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diseases, Rocky Mountain Spotted Fever and Mediterranean Spotted Fever, respectively[5]. Many other *Rickettsia* spp. from SFG, considered to be nonpathogenic for decades, such as *R. helvetica*[5], *R. monacensis*[6] or *R. slovaca*[7], are now associated with human infections. Moreover, novel species (i.e. *Candidatus* R. mendelii[8], *Candidatus* R. thierseensis[9]) of undetermined pathogenicity continue to be detected in or isolated from ticks around the world[10–12].

SFG rickettsioses, like other tick-borne diseases, belong to the emerging and re-emerging infectious diseases[10,13,14]. They are considered some of the most rapidly expanding vector-borne diseases, with potentially severe consequences for public health, particularly in the temperate regions of Europe[15,16]. In humans, spotted fever rickettsioses do not have pathognomonic features; however, the typical clinical symptoms include flu-like signs (fever, headache, muscle pain), skin manifestations (rash or a characteristic inoculation eschar 'tâche noire' at the site of the tick bite), as well as in some cases local lymphadenopathy. These major clinical signs vary depending on the rickettsial species involved and may be absent or unnoticed during an undirected clinical examination[10,17].

The risk of rickettsioses in a given region may depend on the presence of specific tick species and tick population density, the geographical and climatic conditions, as well as the duration of tick attachment to the host and human activity[5]. The main vectors and reservoirs of SFG rickettsiae are hard ticks from the Ixodidae family[12]. In Europe, a significant role in the circulation cycle of SFG rickettsiae is attributed to the most abundant tick species, *Ixodes ricinus*, *Dermacentor reticulatus* and *Rhipicephalus sanguineus* (in Mediterranean countries)[13,18,19]. Monitoring the level of tick infection with pathogens as *Borrelia* spirochetes or microorganisms potentially pathogenic to humans, as well as conducting serological investigations of the general population or selected healthy groups, is one of the key elements in assessing the circulation of tick-borne pathogens and the risk of tick-borne diseases in a given area[20]. Such monitoring is particularly important for SFG *Rickettsia*. It is assumed that this group of microorganisms will continue to evolve and should be closely monitored by data collected from properly designed field surveys of vectors and animal hosts, periodic serosurveys in healthy populations, and improved surveillance systems for human cases[4].

The aims of the study were: (1) to assess the prevalence of *Rickettsia* spp. in questing *I. ricinus* ticks in north-eastern Poland; (2) to determine the species diversity of the *Rickettsia* genus in the study area, examining the impact of subregional conditions, biotope, and year of study on the frequency of *Rickettsia* spp.; (3) to assess contact with SFG rickettsiae among adult residents of the north-eastern region of Poland based on serological investigations; and (4) to determine the level of SFG *Rickettsia* co-infection with *Borrelia* spirochetes in ticks and humans.

Materials and methods

Molecular detection of the spotted fever group rickettsiae in ticks

Study area and tick collection

Rickettsia spp. were examined in a total of 4,090 *I. ricinus* ticks, including 381 females, 450 males, and 3,259 nymphs (in 651 pools of five and one pool of four specimens). Questing ticks were collected between April and June of 2016 and 2017 at 14 sites located in the western, central, and eastern subregion of the Warmia-Mazury province in north-eastern Poland (Fig. 1). The tick collection sites represented forest landscapes and ecotone habitats (zones between grassy and forested areas). At each site, ticks were collected twice per month in each year of the study during the daytime between 9 a.m. and 4 p.m. by two persons for at least 30 min using the standard flagging method[21]. Ticks were preserved in 70% ethanol. In the laboratory, species, sex (female, male) and developmental stage (nymph, adult) of collected ticks were morphologically identified based on a taxonomic key[22]. Subsequently, adult ticks were stored individually, and nymphs in pools at − 80°C for further molecular analyses.

DNA extraction

Genomic DNA from *I. ricinus* ticks was isolated using the universal Sherlock AX kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's protocol. DNA was extracted from individual adult ticks and from pools of nymphs and then frozen at − 80°C for further analysis. The procedure was described in detail in Kubiak et al.[27].

The presence of *Rickettsia* spp. DNA in tick genomic DNA samples was confirmed by the conventional PCR method using two sets of primers: (a) CS409/Rp1258[23] - specific to the citrate synthase (*gltA*) gene and (b) ITS-F/ITS-R[24] - specific to the intergenic spacer region (ITS) between the 23 S rRNA and 5 S rRNA genes. Target regions, expected length of the PCR products and the oligonucleotide sequences of primers are detailed in Table 1.

The total volume of the PCR mixture (25 µL) contained: 12.5 µL of 2 × PCR Master Mix Plus (0.1 U/µL of Taq polymerase supplied in a PCR buffer, 4 mM of $MgCl_2$ and 0.5 mM of each dNTP) (A&A Biotechnology, Gdynia, Poland), 0.5 µL of each primer (10 µM), 1.5/2 µL of template DNA and an appropriate volume of sterile nuclease-free water. Sequence-verified DNA of *R. helvetica*, obtained from an infected *I. ricinus* tick, as a positive control was used. PCR was performed on a Mastercycler® nexus GSX1 thermocycler (Eppendorf AG, Hamburg, Germany). PCR amplicons were analysed by electrophoresis in 1.5% agarose gels stained with Midori Green Stain (Nippon Genetics Europe, Düren, Germany). The PCR products of approximately 769 bp of the *gltA* gene[23] and between 300 and 533 bp of the *rrl-rrf* ITS region[24] were considered *Rickettsia*-positive.

