THE OCCURRENCE OF *TOXOPLASMA GONDII* AND *BORRELIA BURGDORFERI* SENSU LATO IN *IXODES RICINUS* TICKS FROM EASTERN POLAND WITH THE USE OF PCR

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Abstract: 715 *Ixodes ricinus* ticks from 4 sites of Lublin macroregion (Piotrowice, Polesie National Park, Dąbrowa and Łęczyńsko-Włodawskie Lakeland) were examined for the presence of *Borrelia burgdorferi* sensu lato and *Toxoplasma gondi*, using polymerase chain reaction (PCR) and nested-PCR methods. The clonal type of samples tested positive for *T. gondii* was identified by amplification and fragmenting with restrictions enzymes (RFLP-restriction fragment length polymorphism). Positive results were confirmed by sequencing. The overall percentage of tick infection with *B. burgdorferi* sensu lato (s.l.) was 12.7%, and with *T. gondii* – 12.6%. The coincidence of both pathogens was found in 2.4% of the examined specimens. The highest proportions of infections were detected in females, among all the developmental stages, both in the case of single pathogens (23.5% of ticks infected with *B. burgdorferi* s.l., and 19.7% – with *T. gondii*), and their coincidence (3.8% of all the tested females). RFLP PCR test detected that type I and atypical (type A) *T. gondii* strains were most common in ticks. The presence of type II/III was confirmed exclusively in females.

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INTRODUCTION

Toxoplasma gondii infections, like those with *Borrelia* spp., are recorded especially among inhabitants of rural and forest environments. Because Lublin province consists largely of agricultural and forested lands, exposure of the local population to those pathogens seems considerable. Toxoplasmosis is a worldwide disease of many mammals, including man [16], and still constitutes a serious diagnostic and treatment problem.

The incidence of infections with *T. gondii* in the human population differs according to the climate, diet and sanitary conditions, and reaches from 5%–90%; in the population of Poland it is estimated at 40%–50%. In the congenital form, toxoplasmosis may lead to abortion, neonatal death, or foetal abnormalities (e.g. ocular damage). Toxoplasmic encephalitis and disseminated toxoplasmosis have been observed in persons with immunodeficiencies, such as AIDS patients [64]. Human infections are caused mainly by genotypes I and II of *T. gondii*. Type II has been isolated from patients with congenital toxoplasmosis and AIDS, whereas type II and III strains are often isolated from animals [23, 27].

Lyme disease (LD) is a chronic multisymptomatic zoonosis with dermatologic, osteoarticular, neurologic and organ symptoms. LD is common worldwide and is associ-

ated with areas inhabited by ticks. *Borrelia* can be horizontally transmitted from all the developmental stages of ticks to their vertebrate hosts. The co-feeding may play an important role in horizontal transmission of *B. burgdorferi*, creating a threat of the disease transmission to humans. Wild animals were identified as the pathogen reservoirs, especially small forest rodents. In the case of wild animals, the course of disease is chronic, subclinical and sometimes without any pathological signs [65]. Serological studies performed in Europe showed that populations exposed to contact with ticks (forestry workers and farmers) more often have specific antibodies against *B. burgdorferi* than other groups. The annual number of LD cases in Poland is showing a tendency to increase [40], and in 2008 it reached 2,365.

There is no data concerning the coincidence of two pathogens – *T. gondii* and *B. burgdorferii* – in ticks. Thus the objective of this study was to estimate the potential risk of infection for people in Lublin macroregion (eastern Poland) basing on the incidence of those pathogens in ticks, detected by PCR examination. The present study on the range of *Ixodes ricinus* tick infection with *T. gondii* is a continuation of the latest research in the selected areas of West Pomeranian province [59]; it also addresses, as a new problem, the incidence of confections with two dangerous pathogens *T. gondii* and *B. burgdorferii*.

MATERIALS AND METHODS

Tick collection. 715 *Ixodes ricinus* were collected at 4 sites in the Lublin macroregion: Piotrowice, Łęczyńsko-Włodawskie Lakeland, Dąbrowa, and the Polesie National Park. Ticks were harvested by flagging in 3 subsequent vegetation seasons, from April–October, in 2007–2009. The specimens were placed in test tubes (separately: males, females and nymphs) and stored live until DNA isolation.

