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PRELIMINARY STUDY ON THE OCCURRENCE OF *TOXOPLASMA GONDII* IN *IXODES RICINUS* TICKS FROM NORTH-WESTERN POLAND WITH THE USE OF PCR

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Abstract: A batch of 259 *Ixodes ricinus* ticks collected by flagging in woodlands of north-western Poland (Szczecin area) was examined for the presence of *Toxoplasma gondii* DNA with nested polymerase chain reaction (PCR). Positive isolates were genotyped with the restriction fragment length polymorphism (RFLP) analysis of B1 gene marker. Another batch of 340 *I. ricinus* ticks from Szczecin area was homogenized and inoculated into mice. Subsequently, the biological material obtained from inoculated mice was also examined with nested PCR for the presence of *Toxoplasma gondii* DNA and positive isolates were genotyped with RFLP as above. The study revealed that 12.7% of *I. ricinus* ticks were infected with *T. gondii*. Prevalence of *T. gondii* infection was highest in females (27.9%), being significantly greater ($p<0.05$) than in males (12.3%) and nymphs (5.6%). Most of the positive isolates (78.8%) belonged to type I of *T. gondii*. Samples from brains and other organs of 60 inoculated mice showed 44 positive results for *T. gondii* DNA with the dominance of atypical genotype and frequent mixed infections.

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INTRODUCTION

Toxoplasmosis is a widespread zoonotic disease caused by a parasitic protozoan *Toxoplasma gondii*. It may pose a serious public health problem as a congenital infection causing cerebral and ocular damage in newborns, and as an acquired infection in immunocompromised individuals, such as AIDS patients [8, 31]. Usually, infection with this parasite proceeds in a latent, asymptomatic form, manifesting by a positive serologic response (in humans mostly in the range of 30–60%, depending on population). However, recent studies indicate that also the latent toxoplasmosis may exert some, as yet not fully understood impact on the infected persons, including the development of behavioural changes and schizophrenia [32]. *T. gondii* may pose also a veterinary problem as a cause of illnesses in domestic animals, such as abortion and neonatal infections in sheep and goats [8].

Three main genotypes are responsible for the majority of human infections. The type I strains are associated with severe or atypical ocular toxoplasmosis in immunocompetent adults and are represented in congenital infections. The type II responds for most clinical toxoplasmosis cases in immunocompromised patients and has been isolated in cases of congenital toxoplasmosis. Type II and III strains are often isolated from animals. Additional genotypes are also recognised, but appear to be less common [6, 16, 19, 23, 30].

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The sexual reproduction of the parasites occurs in the small intestine of cat which has been demonstrated to be a definitive host of *T. gondii* and the most important link in the epidemiological chain of toxoplasmosis. Nevertheless, the whole picture of the circulation of *T. gondii* in nature and routes of transmission to a variety of warm-blooded hosts have not been fully elucidated. So far, oral transmission, by consumption of raw meat, food, and rarely, water contaminated with cat faeces containing infective *T. gondii* oocysts, is regarded as the only route of primary infection [27, 31). However, this route hardly explains the common occurrence of *T. gondii* in a variety of hosts, such as herbivorous mammals, wild rodents and birds that are unlikely to have contracted primary infection orally with meat or cat faeces [2, 25]. The gaps in the knowledge of epidemiology of the disease do not allow for the application of effective preventive measures, and the rates of human infection with *Toxoplasma* in Europe and other continents do not show the tendency to decline. Thus, some other routes of transmission suggested by earlier authors and partly forgotten should nowadays be considered, including transmission by blood-sucking arthropods.

In a former study [26], the presence of *T. gondii* DNA was found in *Ixodes ricinus* ticks collected in the Lublin region (eastern Poland). The aim of the present work was to study the occurrence of *T. gondii* DNA in *I. ricinus* ticks from the opposite, north-western part of Poland. Choosing of this region was prompted by the fact that in a previous study performed in this area by Kuźna-Grygiel *et al.* [21] the abundant occurrence of parasitic, piroplasma-like protozoa was found microscopically in salivary glands of *I. ricinus* ticks. Therefore, a possibility of infection with other protozoa such as *Toxoplasma gondii* seemed also probable.