Rickettsia species identification

The CleanUp purification kit (A&A Biotechnology, Gdynia, Poland) was used to purify randomly selected PCR products (46 for the *gltA* gene and 4 for the *rrl-rrf* ITS). The amplicons were then bidirectionally sequenced by Macrogen Europe (Amsterdam, The Netherlands). The nucleotide sequences obtained in this study were

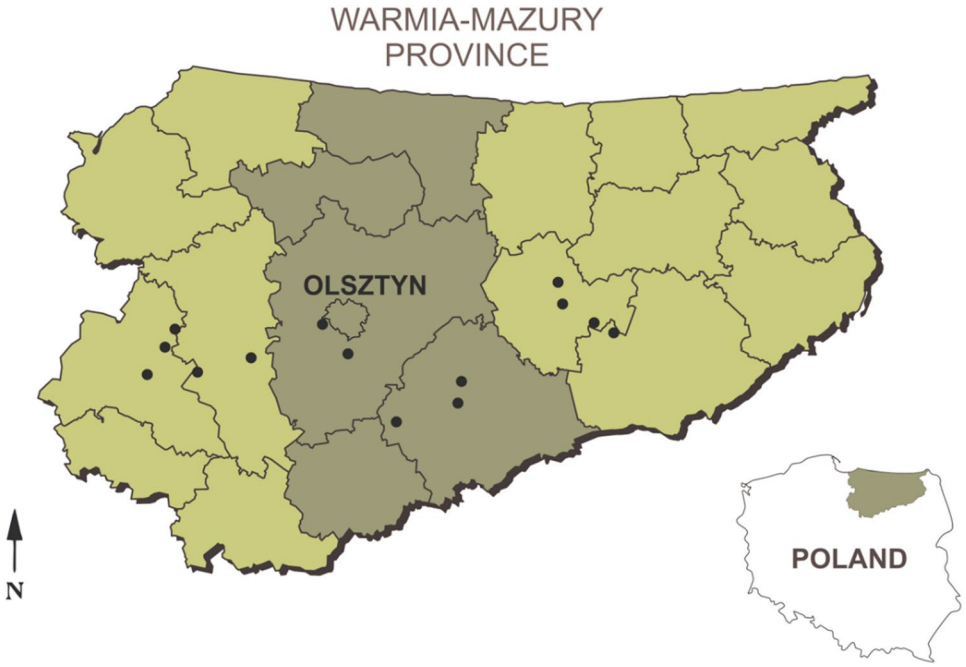

Fig. 1. *Ixodes ricinus* tick collection sites located in the Warmia-Mazury province (north-eastern Poland). The colors indicate the western, central, and eastern subregions of the Warmia-Mazury province, from left to right, respectively. Geographical coordinates and detailed descriptions of the habitats of the tick collection sites were published in Kubiak et al.[27][. The map was created based on Google Maps (https://www.google.pl/maps) in CorelDRAWX5 software.

Locus	Primer name	Primer sequence 5′–3′	Product size [bp]	Annealing temperature [°C]	Reference
gltA[1]	CS409	CCT ATG GCT ATT ATG CTT GC	769	55	Roux et al.[23]
	Rp1258	ATT GCA AAA AGT ACA GTG AAC A			
ITS[2]	ITS-F	GAT AGG TCG GGT GTG GAA	300–533	52	Vitorino et al.[24]
	ITS-R	TCG GGA TGG GTA CGT GTG			

Table 1. Primer sets used for PCR amplification. [1]*gltA*—citrate synthase gene, [2]ITS—intergenic spacer region between the *rrl* (23S rRNA) and *rrf* (5S rRNA) genes.

manually edited using BioEdit, v. 7.2 software[25] (https://bioedit.software.informer.com, accessed July 9, 2024), and then compared using the Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/BLAST/, accessed on 20 July 2024) to nucleotide sequences available in the GenBank database (http://www.ncbi.nih.gov/Genbank/index.html, accessed July 20, 2024). Representative sequences of the *Rickettsia gltA* gene and *rrl-rrf* ITS were registered in the GenBank database.

Phylogenetic analysis

Nucleotide sequences of the *gltA* gene (GenBank: PQ119728-729) obtained in this study were used to demonstrate the molecular relationship between the identified *Rickettsia* species and reference sequences in the GenBank database. The phylogram was constructed using the Maximum Likelihood method based on the Kimura 2-parameter model. The topology of the phylogenetic tree was evaluated using the bootstrap method with 1,000 replicates. Phylogenetic analysis was conducted using MEGA 11 software[26] (https://www.megasoftware.net, accessed on 1 August 2024).

Coinfections of Rickettsia spp. with Borrelia burgdorferi s.l. in ticks

Additionally, co-infections of *Rickettsia* spp. with *Borrelia* spirochaetes were also analysed in the same tick DNA samples. Data on the prevalence of spirochaetes (*B. burgdorferi* s.l. and *B. miyamotoi*) in *I. ricinus* ticks from the same study sites and years were published by Kubiak et al.[27]. Due to the fact that the level of nymph infection was studied in pools of specimens, the analysis of co-infections was conducted only among adults of *I. ricinus*.

IgG seroreactivity to spotted fever group rickettsiae and *Borrelia* spirochaetes in humans

Selection and recruitment of participants

The material for the serological studies consisted of 155 serum samples collected in 2023–2024 from two groups of adult residents (≥ 18 years) of the north-eastern region of Poland, with varying degrees of exposure to tick bites. The high-risk group (HR) consisted of 102 forest workers (22 females, 80 males) from two forest inspectorates: Jedwabno ($n = 61$) and Olsztynek ($n = 41$) in the Warmia-Mazury province. The low-risk group (LR) consisted of 53 volunteers (39 females, 14 males), residents of the Olsztyn city area, who occasionally came into contact with tick-infested environments as part of their recreational activities. The qualification for the LR group was carried out based on an original survey questionnaire, which provided information on socio-demographic data, history of tick bites, as well as previously diagnosed borreliosis or other tick-borne diseases. All study participants had no history of Lyme disease.