DNA isolation from ticks. DNA was isolated with the ammonia method [45]. Each adult form separately and nymphs in pools of 5 were suspended in 100 µl of 0.7 M ammonia hydroxide in sterile Eppendorf test tubes and triturated. The tubes were closed and kept in a heating block at 98ºC for 15–20 min. The obtained lysates were stored at –20ºC for further examination.

***Toxoplasma gondii* DNA identification with chain polimerase reaction (PCR) method.** The reaction mixture (50 µl) contained: 1.5 U Tag DNA polymerase (Qiagen, Syngen Biotech, Wrocław, Poland), 5 µl of the reaction buffer 10× diluted, 0.2 mM dNTPs (Polgen, Łódź, Poland), 1 µl of each of the primers 10 mM [22]: in the first reaction – Pml/S1 (5'-TGTTCTGTCCTATCG-CAACG) and Pml/AS1 (5'-ACGGATGCAGTTCCTT-TCTG), and in the second nested-PCR reaction: Pml/S2 (5'-TCTTCCCAGACGTGGATTTC) and Pml/AS2 (5'-CTCGACAATACGCTGCTTGA) (Eurogentec, Seraing,

Belgium), nuclease-free water (Applied Biosystems, Warsaw, Poland) and 5 µl matrix DNA.

Two-stage PCR reaction consisted of 30 and 20 cycles, respectively. Each cycle included: the proper denaturation at 94ºC for 30 sec., primers annealing at 60ºC for 30 sec. and elongation at 72ºC for 90 sec. The reaction products of stage I amplification (5 µl) were used at stage II PCR. Additionally, at each stage, the initial denaturation (2 min. at 94ºC) and the final elongation (2 min. at 72ºC), were performed. The 531 bp-long products of the second amplification were detected in 2% agarose gel (Prona, Basica LE) after staining in ethidium bromide. The positive control were the following strains of *T. gondii*: RH (type I, mouse virulent), ME49 (type II, mouse avirulent) and C56 (type III, mouse avirulent). The negative control was nuclease-free water (Applied Biosystems, Warsaw, Poland) [59].

To identify the clonal type (I or II/III) of the isolated *T. gondii* strain, RFLP PCR was performed. The PCR amplification products were treated with restriction enzymes: Eco 721 (substitute for PmII) and XhoI (Fermantas, Vilnius, Lithuania), and the reaction products were detected in 2% agarose gel.

DNA sequencing was performed by the DNA Sequencing and Oligonucleotides Laboratory (Institute of Biochemistry and Biophysis, Polish Academy of Sciences, Warsaw, Poland). The results were compared to published sequences in the GenBank database using the BLAST server at the National Center for Biotechnology Information (Bethesda, Maryland, USA).

***Borrelia burgdorferi* sensu lato DNA identification with polymerase chain reaction (PCR) method.** The reaction mixture (20 µl) contained: 0.5 U of Tag DNA polymerase (Qiagen, Syngen Biotech, Wrocław, Poland), 2 µl of reaction buffer 10× diluted, 2 mM of dNTPs (final concentration: 0.05 mM; Fermentas, Lithuania), 400 pmoles of each of the primers: Fla1 (5'-AGAGCAACTTA-CAGACGAAATTAAT) and Fla2 (5'-CAAGTCTATTTT-GGAAAGCAC TACA) (Eurogentec, Seraing, Belgium) complementary to the *fla* gene sequence, nuclease-free water (Applied Biosystems, Warsaw, Poland), and 2 µl of matrix DNA [70].

All PCR reactions were performed in PTC-150 thermal cycler (MJ Research Inc., Waltham, USA). Amplification consisted of initial denaturation (3 min. at 95ºC) and 35 amplification cycles, each including: the proper denaturation at 94ºC for 30 sec., primers annealing at 54ºC for 45 sec., elongation at 72ºC for 45 sec., and final elongation at 72ºC for 7 min.

