Toxoplasma gondii infection in selected species of free-living animals in Poland

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A – Research concept and design, B – Collection and/or assembly of data, C – Data analysis and interpretation, D – Writing the article, E – Critical revision of the article, F – Final approval of article

Abstract

Introduction and objective. Free-living animals can play an important role as a reservoir of Toxoplasma gondii; however, data concerning this issue in Poland are still limited. The aim of the study was to assess the occurrence of T. gondii infection by using molecular methods in free-living animals in selected regions of Poland.

Materials and method. Tissues samples of 396 animals (foxes, muskrats, birds, martens, badgers, polecats, raccoons, minks, raccoon dogs, otters, small rodents and insectivores, and grass snakes) were collected from various regions of Poland. After samples digestion, DNA was isolated using QiAmp DNA Mini Kit (Qiagen). DNA extraction from small rodents and insectivores samples was performed without digestion. Next, nested PCR (B1 gene) and, for a part of nested PCR positive amplicons, RFLP PCR, were performed according to the method by Grigg and Boothroyd (2001). The other part of nested PCR positive DNA isolates were genotyped using 5 genetic markers: SAG1, SAG2 (5’- and 3’), SAG3, BTUB and GRA6, based on the method by Dubey et al. (2006). These PCR products were sequenced and compared with the NCBI database using Blast.

Results. In total, in 50 of the 396 examined animals DNA of T. gondii was detected (12.6%). The highest percentages of positive results in PCR was obtained in martens (40.9%) and badgers (38.5%), lower in birds (27.3%) and the lowest in foxes (7.4%). The RFLP and multiplex PCR analysis showed the dominance of T. gondii clonal type II (or II/III).

Conclusions. The results of this study indicate the frequent T. gondii infection among free-living animals in Poland, especially martens and badgers, which may indirectly indicate that these animals contribute to the spread of the parasite in the sylvatic environment in Poland. The genotyping analysis showed the dominance of T. gondii clonal type II (or II/III).

Key words

Poland, Toxoplasma gondii, nested PCR, genotyping, free-living animals

INTRODUCTION

Toxoplasmosis, caused by the protozoan parasite Toxoplasma gondii, is one of the most common parasitic infections of warm-blooded organisms, including humans [1]. Recently, toxoplasmosis has been ranked in the 4th position among foodborne parasites as disease causing the greatest global concern [2]. T. gondii infection can result in abortion in pregnant women or cerebral and ocular damage in newborns. An acquired infection of T. gondii can cause ocular abnormalities, and in immunocompromised individuals even leads to severe disease [3, 4].

T. gondii strains were classified into 3 major clonal lineages, types I, II, and III; however, other lineages, including atypical and recombinant genotypes, were also detected [5]. Type II is predominantly found in animals in Europe. However, recently, e.g. in Slovakia, Italy and Switzerland, type III and type I have also been detected [6–10]. Genotyping of isolated strains can improve knowledge about the circulation of the parasite, both on the global and a local geographical scale [11].

Free-living animals can play an important role as a reservoir of T. gondii; however, data concerning this issue in Poland are still limited. In the transmission of parasite in the sylvatic environment, an important role can play carnivores, typically infected by feeding on meat or carrion containing tissue cysts, but also herbivores who acquire an infection via food or water contaminated with oocysts excreted by definitive hosts – wild and domestic cats [1]. Small mammals are considered an important reservoir of T. gondii for foxes and raptorial birds, and their role has also been confirmed in the transmission of the parasite to pigs [12]. Rodents are considered to be important paratenic and intermediate hosts of this protozoan, and reservoirs for many bacterial and viral pathogens that can affect humans and animals. Companion animals (dogs and cats) feeding on small rodents contribute to the transmission of these pathogens from sylvatic to synanthropic environment, and to disease incidence in humans.

Since T. gondii is transmitted to a considerable extent by free-living carnivores, their high population densities (e.g. foxes, raccoons), pose increased health risks for humans and animals [13]. In the last decades, in Poland, the density of foxes has dramatically increased. Foxes often considerably exceed their natural habitats and occupy urban and peri-urban areas [14]. In the epidemiology of toxoplasmosis, an important role also seems to be played by birds of prey, which can indicate infections in local rodent populations. Birds also represent a source of infection for cats [15].