Collected serum samples were used to test for the presence of specific IgG antibodies for SFG rickettsiae and *Borrelia* spirochaetes in each sample.

Blood sampling and serological tests

Blood sera (~ 2 ml) were obtained by centrifugation of venous blood collected in a closed system under the applicable procedures by qualified medical personnel of the Epidemiological and Clinical Research Laboratory, the Voivodeship Sanitary and Epidemiological Station in Olsztyn, the Medical Diagnostic Laboratory of the Clinical Hospital of the Ministry of Internal Affairs and the Administration of the Warmia-Mazury Oncology Centre in Olsztyn, for both the HR and LR group. All serum samples were stored at −20°C until further analysis.

To detect IgG antibodies to antigens of SFG rickettsiae, the *Rickettsia* IgG (Spotted Fever Group) ELISA kit (DRG International Inc., Springfield, New Jersey, USA) was used. The assays were performed according to the manufacturer's instructions. The cut-off calibrator was set at an index of 1.0 to provide for discrimination between reactive and non-reactive sera. The index from above 1.1 was considered to be positive and below 0.9 was considered to be negative for *Rickettsia* spp. An index between 0.9 and 1.1 was considered equivocal.

The presence of anti-*B. burgdorferi* s.l. IgG antibodies were examined with the use of the *Borrelia* IgG ELISA Recombinant Antigen kit (Biomedica Laboratories, Vienna, Austria). The test was conducted in accordance with the manufacturer's instructions. Positive or negative results were determined by the manufacturer's recommendation.

Serological tests were conducted and interpreted by qualified laboratory diagnosticians at the Epidemiological and Clinical Research Laboratory of the Voivodeship Sanitary and Epidemiological Station in Olsztyn.

Ethics approval and consent to participate

The study protocol followed the ethical guidelines of the 2013 Declaration of Helsinki. Each person, both from the HR and LR groups, voluntarily expressed their willingness to participate and consented to the use of biological material in this study. The study was approved by the Bioethics Committee of the University of Warmia and Mazury in Olsztyn (No. 35/2024).

Statistical analysis

A chi-square (chi2, χ^2) test (with post-hoc Bonferroni test) and 95% confidence intervals (95% CI) were used to compare *Rickettsia* infection rates according to the developmental stage of ticks, subregions, habitats, and study years. *Rickettsia* spp. prevalence in nymphs (tested in pools) was presented as the Minimum Infection Rate (MIR). MIR was calculated as the percentage obtained by dividing the number of *Rickettsia*-positive pools by the total number of nymphs tested, assuming the presence of only one infected nymph per positive pool. The seroprevalence of *Rickettsia* and *Borrelia* was statistically compared within the human study group (HR vs. LR groups, women vs. men, and the Jedwabno vs. Olsztynek forest inspectorates) based on the chi-square (chi2, χ^2) test and 95% confidence intervals (95% CI). In the analysis, equivocal serological results for the tested pathogens were classified as negative. Statistical analyses were performed using SPSS software, v. 27.0 for Windows (SPSS Inc., Chicago, IL, USA). A p-value of < 0.05 was considered statistically significant in all analyses.

Results

Prevalence of *Rickettsia* spp. In ticks

The presence of *Rickettsia* DNA was confirmed in 7.5% (308/4090) of the tested isolates. *Rickettsia* spp. was detected in at least 6.5% (MIR, minimum infection rate) of *I. ricinus* nymphs, 11.3% (43/381) of females and 11.8% (53/450) of males (χ^2=24.296, $p < 0.001$) (Table 2). The proportion of *Rickettsia*-positive ticks was significantly higher in 2016 (8.4%, 183/2188) than in 2017 (6.6%, 125/1902) (χ^2=4.269, $p = 0.03$). The highest infection rate (9.5%, 129/1351) was recorded in the central subregion of Warmia and Mazury. The type of habitat did not affect the *Rickettsia* spp. prevalence, which was 7.3% (127/1750) in forest areas and 7.7% (181/2340) in ecotones.

Rickettsia species diversity in ticks

To confirm *Rickettsia* species, 46 (15%) randomly selected PCR amplicons of the fragment of *gltA* gene (~ 730 bp) were sequenced and compared with GenBank accessions. Sequence analysis showed the presence of *R. helvetica* and *R. monacensis* (our GenBank submission: PQ119728 and PQ119729, respectively). Both *Rickettsia* species are known as human pathogens. The phylogenetic relationships between the *Rickettsia* species identified in this study and reference accessions from GenBank are presented in the Fig. 2. All obtained nucleotide sequences of *R. helvetica* ($n = 45$) were similar and showed 100% identity to the isolate C9P9 of *R. helvetica* (GenBank: U59723). The same nucleotide sequence was also detected from *I. ricinus* in north-eastern (GenBank: OM927745) and central (GenBank: MH018978) Poland as well as from Serbia (GenBank: MH618386) and Italy (GenBank:

		No. of tested ticks	*Rickettsia*-positive	
			n/% * (95% CI)	*p*-value**
Stage	Nymphs	3259	212/6.5[a] (5.68–7.41)	<0.001
	Females	381	43/11.3[b] (8.29–14.90)	
	Males	450	53/11.8[b] (8.95–15.12)	
Year	2016	2188	183/8.4[a] (7.24–9.60)	0.03
	2017	1902	125/6.6[b] (5.50–7.78)	
Subregion	West	1563	104/6.7[a] (5.47–8.00)	0.003
	Central	1351	129/9.5[b] (8.03–11.24)	
	East	1173	75/6.4[a] (5.05–7.93)	
Habitat	Forest	1750	127/7.3[a] (6.09–8.57)	0.567
	Ecotone	2340	181/7.7[a] (6.68–8.89)	
Total		4090	308/7.5 (6.74–8.38)	

Table 2. Prevalence of *Rickettsia* spp. in *I. ricinus* ticks by developmental stage, year, subregion and habitat, north-eastern Poland. *for nymphs Minimum Infection Rate (MIR) is given (5 nymphs per isolate); **chi2 test, $p < 0.05$; [a] vs.[b]statistically significant difference between groups; [a] vs.[a] or [b] vs.[b]no statistically significant difference (post -hoc Bonferroni test).

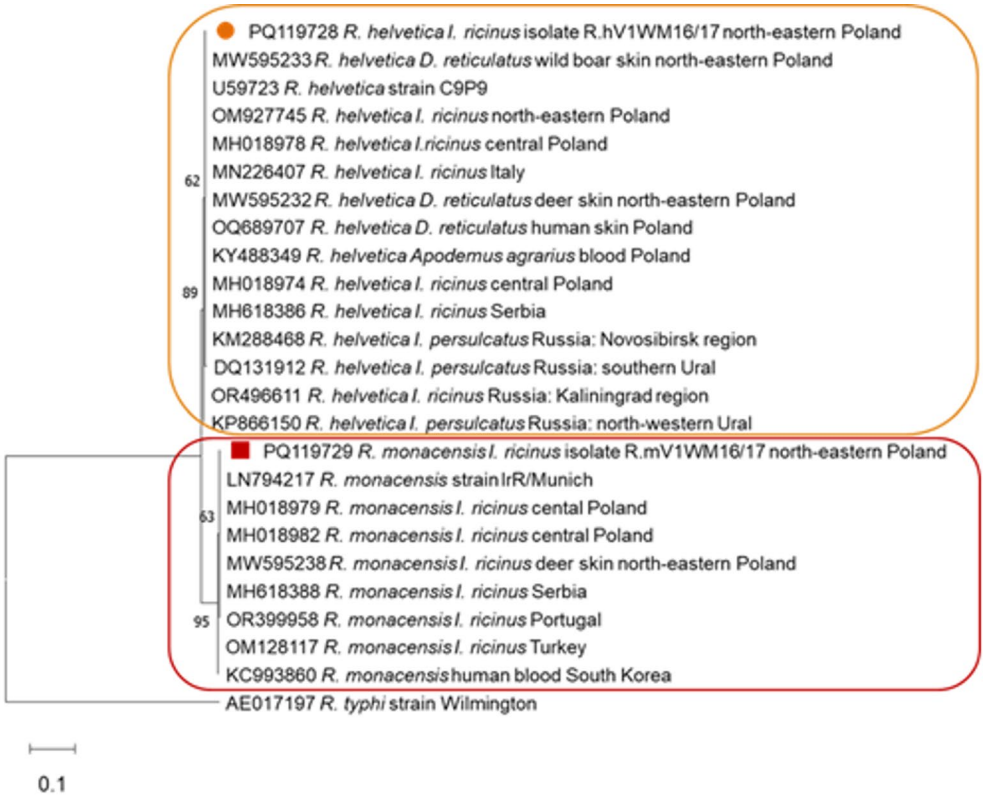

Fig. 2. Phylogenetic relationships between *Rickettsia* species identified in the study and accessions from GenBank, based on the sequences of the *gltA* gene. The phylogram was constructed with MEGA 11 software (https://www.megasoftware.net) using the Maximum Likelihood method and the Kimura 2-parameter method as a distance method. The percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of base substitutions per site. The sequences obtained in this study are labelled with color symbols.

MN226407) (Fig. 2). One obtained sequence identified as *R. monacensis* showed 100% identity with *gltA* sequences of the *R. monacensis* strain IrR/Munich from Germany (GenBank: LN794217) and from questing *I. ricinus* ticks from north-eastern (GenBank: MW595238) and central Poland (GenBank: MH018982) (Fig. 2).

Length analysis of the *rrl-rrf* ITS PCR products showed the presence of amplificons of ~530 bp in 259 *Rickettsia*-positive DNA samples and ~350 bp in three samples. Such lengths of ITS fragments are characteristic

for *R. helvetica* and *R. monacensis* , respectively. In order to confirm the specificity of PCR-screening, a randomly selected PCR product corresponding to *R. helvetica* and all sequences corresponding to *R. monacensis* were sequenced and deposited in GenBank under the accession numbers PV528813 and PV528814, respectively. The 505 bp sequence (our submission: PV528813) was 100% identical to the sequence of isolate BO144 of *R. helvetica* (GenBank: OZ018776) and *R. helvetica* previously identified in *I. ricinus* ticks in Poland (GenBank: JQ796866) and Austria (GenBank: EU057990). The nucleotide sequences of PCR products initially identified as *R. monacensis* were monomorphic (our submission: PV528814) and showed 100% identity to the IrR/Munich strain of *R. monacensis* (GenBank: LN794217) and sequences isolated from *I. ricinus* in Poland (GenBank: JQ796867), Bosnia and Herzegovina (GenBank: PV231331) and Switzerland (GenBank: JQ670878).