MATERIALS AND METHODS

Tick collection. The *Ixodes ricinus* ticks (females, males and nymphs) were collected by flagging of lower vegetation in May 2007 in 3 suburban woodland localities: Bartoszewo, Kijewo and Szczecin Landscape Park, near the city of Szczecin (north-western Poland). Ticks were collected at peripheral areas of deciduous and mixed forests, mostly along the forest paths. Live females, males and nymphs of *I. ricinus* were collected separately in test tubes for further examination. A batch of 259 ticks (61 females, 73 males, and 125 nymphs) was fixed in 70% ethanol and preserved until examination by nested-PCR for the presence of *T. gondii* DNA (Tab. 1), while another batch of 340 live ticks (70 females, 60 males, and 210 nymphs) was homogenized and inoculated into mice.

Bioassay on mice. Ticks were homogenized in mortar in pools (10 females or 10 males or 30 nymphs), each in 1.5 ml of 0.9% NaCl containing antibiotics (1000 U of penicillin and 100 µg of streptomycin per 1 ml). Seronegative for *T. gondii* female Swiss-Webster mice, at the age of 8-10 weeks, were injected intraperitoneally (i.p.) with the homogenate of ticks in antibiotic solution (0.5 ml per mouse). Mice were observed daily and in the case of appearance of clinical symptoms (apathy, depression, diarrhoea) they were killed and sectioned. Several blind passages were performed, in which the next batch of mice were injected i.p. with homogenized organs (brain, lung, heart, liver, spleen) from sectioned mice. The remaining, healthy mice were killed and sectioned 6 weeks after inoculation. Samples of mice organs were stored at -20°C, and prior to the extraction of DNA, were cut into small pieces and homogenized. Altogether, organs from 60 inoculated mice were examined for the presence of *T. gondii* DNA, separately homogenates of brains and separately mixed homogenates of 4 other organs (lung, heart, liver and spleen). In parallel, homogenates of organs of 10 healthy, not inoculated mice were examined as controls.

Polymerase chain reaction (PCR). Adult ticks (females and males) were examined singly, while nymphs were examined in pools of 5 specimens. DNA was isolated from the examined ticks by lysis in ammonium hydroxide, according to the method described previously [26]. DNA from mice tissue samples was isolated using QIAmp DNA Mini Kit (Qiagen, Syngen Biotech, Wrocław, Poland) according to manufacturer's protocol. All samples of isolated DNA were stored at or below -20°C until the assays were performed. Detection of *T. gondii* DNA based on amplification of 35-fold-repetitive gene B1 fragment in 2 following nested PCR reactions was performed with the method described by Grigg and Boothroyd [15]. The following primers were used: 5'-TGTTCTGTCCTATCGCAACG, 5'-TCTTCCCA-GACGTGGATTTC, 5'-ACGGATGCAGTTCCTTTCTG and 5'-CTCGACAATACGCTGCTTGA. The following strains of *T. gondii* were used in PCR as controls: RH (type I, mouse virulent), ME49 (type II, mouse avirulent), and C56 (type III, mouse avirulent). 5 µl of target DNA were mixed with 5 µl of 10 × PCR Buffer containing 1.5 mM $MgCl_2$ (Finnzymes Oy, Espoo, Finland), 0.1 mM deoxynucleoside triphosphate mix (Polgen, Łódź, Poland), 10 pmol of each primer (Eurogentec, Seraing, Belgium), and 1.5 U of DNA polymerase (DyNAzyme™ II DNA Polymerase, Finnzymes Oy, Espoo, Finland) in a total reaction volume of 50 µl. 30 and 20 cycles were performed respectively in 2 stages reaction. Each of the cycles was carried out at 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s. 5 µl of specimen from the 1st amplification was used in the 2nd stage of reaction. Additionally, stages of initial denaturation at 94°C for 120 s and final elongation at 72°C for 120 s were performed. PCR amplification was carried out using an automated thermocycler (PTC-150, MJ Research Inc., Waltham, USA). For the analysis of PCR amplification products, 12 µl aliquots of reaction mixtures, marker pUC19 DNA/MIPS (Fermentas, Vilnius, Lithuania), positive control and negative control (redistilled water) were