The 482 bp-long amplification products were detected in 2% agarose gel (Prona, Basica LE) after staining in ethidium bromide.

The positive control was strain *B. burgdorferi* s.l. Bo-148c/2 (obtained by courtesy of Dr. habil. Joanna Stańczak, Interdisciplinary Institute of Maritime and Tropical Medicine, Medical University of Gdańsk, Poland). Additionally, the control obtained from the Deaprtment of Biological

Table 1. Results for various developmental stages of *Ixodes ricinus* ticks, collected at individual sites, tested for the presence of B1 *Toxoplasma gondii* gene.

Site	Examined ticks							
	Nymphs[a]		Males		Females		Total	
	Ni/Ne	%[b]	Ni/Ne	%	Ni/Ne	%	Ni/Ne	%
Piotrowice	7/150	4.7	2/54	3.7	13/53	24.5	22/257	8.6
Łęczyńsko-Włodawskie Lakeland	4/40	10.0	14/57	24.6	8/56	14.3	26/153	17.0
Dąbrowa	1/85	1.2	3/49	6.1	12/56	21.4	16/190	8.4
Polesie National Park	1/20	5.0	9/52	17.3	16/43	37.2	26/115	22.6
Total	13/295	4.4	28/212	13.2	49/208	23.5*	90/715	12.6

Ni (number of infected ticks); Ne (number of examined ticks); *significantly greater compared to males and nymphs: $p<0.05$ and $p<0.0001$ respectively (χ^2 test); [a] examined in pools, [b] minimum infection rate.

Occupational Hazards, Institute of Agricultural Medicine, Lublin, Poland, was used.

The negative control was nuclease-free water (Applied Biosystems, Warsaw, Poland).

Statistical analysis. The data was analysed with chi-square (χ^2) test, using Statistica 8.0 (StatSoft Inc., Tulsa, OK, USA).

RESULTS

Infection of *Ixodes ricinus* ticks with *Toxoplasma gondii*. The overall percentage of infections in *Ixodes ricinus* ticks in Lublin macroregion was 12.6%. The most frequently infected developmental stage proved to be females (23.5%). The proportion of infected male ticks was approximately half as high (13.2%), and the infections were at the lowest level in nymphs (4.4%). Statistically significant differences were found for individual sites in the Lublin macroregion where the study was conducted. The majority of tick infections with *T. gondii* was found in the Polesie National Park (22.6%), while in the Łęczyńsko-Włodawskie Lakeland there were less infections (17.0%). The percentages found in Dąbrowa and Piotrowice were similar (8.4% and 8.6%, respectively) (Tab. 1). The nested PCR products were sequence analyzed, which confirmed that the amplified products were identical with the sequence of the *T. gondii* B1 gene published in GenBank.

Strain typing of *Toxoplasma gondii*. To identify the clonal type of *T. gondii* strain, the nested-PCR reaction products were treated with restriction enzymes, which allowed to distinguish type I from type II/III. In the examined samples, the detected levels of type I and atypical genotype were the same – 45.5% positive test for each type (41 for each type). Type II/III was found in 8 samples (9.0%), and only in females (Tab. 2).

Infection of *Ixodes ricinus* ticks with *Borrelia burgdorferi* sensu lato. The overall percentage of *Ixodes ricinus* ticks infections at the studied sites in the Lublin macroregion was 12.7%. The highest proportion of infections was found in females (19.7%), it was lower in males (13.7%), and the lowest – in nymphs (7.1%). The majority of infected specimens was found in Dąbrowa (22.6%), while in the Łęczyńsko-Włodawskie Lakeland they were the fewest (4.6%). In Piotrowice and in the Polesie National Park the proportions of infections with *B. burgdorferi* sensu lato were similar (10.1% and 13.0%, respectively) (Tab. 3). The sequence analysis confirmed that the amplified products PCR were identical with the *fla* gene *B. bugdorferi* sequence published in GenBank.

The coincidence of *Borrelia burgdorferi* sensu lato and *Toxoplasma gondii* in *Ixodes ricinus* ticks in the Lublin macroregion. The overall percentage of *Borrelia burgdorferi* s.l. and *Toxoplasma gondii* coincidence was

Table 2. Results of *T. gondii* genotype analysis (RFLP-PCR) of positive samples obtained directly from *Ixodes ricinus* ticks.