Rickettsia spp. and *Borrelia* spirochaete co-infections in adult ticks

In a previous study[27], an infection rate of *Borrelia* spirochaetes in adult *I. ricinus* ticks was 18.2% (151/831) and was higher compared to the level of *Rickettsia* spp. infection 11.6% (96/831) (Table 2). A total of 27.1% (225/831) of adult *I. ricinus* ticks tested positive for *Rickettsia* spp. and/or *B. burgdorferi* s.l. Co-infections with both bacteria were detected in 2.6% (22/831) of the examined ticks, which was significantly less frequent compared to non-infections and mono-infections (χ^2=645.28, $p < 0.001$) (Table 3). The co-infection rate in females was 2.6% (10/381) and did not differ significantly from that in males, which was 2.7% (12/450). In the *I. ricinus* population from the central part of the Warmia-Mazury province, the level of co-infection was significantly higher compared to the tick population from the western part, with rates of 4.7% (13/279) and 0.5% (1/208), respectively (Table 3). The co-infection rate of *Rickettsia* spp. and *Borrelia* spirochaetes in adult *I. ricinus* ticks was not significantly influenced by either the year of the study or the habitat (Table 3).

Rickettsia spotted-fever group seroprevalence in humans

Of the 155 serum samples tested with ELISA, positive results of IgG antibodies against *Rickettsia* spp. were found in 38.7% (60/155) of the total human study group (Table 4). The overall seroprevalence was significantly different between men 56.4% (53/94) and women 11.5% (7/61) ($\chi^2 = 31.445$, $p < 0.001$). The IgG seroprevalence for *Rickettsia* SFG was significantly higher in the HR group (forestry workers) than the LR group (city residence), 52.9% (54/102) and 11.3% (6/53) ($\chi^2 = 25.465$, $p < 0.001$), respectively (Table 4). In both examined groups the positive results were significantly higher in men (61.3% - HR and 28.6% - LR) than women (22.7% - HR and 5.1% - LR) (Table 4). Among forestry workers (HR), no significant differences in *Rickettsia*-positive results between Jedwabno (55.7%) and Olsztynek (48.8%) ($\chi^2 = 0.476$, $p = 0.490$) forest inspectorates were noted.

Borrelia spirochaetes seroprevalence in humans

The overall *Borrelia* IgG seroprevalence was 21.3% (33/155) which was nearly two times lower than the *Rickettsia* seroprevalence (Table 4). Significantly more positive results for *Borrelia*-IgG were found in males (33.0%, 31/94) than females (3.3%, 2/61) ($\chi^2 = 19.473$, $p < 0.001$). The IgG anti-*Borrelia* rate was significantly higher in the HR group (30.4%, 31/102) compared to the LR group (3.8%, 2/53) (Table 4). In both the HR and LR groups, *Borrelia*-positive results were significantly more frequent in men than in women, 36.3% and 14.3% vs. 9.1 and 0.0%, respectively (Table 4). 29.5% (18/61) of workers from the Jedwabno forest inspectorate and 31.7% (13/41) from the Olsztynek forest inspectorate were IgG *Borrelia*-positive ($\chi^2 = 0.056$, $p < 0.813$).

		Noninfection	Monoinfection *Rickettsia* spp. or *Borrelia burgdorferi* s.l.	Co-infection *Rickettsia* spp. and *Borrelia burgdorferi* s.l.	
		n/% (95% CI)			*p*-value*
Sex	Females	262/68.8[a] (63.85–73.39)	109/28.6[a] (24.12–33.43)	10/2.6[a] (1.27–4.77)	0.035
	Males	344/76.4[b] (72.25–80.29)	94/20.9[b] (17.22–24.94)	12/2.7[a] (1.39–4.61)	
Year	2016	242/72.7[a] (67.55–77.39)	80/24.0[a] (19.53–28.98)	11/3.3[a] (1.66–5.83)	0.623
	2017	364/73.1[a] (68.97–76.94)	123/24.7[a] (20.97–28.73)	11/2.2[a] (1.11–3.92)	
Subregion	West	167/80.3[a] (74.22–85.47)	40/19.2[a] (41.11–25.25)	1/0.5[a] (0.01–2.65)	0.009
	Central	191/68.5[b] (62.65–73.87)	75/26.9[b] (21.77–32.49)	13/4.7[b] (2.50–7.84)	
	East	248/72.1[b] (67.03–76.77)	88/25.6[a, b] (21.05–30.54)	8/2.3[a, b] (1.01–4.53)	
Habitat	Forest	272/70.8[a] (66.01–75.33)	101/26.3[a] (21.97–31.01)	11/2.9[a] (1.44–5.07)	0.454
	Ecotone	334/74.7[a] (70.42–78.69)	102/22.8[a] (19.01–26.99)	11/2.5[a] (1.23–4.36)	
Total		606/72.9 (69.76–75.92)	203/24.4 (21.54–27.50)	22/2.6 (1.67–3.98)	< 0.001

Table 3. *Rickettsia* spp. and *Borrelia burgdorferi* s.l. infection in *I. ricinus* ticks by sex, year, subregion and habitat, north-eastern Poland. * - chi2 test, $p < 0.05$; [a] vs. [b] - statistically significant difference between groups; [a] vs. [a] or [b] vs. [b] - no statistically significant difference (post-hoc Bonferroni test).