Table 1. Frequency of occurrence of *Toxoplasma gondii* DNA in *Ixodes ricinus* ticks collected in 3 localities of Szczecin region (north-western Poland), examined by polymerase chain reaction (PCR).

Locality	Females		Males		Nymphs[a]		Total	
	+/N	%	+/N	%	+/N	%	+/N	%
Bartoszewo	1/7	14.3	3/11	27.3	0/45[a]	0[b]	4/63	6.4
Kijewo	9/25	36.0	2/25	8.0	2/20[a]	10.0[b]	13/70	18.6[#]
Szczecin Landscape Park	7/29	24.1	4/37	10.8	5/60[a]	8.3[b]	16/126	12.7
Total	17/61	27.9*	9/73	12.3	7/125[a]	5.6[b]	33/259	12.7

+/N: number of infected/examined ticks; [#] significantly greater compared to Bartoszewo and Szczecin Landscape Park: $p<0.05$; *significantly greater compared to males and nymphs: $p<0.05$ and $p<0.0001$, respectively; [a] examined in pools, [b] minimum infection rate.

applied to 2% agarose gel (Basica LE, Prona, EU) with Tris-Borate-EDTA (pH 8.2) as running buffer, and electrophoresis was performed for 60 min at 110 V. DNA bands were stained with ethidium bromide. Achieved specific products of 531 base pairs (bp) were visualized and analyzed using System Gel Documentation (Syngene, InGenius, Cambridge, UK).

To genotype strains of *T. gondii* detected in infected ticks and bioassayed mice, the restriction fragment length polymorphism (RFLP) analyses of PCR-amplified parasite DNA were performed. The amplified B1 product was digested with restriction enzymes XhoI and Eco72I (substitute for PmlI) (Fermentas, Vilnius, Lithuania) and identified on agarose gel [15].

DNA sequencing was performed by the DNA Sequencing and Oligonucleotides Laboratory (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland) with a capillary DNA Analyzer 3730xl (ABI/Hitachi, Tokyo, Japan) using primers for the nested reaction and a Big Dye Terminator v. 3.1 chemistry kit (Applied Biosystems, Warsaw, Poland). Results were compared to published sequences in the GenBank database using the BLAST server at the National Center for Biotechnology Information (Bethesda, Maryland, USA).

Statistical analysis. The data were analysed by chi-square (χ^2) test with the use of Statistica 8.0 (StatSoft Inc., Tulsa, OK, USA).

RESULTS

Tick infection rate. The total infection rate with *T. gondii* parasites among *I. ricinus* ticks collected in north-western Poland was 12.7% (Tab. 1). The majority of positive results were detected in females (27.9%), then in males (12.3%) and nymphs (5.6%). The highest frequency of the *T. gondii* infection was found in *I. ricinus* ticks collected in Kijewo, followed by those collected in the Szczecin Landscape Park and in Bartoszewo (Tab. 1). Nested PCR products were sequence analyzed, which confirmed that the amplified products were identical with the published in the GenBank sequence of the *T. gondii* B1 gene.

Table 2. Frequency of occurrence of *Toxoplasma gondii* DNA in brains and other organs of mice inoculated with homogenates of pooled *I. ricinus* ticks, examined by polymerase chain reaction (PCR).