Site	*T. gondii* genotype											
	Nymphs[a]			Males			Females			Total		
	I	II/III	A	I	II/III	A	I	II/III	A	I	II/III	A
Piotrowice	2	0	5	1	0	1	7	0	6	10	0	12
Łęczyńsko-Włodawskie Lakeland	4	0	0	7	0	7	3	2	3	14	2	10
Dąbrowa	1	0	0	2	0	1	6	3	3	9	3	4
Polesian National Park	1	0	0	4	0	5	3	3	10	8	3	15
Total	8	0	5	14	0	14	19	8	22	41	8	41

[a] examined in pools, A – atypical genotype.

Table 3. Results for various developmental stages of *Ixodes ricinus* ticks, collected at individual sites, tested for the presence of *Borrelia burgdorferi* sensu lato *fla* gene.

Site	Developmental forms of *Ixodes ricinus* ticks							
	Nymphs[a]		Males		Females		Total	
	Ni/Ne	%[b]	Ni/Ne	%	Ni/Ne	%	Ni/Ne	%
Piotrowice	8/150	5.3	7/54	13.0	11/53	20.7	26/257	10.1
Łęczyńsko-Włodawskie Lakeland	0/40	0.0	1/57	1.7	6/56	10.7	7/153	4.6
Dąbrowa	11/85	12.9	13/49	26.5	19/56	34.0	43/190	22.6*
Polesie National Park	2/20	10.0	8/52	15.4	5/43	11.6	15/115	13.0
Total	21/295	7.1	29/212	13.7	41/208	19.7	91/715	12.7

Ni (number of infected ticks); Ne (number of examined ticks); * significantly greater compared to Piotrowice and Łęczyńsko-Włodawskie Lakeland, and Polesie National Park: $p<0.001$ and $p<0.05$ respectively (χ^2 test); [a] examined in pools, [b] minimum infection rate.

2.4%. The highest proportion of coinfections was recorded in Dąbrowa (in 3.1% of ticks collected in this district), the lowest – in the Łęczyńsko-Włodawskie Lakeland (1.3%). Among 715 examined specimens, similar percentages of infections with one pathogen were detected (10.3% for *B. burgdorferi* s.l. and 10.2% for *T. gondii*) (Tab. 4).

The highest percentage of coinfections was found in females (3.8%), the lowest – in males (1.4%). The level of coincidence of the 2 pathogens in the examined nymphs was 2.0% (Tab. 5).

DISCUSSION

T. gondii is a parasitic protozoan widespread in the environment. The presence of *T. gondii* oocysts was confirmed, eg. in water samples [34, 35, 58]. It is generally believed that humans are infected with *T. gondii* usually by eating raw or half-cooked meat with the protozoan cysts, or food contaminated with faeces of an infected cat [16, 64]. However, a high incidence of *T. gondii* found, among others, in free-living ruminants suggests a possibility of other, so far unknown, paths of transmission of this protozoan. Infection with *T. gondii* is usually asymptomatic, but it may be dangerous during pregnancy and in immunocompromised patients, leading to congenital defects in a foetus or abnormalities concerning many systems (eg. in AIDS patients). Because the incidence of *T. gondii* infections in humans and in animals (including farm animals) is high, it is important to identify potential sources and pathways of infection with this protozoan. Our own study results and the literature [16, 17, 56, 60, 64] indicate that infection with *T. gondii* can constitute a serious epidemiological problem, especially in the rural environment where various transmission paths of the protozoan intersect. Due to the fact that they are widespread, and tick-bites occur frequently both in humans and in animals, ticks might play an important role in toxoplasmosis transmission. This pathway has been suggested by some authors [7, 8, 12, 13, 20, 50, 59, 71], while others were of different opinion [6, 21, 31]. Possible transmission of *T. gondii* ticks seems to be confirmed by the results of experimental tests [13], where *T. gondii* was transmitted by nymphs during interrupted feeding on mice. This possibility was also preliminarily corroborated in our own study, where *T. gondii* DNA was found in mice tissues inoculated with homogenate of ticks harvested from the natural environment [59].