	Sex	HR ($n=102$)		LR ($n=53$)		Total	
		n/% (95% CI)					
IgG SFG *Rickettsia* - positive	Women	5/22.7 (7.82–45.37)	0.001*	2/5.1 (0.63–17.32)	0.018*	7/11.5 (4.74–22.23)	< 0.001*
	Men	49/61.3 (49.70–71.94)		4/28.6 (8.39–58.10)		53/56.4 (45.76–66.59)	
	Total	54/52.9 (42.8–62.9)		6/11.3 (4.27–23.03)		60/38.7 (31.00–69.00)	
		< 0.001*					
IgG *Borrelia burgdorferi* s.l. - positive	Women	2/9.1 (1.12–29.16)	0.014*	0/0.0 (0.0–9.03)	0.016*	2/3.3 (0.40–11.35)	< 0.001*
	Men	29/36.3 (25.79–47.76)		2/14.3 (17.8–42.81)		31/33.0 (23.62–43.44)	
	Total	31/30.4 (21.67–40.29)		2/3.8 (0.45–12.98)		33/21.3 (15.13–28.58)	
		< 0.001*					

Table 4. Prevalence of anti-*Rickettsia* spp. and anti-*B. burgdorferi* s.l. IgG antibodies in adult residents of north-eastern Poland according to sex. * - chi2 test, $p < 0.05$, HR - the high-risk group (forest workers); LR - the low-risk group (residences of Olsztyn city).

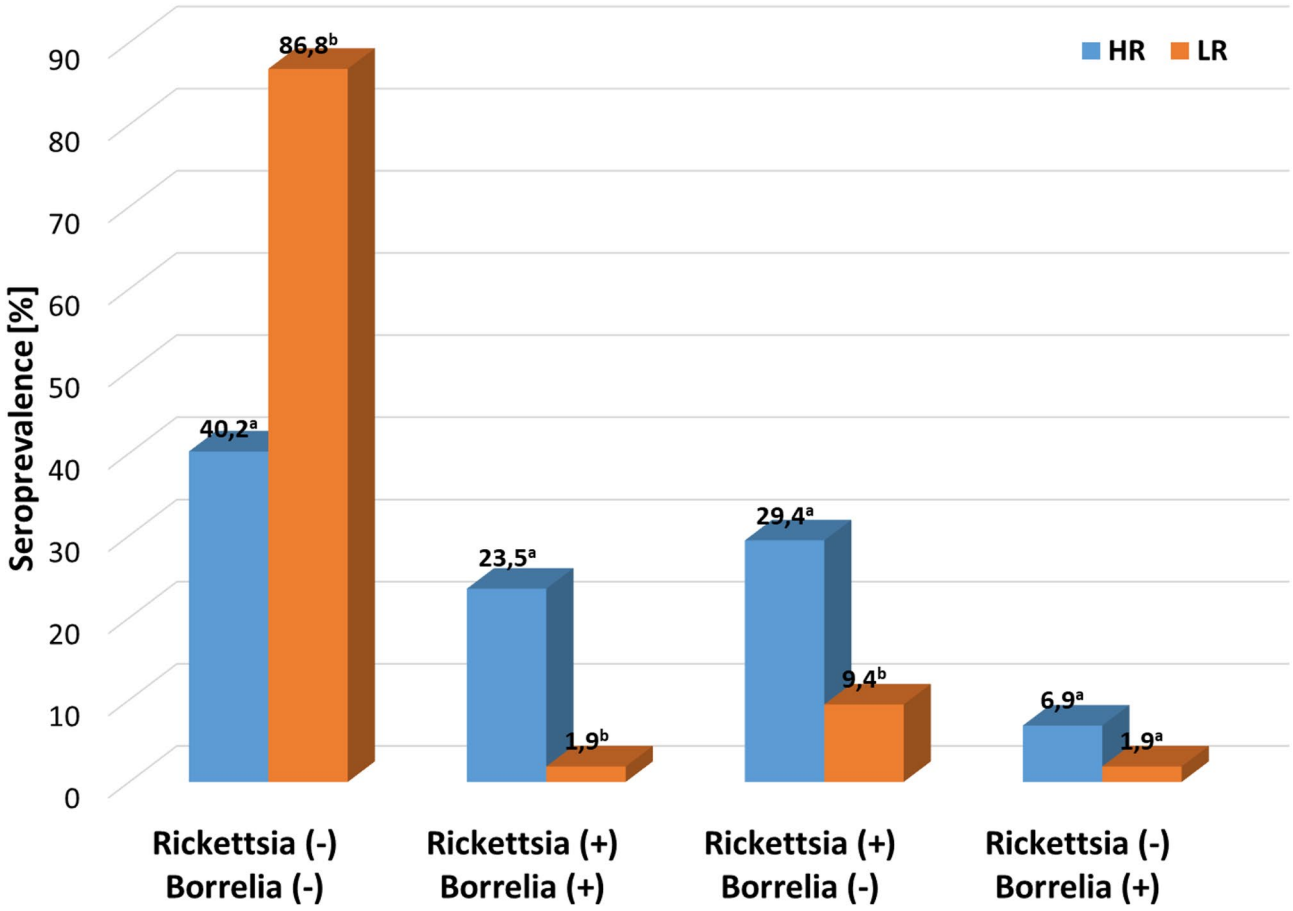

Fig. 3. Seroprevalence of IgG antibodies in single infection and coinfection with *Rickettsia* spp. and *B. burgdorferi* s.l. in adult residents of north-eastern Poland. chi2 test, $p < 0.001$, [a] vs. [b] - statistically significant difference between groups; [a] vs. [a] or [b] vs. [b] - no statistically significant (post-hoc Bonferroni test); HR - the high-risk group (forest workers); LR - the low-risk group - residences of Olsztyn city.