Locality	Tick stage	No. of ticks' pools	No. of injected mice	Positive for *T. gondii* DNA			
				Brains	Other organs	Brains and other organs	Total
Bartoszewo	Females[a]	1	3	0	0	1	1
	Males	not examined	-	-	-	-	-
	Nymphs[b]	2	6	1	1	4	6
	Total	3	9	1	1	5	7
Kijewo	Females[a]	3	9	1	2	5	8
	Males[a]	3	9	0	2	5	7
	Nymphs	not examined	-	-	-	-	-
	Total	6	18	1	4	10	15
Szczecin Landscape Park	Females[a]	3	9	1	0	5	6
	Males[a]	3	9	0	1	8	9
	Nymphs[b]	5	15	0	1	6	7
	Total	11	33	1	2	19	22
Total		20	60	3	7	34	44

[a] pooled in 10 specimens; [b] pooled in 30 specimens.

Table 3. Results of *T. gondii* genotype analysis (RFLP-PCR) of positive samples obtained directly from *Ixodes ricinus* ticks.

T. gondii genotype Locality	Females			Males			Nymphs*			Total		
	I	II/III	A	I	II/III	A	I	II/III	A	I	II/III	A
Bartoszewo	1	0	0	3	0	0	n.e.	n.e.	n.e.	4	0	0
Kijewo	5	1	3	2	0	0	2	0	0	9	1	3
Szczecin Landscape Park	4	1	2	4	0	0	5	0	0	13	1	2
Total	10	2	5	9	0	0	7	0	0	26	2	5

* examined in pools of 5 specimens; n.e. - not examined; A – atypical genotype.

Mice infection rate. Samples from brains and/or other organs of 60 inoculated mice showed 44 positive results for the presence of *T. gondii* DNA (73.3%) (Tab. 2). Similar to in ticks, sequence analysis confirmed the identity of the *T. gondii* DNA. All samples from brains and other organs of 10 healthy, not inoculated mice showed negative results for the presence of *T. gondii* DNA.

Strain typing. To identify the *T. gondii* genotype in the B1-positive samples, RFLP analyses of PCR-amplified parasite DNA isolated from ticks and mice were carried out. The majority of types revealed in RFLP analysis on amplicons obtained directly from ticks were of type I (26/33=78.8%), followed by of atypical genotype (not digested by XhoI nor Eco72I) (5/33=15.1%) and of type II/III (2/33=6.1%) (Tab. 3).

In mice, DNA stripes common only to type I were found in the brain and/or other organs of 7 mice, and common only to types II and/or III in another 4 mice. In 15 mice, the type was not classified. Mixed infections of type I and II/III were detected in 3 mice, of type I and atypical in 4 mice, and of type II/III and atypical in 11 mice (Tab. 4). The isolation of various types from the single mouse was probably due to inoculation with the homogenate of pooled ticks. Moreover, it is possible that the single ticks were infected with mixed types of *T. gondii*.

DISCUSSION

Ixodes ricinus is the most common tick species in the forest areas of Europe. An epidemiological study carried out among the rural society in Poland showed frequent cases of people and animals bitten by ticks [25]. There is a lack of actual information about the occurrence of *T. gondii* in ticks, both in Poland and worldwide. A few previous studies have shown the possibility of experimental transmission of infection with *T. gondii* by ticks, and *Toxoplasma* strains were isolated from different tick species [13, 14]. Deryło *et al.* [7] have demonstrated experimentally the transmission of *Toxoplasma gondii* infection by nymphs of *Ixodes ricinus* and found microscopically the presence of

Table 4. Results of *T. gondii* genotype analysis (RFLP-PCR) of positive samples obtained from brains and other organs of mice inoculated with homogenates of pooled *I. ricinus* ticks.

Locality	Homogenate#	Numbers of mice with identified *T. gondii* genotype(s)						
		I	II/III	A	I+II/III	I+A	II/III+A	Total
Bartoszewo	Females[a]	0	0	0	0	0	1	1
	Males	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
	Nymphs[b]	1	1	2	1	0	1	6
Subtotal		1	1	2	1	0	2	7
Kijewo	Females[a]	4	1	1	0	1	1	8
	Males[a]	1	1	2	0	1	2	7
	Nymphs	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
Subtotal		5	2	3	0	2	3	15
Szczecin/Landscape/Park	Females[a]	1	0	1	1	1	2	6
	Males[a]	0	1	4	1	1	2	9
	Nymphs[b]	0	0	5	0	0	2	7
Subtotal		1	1	10	2	2	6	22
TOTAL		7	4	15	3	4	11	44

homogenate from: Females, Males and Nymphs of ticks; [a] pooled in 10 specimens; [b] pooled in 30 specimens; A – atypical genotype; n.e. – not examined.