The percentage of tick infections with *T. gondii* (12.6%) found in Lublin macroregion is comparable to that (12.7%) obtained in the study conducted in the West Pomeranian province [59], and much higher than the proportion detected in the Lublin province in earlier studies (2.2% of 92 examined specimens) [57]. Three (type I, II and III) of *T. gondii* genotypes play the main role in pathogenesis in humans and animals. In the Lublin macroregion, the ticks tested positive for *T. gondii* proved to represent type I and type A (atypical genotype) (45.5% of all positive results for each type). In a 2008 study, in ticks harvested from the

Table 4. Number of *Ixodes ricinus* ticks infected with *Borrelia burgdorferi* sensu lato and *Toxoplasma gondii*, at various sites in Lublin macroregion.

Site	Numer of pathogens in *Ixodes ricinus* ticks				
	No pathogens	1 pathogen		2 pathogens	Total
		Borrelia burgdorferi sensu lato	*Toxoplasma gondii*	*Borrelia burgdorferi* sensu lato + *Toxoplasma gondii*	
Piotrowice	215 (83.7%)	20 (7.8%)	16 (6.2%)	6 (2.3%)	257 (100%)
Łęczyńsko-Włodawskie Lakeland	122 (79.7%)	5 (3.3%)	24 (15.7%)	2 (1.3%)	153 (100%)
Dąbrowa	137 (72.1%)	37 (19.5%)	10 (5.3%)	6 (3.1%)	190 (100%)
Polesie National Park	77 (67.0%)	12 (10.4%)	23 (20.0%)	3 (2.6%)	115 (100%)
Total	551 (77.1%)	74 (10.3%)	73 (10.2%)	17 (2.4%)	715 (100%)

Table 5. Number of *Ixodes ricinus* ticks at various developmental stages infected with *Borrelia burgdorferi* sensu lato and *Toxoplasma gondii* in Lublin macroregion.

Stage	Number of pathogens in *Ixodes ricinus* ticks				
	No pathogens	1 pathogen		2 pathogens	Total
		Borrelia burgdorferi sensu lato	*Toxoplasma gondii*	*Borrelia burgdorferi* sensu lato + *Toxoplasma gondii*	
Nymphs	267 (90.5%)	15 (5.1%)	7 (2.4%)	6 (2.0%)	295 (100%)
Males	158 (74.5%)	26 (12.3%)	25 (11.8%)	3 (1.4%)	212 (100%)
Females	126 (60.6%)	33 (15.9%)	41 (19.7%)	8 (3.8%)	208 (100%)
Total	551 (77.1%)	74 (10.3%)	73 (10.2%)	17 (2.4%)	715 (100%)

environs of Szczecin, type I clearly prevailed (78.8%). To corroborate transmission pathways and sources of infection for people living in a given area, it would be advisable to compare the clonal type of the parasite isolated from patients with active toxoplasmosis, with that of the parasite isolated from the environment, among others, from ticks themselves. This, however, requires, further studies.

Ixodes ricinus is the most common species of ticks both in Poland and in Europe, and it plays the key role in epidemiology of Lyme borreliosis as vector and reservoir of *B. burgdorferi* spirochetes [69]. To-date, studies show that the incidence of tick infections with *Borrelia* spp. reaches, eg., 22% in Slovakia [36], 18% in Germany [39], and 9.3% in Spain [3]. In Poland, the average percentage of ticks infected with *Borrelia* spp. varies from 5.4%–12.4% [10]. The present detected proportion of infections (12.7%) is higher than the result obtained for the Lublin macroregion in 2006 (5.4%), which may be due to the number of examined nymphs that presented the lowest level of infections with *B. burgdorferi* [10, 72]. The results of tick examination may be reflected in serological tests on persons occupationally exposed to tick bites (e.g. forestry workers and farmers). In European countries, the proportion of forestry workers with antibodies against *B. burgdorferi* is 30% in Germany [46], 12.8% in Slovakia [5], and 7.5% in Italy [47]. In Poland, the average level of positive results reaches 30%–40% [9, 73].