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Identification and genetic characteristics of *T. gondii* isolated from free-living animals can help in understanding the ways of circulating of the parasite in the environment. Thus, the aim of this study was to assess the occurrence of *T. gondii* infection in free-living animals in selected regions of Poland using molecular methods.

**MATERIALS AND METHOD**

**Samples collection.** Tissues samples from 396 animals: 148 red foxes (*Vulpes vulpes*), 16 muskrats (*Ondatra zibethicus*), 11 birds (species listed in Tab. 1), 22 European pine martens (*Martes martes*), 13 European badgers (*Meles meles*), 3 European polecats (*Mustela putorius*), 61 raccoons (*Procyon lotor*), 16 American minks (*Neovison vison*), 13 raccoon dogs (*Nyctereutes procyonoides*), 7 otters (*Lutra lutra*), 70 small rodents and insectivores (species listed in Tab. 1) and 16 grass snakes (*Natrix natrix*), were collected from various regions of Poland in 2013 – 2017 (Tab. 1).

Samples from birds and small carnivores (liver, muscles, heart, brain), as well as samples from snakes (heart, muscles), were collected in cooperation with the Polish Hunting Association; these animals had been shot by hunters or found dead on roads.

Samples foxes (muscles, heart, liver, tongue) were collected from animals shot during an official survey concerning the efficacy of an anti-rabies vaccination in different parts of Poland.

Samples from small rodents and insectivores (liver, kidney, lung) were collected from dead animals found in the vicinity of human settlements in Lublin province.

**Sample processing and DNA extraction.** Tissue samples (10–50 g) from birds, carnivores, and snakes were digested according to the method described by Dubey and Beattie (1988) [16]. Samples were cut and homogenized in 25–125 mL of 0.9% NaCl, followed by mixing with 50–250 mL of acid-pepsin solution (2.6 g pepsin, 7 mL HCl, and 0.9% NaCl filled-up to 500 mL, pH 1.1–1.2). After incubation at 37°C for 90 min in a shaker bath, the solution was poured out through the gauze, and centrifuged at 1,200 g for 10 min.

The pellets were collected, resuspended in 4–20 mL of phosphate buffered saline (PBS, pH 7.4), neutralized with 3–15 mL of freshly prepared 1.2% sodium bicarbonate (pH 8.3) and centrifuged (1,200 g × 10 min). Supernatant was removed and the pellet resuspended in 1–5 mL of 0.9% NaCl.

Each pellet sample was divided into 2 sub-samples (100 mL) from which DNA was extracted using a commercial kit (QiAmp DNA Mini Kit, Qiagen), according to the manufacturer’s instructions, except for an overnight incubation with proteinase K. Brain samples and samples from small rodents and insectivores (liver, kidney, lung) were homogenized, and approximately 25 mg of each sample (without digestion step) was destined for DNA extraction, as described above. All DNA samples were stored at −20°C until examination.

**Polymerase chain reaction (PCR).** *T. gondii* DNA was detected by amplification of 35-fold-repetitive B1 fragment gene in nested polymerase chain reaction (PCR), according to the method by Grigg and Boothroyd (2001) [17]. PCR were carried out in a C1000 Thermal Cycler (Bio-Rad).

**Genetic characterization.** For a part of PCR positive amplicons, restriction fragment length polymorphism (RFLP) PCR was performed according to the method by Grigg and Boothroyd (2001) [17], where an amplicons were digested with restriction enzymes Eco 721 (PmII) and Xhol (Thermo Scientific). For the other part of B1 positive DNA isolates the genotyping was performed using 5 genotyping markers (SAG1, SAG2 (5’- and 3’), SAG3, BTUB and GRA6), based on the method by Dubey et al. (2006) [18].