Co-exposure in humans

IgG antibodies against SFG *Rickettsia* and/or *B. burgdorferi* s.l were detected in 43.9% (68/155) of the total study group. Co-exposure to both bacteria was detected in 16.1% (25/155) of the participants. The IgG seroprevalence rate for co-infection with both pathogens was significantly higher among forest workers (HR group) than among residents of Olsztyn city (LR group) (23.5% [24/102] vs. 1.9% [1/53]) (Fig. 3).

Discussion

Bacteria of the genus *Rickettsia* are the second most frequently detected microorganisms in questing ticks and those feeding on hosts in urban and natural areas of north-eastern Poland, following *Borrelia* spirochaetes[28,29]. The overall prevalence of SFG *Rickettsia*, determined at 7.5% (including MIR in nymphs), falls within the lower values of the reported prevalence range (4.4–42.3%) observed in other regions of Poland[30–34]. In the studied *I. ricinus* tick population, we observed significant variations in SFG *Rickettsia* prevalence between the study years and among the subregions of the Warmia-Mazury province. A highly variable prevalence of *Rickettsia* spp. in *I. ricinus* ticks, ranging from 0.5% to 66%, has also been reported in other European countries[35–37]. Differences in the prevalence of *Rickettsia* spp. in *I. ricinus* ticks are influenced by a number of factors, such as geographic region, habitat type, and changes in environmental conditions throughout the year[30,32,34,38]. However, microclimatic factors[37,39,40] and the availability of suitable hosts that maintain and spread *Rickettsia* spp. in ecosystems[41,42] are also important.

In the studied *I. ricinus* tick population, the SFG *Rickettsia* infection rate in adults was almost twice as high as in nymphs (11.6% vs. 6.5%). Higher infection rates in females and males may result from more frequent contact with infected hosts during a greater number of blood meals compared to nymphs[32,40]. All developmental stages of *I. ricinus* can harbor SFG *Rickettsia*, including the most frequently identified species in Europe such as *R. helvetica* and *R. monacensis*[35,41]. Transstadial transmission of these species should be taken into account, as well as the transovarial transmission of *R. helvetica* observed under field conditions[43]. It is therefore possible that the vector *I. ricinus*, like the vertebrate hosts, serves as a reservoir for these two *Rickettsia* species[18,35,44].

The influence of geographical, ecological, and environmental factors on the variability in *Rickettsia* spp. prevalence in *I. ricinus* populations highlights the need for systematic monitoring of pathogens (or potentially pathogenic species) transmitted by ticks, other than *Borrelia* spirochetes and the tick-borne encephalitis virus. From a medical point of view, data on the diversity of microorganism species and their prevalence in ticks as their vectors are of great importance. Molecular analyses revealed the presence of only two species, *R. helvetica* and *R. monacensis*, in the *I. ricinus* tick population in northeastern Poland. These two species seem to be typical for the *I. ricinus* microbiota in Poland[30,31,34,45]. Only a few scientific reports have indicated the occurrence of another species *Candidatus* R. mendelii[30,45] and *R. slovaca*[46] in Polish *I. ricinus* ticks. *R. helvetica* and *R. monacensis* are also most frequently identified in other *I. ricinus* populations from Europe[35]. *Rickettsia helvetica* found in *I. ricinus* ticks occurs from 0.5% (Islands in the Baltic Sea) to a maximum as high as 66% in the Netherlands[13]. Similarly wide fluctuations in prevalence have been observed for *R. monacensis*, varying from 1% in Germany to 57% in Italy[10]. *Rickettsia helvetica* is considered the predominant species in *I. ricinus* in central and northern Europe, whereas *R. monacensis* is more frequently detected in southern Europe[13,37]. Notably, both species have been recognized as human pathogens[6,47–49]. In patients suspected of infection with these species, a relatively mild clinical course has typically been observed, including symptoms such as headache, rash, and occasionally an inoculation eschar[3]. In some cases, neurological complications such as meningitis and meningoencephalitis have been diagnosed[50,51]. Although both *R. helvetica* and *R. monacensis* have been isolated from symptomatic patients, no experimental studies have yet been conducted to unequivocally confirm their transmission through the tick-to-host route[52].

In Poland, spotted fever and other rickettsioses are subject to national epidemiological surveillance. However, the number of reported cases does not exceed 10 per year[53] (accessed April 30, 2025). The data do not specify whether the infections were caused by *R. helvetica* or by other *Rickettsia* species.

One approach to estimating the burden and risk of tick-borne diseases (TBDs), as well as the circulation of tick-borne pathogens in the environment, involves serological testing of the general population or healthy groups, such as blood donors, to detect specific antibodies in blood serum produced as a result of contact with antigens of microorganisms transmitted by ticks[20]. However, the distribution of TBDs is clearly linked to the occurrence of ticks in specific biotopes. The results of serological tests in the general population may be unreliable and very expensive because most people are not commonly exposed to tick bites. Therefore, groups at increased risk of contact with ticks, such as forest workers, farmers or patients already diagnosed with TBD as well as people working, living or engaging in recreational activities in habitats favourable to tick occurrence[20], are indicated as populations that can act as "sentinels". Among them, the emergence of new or rare tick-borne pathogens will be identified before they spread in the general population. This strategy can also reveal the co-occurrence of two or more TBDs in the same patient[20,52].