T. gondii in the tissues of nymphs and females. Cases of human toxoplasmosis associated with tick bites have also been described [4, 24]. However, there are some scientists who do not believe that *T. gondii* is able to propagate in tissues of poikilothermic animals [8, 20].

Despite the probably significant role of ticks in epidemiology of toxoplasmosis, this problem has not been a popular subject of investigation in recent years, with the exception of our own single study [26]. It seems that the present study provides new information about the occurrence of *T. gondii* in ticks, as well as confirming the possibility of live parasite transmission from ticks to mice. DNA of *T. gondii* was detected in 12.7% of examined ticks (females, males and nymphs) collected in the Szczecin locality. A previous study carried out in Lublin region [26] showed a lower (2.8%) frequency of *T. gondii* infection in *I. ricinus*, which occurred only in tick females. Based on data from available literature, similar studies in other parts of the world are unknown. Detection of parasite DNA in mice inoculated with homogenate of ticks collected in the natural environment may confirm the possibility of transmission of live *T. gondii* from ticks to mammals.

Because exact data concerning the occurrence of *T. gondii* infection among people and animals from north-western Poland are unknown, a direct correlation between the prevalence of infection in ticks, people and animals in this area is difficult to estimate.

On the basis of pathogenicity in mice and variability of genetic markers (including B1 gene) *T. gondii* strains were divided into types. Type I strains are mouse virulent, whereas type II and type III strains are mouse avirulent [15, 28, 34]. The majority of known strains are one of these 3 types.

Many authors have reported that the majority of infections in animals are of type II *T. gondii*, while the rest of the isolates are dominantly type I or III [3, 6, 18, 19]. However, reports of "atypical" genotypes have increased in recent years as a wide range of unique places have been studied [1, 12, 17, 22, 29]. In these areas, atypical forms of *T. gondii* often dominate [5, 9, 10, 11].

In our preliminary study on genotyping of isolates of *T. gondii* DNA, the single locus B1 gene PCR-RFLP method was implemented [15]. This analysis permits differentiation of type I from types II and/or III. In our study, most of the *T. gondii* strains isolated directly from ticks belonged to type I, and mixed infections were absent, whereas in mice inoculated with ticks homogenates (indirect isolation) the majority of genotyped strains were atypical (15/44), and mixed infections were frequent (18/44). The difference between *T. gondii* types recovered by direct and indirect isolation could be explained by known inhibition of the development of *T. gondii* type I strains in inoculated mice while development of the strains belonging to other types is promoted [33]. The prevalence of mixed infections in inoculated mice could be explained by heterogeneity of inocula and mice organ samples.

Atypical strains have recently been detected more frequently, especially in environmental niches. B1 gene analysis does not differentiate between type II and III but may detect atypical strain. For full characterization of a *T. gondii* strain, it is the best to perform multilocus analysis where many markers are used (eg. B1; SAG1, SAG3; GRA6; L363).

The opinion that the diagnosis of the type predicts disease outcome, and may be helpful in treatment, seems justified but the information on strain biology is still incomplete [3]. Recently, it has been reported that multi-genotype infections are common in animals and humans [33]. Villena *et al.* [33] suggest strain typing to study disease severity and a panel of tests to avoid misinterpretation of results.

Our preliminary studies on the occurrence of *T. gondii* in ticks showed that it is a common invasive factor in ticks, and successful inoculation in mice suggests the possibility of infecting animals and humans by ticks. In further studies, the use of more genetic markers is projected for a better description of *T. gondii* genotypes.

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