In all PCR reactions as positive controls RH (type I), ME49 (type II), and CS6 (type III) DNA isolates of *T. gondii* strains, and as a negative control nuclease-free water, were used. Nested PCR of B1 products after electrophoresis were identified on an agarose gel under ultraviolet light. The amplificons of PCRs with 5 genetic markers were sequenced by an external company (Genomed S.A.), and sequences analyzed using Geneious v. 11.1.4. software (Biomatters) and compared with the sequences deposited in NCBI database using Blast.

**Statistical analysis.** The differences between the prevalence of *T. gondii* were assessed by chi-square (χ2) test with Yates correction, and Student’s t-test. The differences in all analyses were considered statistically significant when p < 0.05. Statistical analyses were performed using Statistica v.9.1 (StatSoft Inc.).

**RESULTS**

**PCR and genotyping results.** In total, in 50 of the 396 examined animals DNA of *T. gondii* was detected (12.6%). Among wild birds, the positive results in PCR was found in 3 of 11 birds (27.3%), including: one kestrel, one seagull and one buzzard. The percentage of positive results in rodents and insectivores was 8.1%; in small rodents and insectivores 8.6%, and in muskrats 6.3%. Among carnivores, the percentage of positive results was 14.1%; the highest *T. gondii* prevalence was found in martens (40.9%) and badgers (38.5%), lower in foxes (26.0%) and raccoons (21.3%) (Tab. 1). RFLP PCR results for selected B1 positive samples (birds, foxes, raccoons) showed pre-dominance II and/or III genotyp(e(s).

Genotyping with 5 genetic markers performed for selected B1 positive DNA isolates also showed pre-dominance of type II (including indistinguishable at SAG1 marker type(s) II/III) (78.6%) and less extent type III (21.4%) (Tab. 1).

**DISCUSSION**

In the presented study, the molecular screening of selected species of wild animals from various regions of Poland showed 12.6% *T. gondii* infection. Studies regarding *T. gondii* prevalence in wildlife in Poland are limited and mostly based on serology. To-date, *T. gondii* DNA has been detected in Poland in humans [19], raw meat products [20], goat milk [21], and in environmental matrices [22, 23, 24]. However, little is known about an importance of small, free-living animals in the epidemiological context of *T. gondii* infection.

Among free-living animals, wild birds are regarded as important reservoir of *T. gondii* for carnivores. Moreover, because of migration behaviour, wild birds may spread the
parasite worldwide. In the current study, DNA of parasite was detected in 3 of 11 wild birds; mostly in birds of prey (common kestrel and common buzzard).

To the best of the authors’ knowledge, this is the first study on *T. gondii* prevalence in wild birds in Poland. In other countries, detection of DNA or live *T. gondii* have been recorded, e.g. in the Czech Republic [25] viable *T. gondii* was isolated from 8% of common buzzards, while in the USA, Lindsay et al. [26] isolated live *T. gondii* from 26.7% of examined raptors, including 11 of 27 red tailed hawks and 1 of 3 kestrels. Dubey et al. [2010] [15], also in the USA, isolated viable *T. gondii* from 6 of 51 raptors or carnivorous birds. In Slovakia, a total of 10 birds from wildlife were examined and the prevalence of *T. gondii* DNA was 40% (types II and III strains were detected) [27]. However, the highest *T. gondii* infection rate (up to 92%) was found in raptors in Turkey [11].

Small rodents and insectivores, being the main prey for many species of carnivores (including cats) and omnivores, are probably the most important *T. gondii* reservoir and may potentially contribute to the transmission of this parasite in both synantropic and sylvatic fauna. In Poland, there are no current data concerning *T. gondii* prevalence in wild, small rodents, thus the results of the present study can have new epidemiological value.

The results of nested PCR revealed the presence of the *T. gondii* DNA in 1/2 (6.3%) of examined birds. By RFLP PCR (B1 gene), DNA of parasite was detected in 2/3 (67%) of examined birds. The results of genotyping by RFLP PCR (B1 gene) or PCR's with 5 genetic markers (types II and III) are shown in Table 1.