Although cases of SFG rickettsiae infections in humans are not commonly reported in the temperate region of Europe[19], studies on the presence of anti-spotted fever group rickettsiae antibodies indicate frequent contact with bacteria of the genus *Rickettsia*, especially in groups occupationally exposed to tick bite[54,55]. In Poland, previous serological surveys among such high-risk groups have shown antibody prevalence rates ranging from 14.7% to 39% in forestry and agricultural workers, depending on the region[33,56–58]. Our serological study also confirmed the reaction to *Rickettsia* antigen in residents of north-eastern Poland, both the high- and low-risk groups of exposure to tick bites. The IgG seroprevalence for SFG *Rickettsia* in forestry workers was almost five times higher than in the city residence group (52.9% vs. 11.3%). A very high percentage (~ 50%) of positive serological results confirming contact with *Rickettsia* bacteria was noted in the group of forestry workers performing outdoor work[33,57]. In other European countries, the seroprevalence rates for SFG *Rickettsia* have been reported to be 3.9–27% for similar occupational groups[48,54,55]. Although the overall level of infection with *Borrelia* spirochetes and SFG *Rickettsia* in the same population of *I. ricinus* ticks studied by us was comparable (8.1%[23] vs. 7.5%), the overall seroprevalence of SFG *Rickettsia* in the group of residents of north-eastern Poland was nearly twice as high as that of *Borrelia* (38.7% vs. 21.3%). We also demonstrated similar large differences when analysing separately subgroups of residents with high and low risk of exposure to ticks. Such large differences in the frequency of seropositive reactions to SFG rickettsiae and *Borrelia* spirochetes undoubtedly result from the fact that *I. ricinus* is not the only vector of rickettsiae. Our multi-year research has demonstrated the presence of *D.*

reticulatus in northeastern Poland, which is a vector of the human pathogenic *R. raoultii*[59,60], responsible for DEBONEL/TIBOL (tick-borne lymphadenopathy/erythematous necrosis-lymphadenopathy) cases in Europe, including Poland[61–63]. It is noteworthy that in both high- and low-risk groups of residents from northeastern Poland, men showed significantly higher rates of *Rickettsia*-positive and *Borrelia*-positive results compared with women. These observations suggest that men may experience more frequent exposure to tick bites, most likely due to occupational activities or outdoor recreation.

However, it should be emphasized that SFG rickettsial infection is not straightforward and novel serological and molecular approaches are still in development for diagnostic applications[64,65]. At present, the detection of antibodies against rickettsiae in the serum by indirect immunofluorescence assay (IFA) is the gold standard for diagnosis of tick-borne rickettsioses[11,66] but ELISA tests based on the purified whole bacteria or antigens, such as lipopolysaccharides from bacterial cultures (used in this study) have also been applied[56,67,68]. Depending on the purpose of the study, the use of ELISA is advantageous over IFA as a large number of samples can be screened in a single run[64]. However, a significant limitation of both methods is the inability to identify the SFG rickettsia species as a potential factor causing seroconversion and/or clinical symptoms[66].

In the context of research on ticks and tick-borne diseases, increasing attention is being given to the possibility of co-infection with genetically distinct pathogens or variants within the same species[69]. In our study, the co-infection rate of SFG *Rickettsia* with *Borrelia* spirochaetes in questing adult *I. ricinus* ticks was low (2.6%) compared to a significantly higher rate (16.1%) observed based on the presence of immunological responses in the study group of residents from north-eastern Poland. The high proportion of individuals with simultaneous IgG antibodies against antigens of both tick-borne microorganisms may result not only from the bite of a single tick infected with multiple pathogens, but also from bites by more ticks either simultaneously or following sequential transmission events. This scenario is supported by our serological results, which indicate that co-infection with SFG *Rickettsia* and *Borrelia* spirochaetes among forest workers (23.5%) occupationally exposed to multiple tick bites was significantly higher than in the group of urban residents (1.9% - low-risk group). Despite the limitations of serological testing in diagnosing co-infections with multiple tick-borne pathogens in humans, numerous studies do not exclude their occurrence in symptomatic patients following tick bites. Moreover, simultaneous infection with multiple tick-borne agents can be associated with greater clinical severity than would be expected from each infection occurring independently[70,71]. Co-infections in ticks and their impact on the pathogenesis of tick-borne diseases are intricate and not yet fully understood. Nevertheless, co-infections should be taken into account in clinical practice, particularly in patients with immunodeficiencies[70–72].

Conclusions

In summary, our findings provide insight into the prevalence of SFG *Rickettsia* in *I. ricinus* ticks and their seroprevalence among residents of north-eastern Poland. The prevalence in ticks, comparable to that of *Borrelia* spirochetes, together with high levels of antibodies in humans, confirms the circulation of these pathogens in the environment and human exposure to infection. Although limited by the assessment of minimum prevalence in nymphs, the developmental stage of *I. ricinus* most frequently biting humans, and by the serological assay, which did not allow precise identification of the specific SFG *Rickettsia* species responsible for seroconversion, our results emphasize the need for surveillance of tick-borne infections, other than Lyme borreliosis and TBE.

Data availability

The datasets used and analyzed during this study are available from the corresponding author (K.K.) upon reasonable request. Sequence data that support the findings of this study have been deposited in the GenBank database under the accession numbers PQ119728-729 and PV528813-814.

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Author contributions

Conceptualization: K.K., methodology: K.K.; formal analysis: K.K. and H.S., investigation: K.K., H.S., S.K. B.W-B., B.D., M.Ł., A.B. P.K.; resources: K.K., H.S., S.K., B.W-B., A.B.; writing—original draft preparation: K.K.; writing—review and editing: K.K., H.S., S.K. B.W-B.; visualization: K.K. All authors have read and agreed to the published version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Ethics statement

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Additional information

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