Ixodes ricinus ticks, are not only the vector and reservoir for bacteria, but also for viruses (eg. viral tick-borne encephalitis) and protozoa. The latter include *Babesia* spp. – the pathogen phylogenetically related to other genera of protozoa, such as *Toxoplasma* or *Plasmodium*, belonging to the *Apicomplexa* type [28].

In recent years, an increasing number of tick coinfections with various pathogens, among others with *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, *Babesia microti*, has been detected [32, 61]. The incidence of mixed infections transmitted by *Ixodes ricinus* is a phenomenon of great epidemiological importance and can affect the course of diseases caused by the transmitted pathogens [48, 63].

The percentage of *B. burgdorferi* and *T. gondii* coinfections, found in the present study, was 2.4%, and was higher that the proportion of coincidence of *B. burgdorferi* with other pathogens detected both in Poland (Lublin macroregion) and in other countries. The coincidence of *Borrelia*

spirochetes and *A. phagocytophilum* was identified in 1.0% of the examined specimens, while the coincidence with *B. microti* – in 0.2% of the ticks. Coinfection with three pathogens: *B. burgdorferi* s.l., *A. phagocytophilum* and *B. microti* in Lublin macroregion was 2.2% [72]. In Germany the coincidence of *B. burgdorferi* and *A. phagocytophilum* reached the level of 0.7% to 0.8% [26, 43], in Moldova – 2.5% [32], and in the Netherlands – from 1.6%–2.0% [52, 69]. The percentage of double tick infections with *B. burgdorferi* s.l. and *B. microti* reached 1% in the Netherlands [24], 0.5% in Italy [42], and 0.9% in Russia [1].

The phenomenon of coinfection should be approached holistically in a given ecosystem, and the reservoir of pathogens – not only their vector – should be considered. In every ecosystem, there are usually several pathogens, or conditionally pathogenic microorganisms, which can form micropopulations (parasitocenoses) in individual ticks. The transmission of pathogens in a given area is determined by the activity of the local tick-hosting animal species [29, 33, 51].

Reservoir hosts for *Borrelia* spirochetes are vertebrate species able to preserve the pathogenic factor, thus becoming a long-lasting source of infection for ticks feeding on them. In Europe those species include mainly small mammals and birds that play a particular role in spreading both ticks themselves and pathogens, as their vectors [54, 55]. Most large mammals are not *B. burgdorferi* s.l. reservoir, although their part in the circulation of this microorganism in nature cannot be ruled out. An important role may be played by domestic animals, especially dogs which can show clinical symptoms of borreliosis [53]. Reservoir hosts for *T. gondii* among wild animals are eg. boars, which was confirmed by research in Slovakia, the Czech Republic and Spain [2, 4, 19]. The protozoan was also detected in the cervids, among others, in deers, roes and elks [68]. It is also not excluded that small mammals [25] and birds [15] play a part as *T. gondii* reservoir. The protozoan was identified in all species of farm animals, among which pigs are epidemiologically most important as a potential source of infection for humans, due to widespread consumption of pork [38, 49]. Serological tests showed that the proportion of infected pigs in Poland and in Europe varies according to the farming type and zoohygienic conditions [14, 41,

56, 60, 64, 66]. Anti-*T. gondii* antibodies were also indetified in sheep [18, 62, 67] and in cattle [30]. Farm animals with *T. gondii* parasitemia may be a potential source of infection for ticks, contributing to broader spreading of infection onto further animals and humans.

On the basis of numerous studies, multiple coinfections were also found in free-living mammals, deer, and tick-hosting birds [44]. Ticks can thus be infected with various pathogens, both during a single bite of those hosts and become infected during the subsequent developmental stages [11, 37].

Examination of ticks to detect multiple infections in a given ecosystem can help to predict such coinfections in humans, which is of great importance for the correct diagnosis and prophylactics of tick-borne diseases. When disease symptoms are unclear, knowledge of the area where a patient was bitten and of the local endemic foci, can be useful, making the identification of a pathogenic factor much more likely.

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