### Table 1. Results of nested and RFLP PCR, and multilocus genotyping of *Toxoplasma gondii*.

<table>
<thead>
<tr>
<th>Group of animals</th>
<th>Species</th>
<th>Locality (Province)</th>
<th>Results of nested PCR N positive/ N examined (%)</th>
<th>Results of genotyping by RFLP PCR (B1 gene) or PCR's with 5 genetic markers Type (N of samples) (marker)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Birds (Class: Aves)</strong></td>
<td>Common kestrel (<em>Falco tinnunculus</em>)</td>
<td>Lubelskie</td>
<td>1/3 (II/III (1) (B1))</td>
<td>II/III (4) (SAG1); II (1) (SAG2, GRA6, BTUB, SAG3); III (1) (GRA6, SAG3)</td>
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<tr>
<td></td>
<td>Black-headed gull (<em>Chroicocephalus ridibundus</em>)</td>
<td>Lubelskie</td>
<td>1/2 (II/III (1) (B1))</td>
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<tr>
<td></td>
<td>Long-eared owl (<em>Asio otus</em>)</td>
<td>Lubelskie</td>
<td>0/1</td>
<td></td>
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<tr>
<td></td>
<td>Northern goshawk (<em>Accipiter gentilis</em>)</td>
<td>Lubelskie</td>
<td>0/1</td>
<td></td>
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<tr>
<td></td>
<td>Peregrine falcon (<em>Falco peregrinus</em>)</td>
<td>Lubelskie</td>
<td>0/1</td>
<td></td>
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<td></td>
<td>White stork (<em>Ciconia ciconia</em>)</td>
<td>Lubelskie</td>
<td>0/1</td>
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<tr>
<td></td>
<td>Common buzzard (<em>Buteo buteo</em>)</td>
<td>Lubelskie</td>
<td>1/1 (II/III (1) (B1))</td>
<td></td>
</tr>
<tr>
<td><strong>Subtotal</strong></td>
<td></td>
<td></td>
<td>3/11 (27.3%)</td>
<td></td>
</tr>
<tr>
<td><strong>Mammals (Class: Mammalia)</strong></td>
<td>Small rodents:</td>
<td>Lubelskie</td>
<td>6/70 (8.6%)</td>
<td>II/III (4) (SAG1); II (1) (SAG2, GRA6, BTUB, SAG3); III (1) (GRA6, SAG3)</td>
</tr>
<tr>
<td></td>
<td>Mice (<em>N of examined animals</em>):</td>
<td>Lubelskie</td>
<td>6/70 (8.6%)</td>
<td></td>
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<tr>
<td></td>
<td><em>Apodemus agrarius</em> (43),</td>
<td>Lubelskie</td>
<td>6/70 (8.6%)</td>
<td>II (1) (SAG3, 3'SAG-G2); II (1) (GRA6); III (1) (GRA6)</td>
</tr>
<tr>
<td></td>
<td><em>A. flavicollis</em> (1),</td>
<td>Lubelskie</td>
<td>6/70 (8.6%)</td>
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<tr>
<td></td>
<td><em>A. sylvaticus</em> (5);</td>
<td>Lubelskie</td>
<td>6/70 (8.6%)</td>
<td></td>
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<td></td>
<td><em>Voles (Microtus arvalis</em>) (2),</td>
<td>Lubelskie</td>
<td>6/70 (8.6%)</td>
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<td></td>
<td><em>Myodes glareolus</em> (4);</td>
<td>Lubelskie</td>
<td>6/70 (8.6%)</td>
<td></td>
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<td></td>
<td><em>Insectivores: Common shrew (Sorex araneus</em>) (8),</td>
<td>Lubelskie</td>
<td>6/70 (8.6%)</td>
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<tr>
<td></td>
<td><em>European mole (Talpa europaea</em>) (7)</td>
<td>Lubelskie</td>
<td>6/70 (8.6%)</td>
<td></td>
</tr>
<tr>
<td><strong>Subtotal</strong></td>
<td></td>
<td></td>
<td>7/86 (8.1%)</td>
<td></td>
</tr>
<tr>
<td><strong>Mammals (Class: Mammalia)</strong></td>
<td>American mink (<em>Neovison vison</em>)</td>
<td>Lubelskie</td>
<td>4/16 (25%)</td>
<td></td>
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<tr>
<td></td>
<td>European otter (<em>Lutra lutra</em>)</td>
<td>Lubelskie</td>
<td>1/7 (14.3%)</td>
<td></td>
</tr>
<tr>
<td><strong>Subtotal</strong></td>
<td></td>
<td></td>
<td>5/23 (21.7%)</td>
<td></td>
</tr>
<tr>
<td><strong>Mammals (Class: Mammalia)</strong></td>
<td>European marten (<em>Martes martes</em>)</td>
<td>Lubelskie, Lubuskie, Mazowieckie</td>
<td>9/22 (40.9%)*</td>
<td>II/III (1) (B1); I and II/III (mixed) (3) (B1)</td>
</tr>
<tr>
<td></td>
<td>European polecat (<em>Mustela putorius</em>)</td>
<td>Lubelskie</td>
<td>1/3</td>
<td></td>
</tr>
<tr>
<td><strong>Subtotal</strong></td>
<td></td>
<td></td>
<td>10/25 (40%)</td>
<td></td>
</tr>
<tr>
<td><strong>Mammals (Class: Mammalia)</strong></td>
<td>Red fox (<em>Vulpes vulpes</em>)</td>
<td>Lubelskie, Warmińsko-Mazurskie, Kujawsko-Pomorskie</td>
<td>11/148 (7.4%)*</td>
<td>II/III (1) (B1); I and II/III (mixed) (3) (B1)</td>
</tr>
<tr>
<td></td>
<td>Raccoon dog (<em>Nyctereutes procyonoides</em>)</td>
<td>Lubelskie</td>
<td>8/61 (13.1%)</td>
<td></td>
</tr>
<tr>
<td><strong>Subtotal</strong></td>
<td></td>
<td></td>
<td>19/109 (17.4%)</td>
<td></td>
</tr>
<tr>
<td><strong>Mammals (Class: Mammalia)</strong></td>
<td>European badger (<em>Meles meles</em>)</td>
<td>Lubelskie, Mazowieckie, Pomorskie</td>
<td>5/13 (38.5%)**</td>
<td>II/III (1) (B1); I and II/III (mixed) (3) (B1)</td>
</tr>
<tr>
<td><strong>Subtotal</strong></td>
<td></td>
<td></td>
<td>24/122 (19.8%)</td>
<td></td>
</tr>
<tr>
<td><strong>Class: Reptilia</strong></td>
<td>Grass snake (<em>Natrix natrix</em>)</td>
<td>Lubelskie</td>
<td>0/16</td>
<td></td>
</tr>
<tr>
<td><strong>Subtotal</strong></td>
<td></td>
<td></td>
<td>0/16</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td>50/396 (12.6%)</td>
<td></td>
</tr>
</tbody>
</table>

N/a – not amplified  
* prevalence significantly smaller compared to other species of this group of animals (p<0.05)  
** prevalence significantly greater compared to other species of this group of animals (p<0.05)
Carnivores are another group of free-living animals important in epidemiology of toxoplasmosis. Carnivores can be infected with tissue cysts when feeding on infected animals, or by ingestion of oocysts from the environment. High population densities of carnivores, in particular of foxes and raccoons, certainly pose increased health risks, not only for companion animals, but also for humans since important parasites of carnivores, such as *T. gondii*, *Toxocara* spp. and *Echinococcus multilocularis*, are also causative agents of zoonoses [13, 31]. Raccoon dogs (*Nyctereutes procyonoides*) are a neozoan species that has rapidly expanded its geographical range and shares many parasites [32]. In Poland, raccoon dogs are observed more often in the western regions of the country (Lubuskie and Zachodniopomorskie Provinces) [33, 34], hence these areas were chosen for the current research.

In the presented study, the highest prevalence among carnivores was found in martens (40.9%), badgers (38.5%), and American minks (25%); also one of 3 European polecats was PCR positive. A lower prevalence was found in European otters (14.3%), raccoons (13.1%), raccoon dogs (7.7%) and red foxes (7.4%). It is noteworthy that the aggregated prevalence in the weasel–like animals belonging to family Mustelidae (badgers, martens, minks, otters, poles) equal to 32.8% was significantly greater (p<0.001), compared to aggregated values stated in other carnivores belonging to the families Canidae and Procyonidae (9.0%), as well as in rodents and insectivores (8.9%). This seems to indicate that Mustelidae form an important reservoir in the circulation of *T. gondii* in nature.

In another study in Poland, *T. gondii* DNA was found, depending on the methods used, in 13.8–19% of red foxes, 25–32% of badgers, 24–29% of raccoon dogs and 28–33% of martens [35]. In the UK, the prevalence of *T. gondii* DNA in brains collected from wild carnivores was detected in 19 of 65 Eurasian badgers (29.2%) [36]. In the Czech Republic, Turčeková et al. [27] showed a high *T. gondii* prevalence in martens (50%), in which revealed genotypes II and III. The results of another study in the Czech Republic showed a much lower *T. gondii* prevalence (5%) in martens than recorded in the presented study (40.9%) [37]. Turčeková et al. [27] found one *T. gondii* DNA sample (type II) out of 7 tested samples of badgers, whereas *T. gondii* DNA was not detected in the tissues of raccoon dogs.

In red foxes, a higher prevalence (18.8%) than that recorded in the current study (7.4%) was found in Belgium [38], similarly in Germany 13.4–18.4% foxes were identified as *T. gondii* positive [39]. In France, *T. gondii* was isolated from 9 of 14 seropositive red foxes [40], whereas in the UK, only 6.0% of foxes were PCR positive and type II lineage was demonstrated [36]. A study on *T. gondii* prevalence in minks in the UK [36] showed similar results to those obtained in the current study (20% and 25%, respectively), whereas in Chile, *T. gondii* DNA was demonstrated only in one of 73 American minks [41].

Little is known about the possibility of *T. gondii* infection in poikilothermic animals. According to the scientific literature, experimental transmission of *T. gondii* to reptiles failed under normal temperatures, but it has been shown that *T. gondii* can be successfully transferred to reptiles when kept at higher temperatures. The results of the presented study have not confirmed the presence of *T. gondii* DNA in grass snakes tissues.

Since a part of the research was carried out in the past, the method then in use and based on a single genetic marker (BI), it allowed only for preliminary genotype assessment. This method can distinguish type I contrary types II and/or III. Moreover, not all DNA samples, positive in BI PCR, were successfully amplified by multilocus PCR used in subsequent years. No amplification with the use of other markers may be caused by the existence of a lower number of copies of particular markers in the genome, which resulted in lower sensitivity.

Summarized, 8 of 22 (36.4%) and 9 of 15 (60%) of positive samples (BI) were successfully genotyped by RFLP PCR and multilocus PCR methods, respectively. By multilocus PCR, successful amplifications enabled at one marker for 4 DNA isolates, at 2 markers for 2 isolates, and at 4 markers for one isolate. The obtained results showed a predominance of *T. gondii* clonal type(s) II and III/III, followed by type III; however, mixed types I and II/III was also found. These results obtained in wild animals seem to correspond with the results of genotyping of *T. gondii* DNA isolated from the raw meat of pigs and cattle in Poland (Sroka et al., article in press); however, another own study showed a high prevalence of type III among *T. gondii* DNA samples isolated from goat milk and raw meat products in Poland, while types I and II were less common [20, 21].

**CONCLUSION**

The results of this study indicate frequent *T. gondii* infection among free-living animals in Poland, especially martens and badgers, which may indirectly indicate that these animals contribute to the spread of the parasite in the sylvatic environment in Poland. RFLP and multilocus PCR analysis showed the dominance of *T. gondii* clonal type II (or II/III). Although the presented results may correspond with the results of research in other European countries, further research is needed on the prevalence, distribution and virulence of *T. gondii* in free-living animals in Poland, as well as genetic characterization of the isolated parasite.

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