SHORT COMMUNICATION

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Identification of *Toxoplasma gondii* in wild rodents in Poland by molecular and serological techniques

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Abstract

Rodents are recognized as reservoirs for *Toxoplasma gondii*, playing a crucial role in maintaining the parasite's presence in the environment. Biomonitoring was conducted to assess the role of sylvatic rodents in maintaining *T. gondii,* and to analyse the prevalence and seroprevalence of the parasite in seven wild rodent species. Rodents were collected in an open grassland study site located in northeastern Poland, and dissected. Brain, spleen, blood and serum samples were collected. Molecular (PCR assay, nested-PCR assay) and serological (ELISA and agglutination tests) methods were applied to indicate the best approach for application in the biomonitoring of *T. gondii* in small mammals. Samples were screened from 68 individuals using PCR assays but no T. gondii DNA were found. The agglutination test showed no signal. Antibodies against *T. gondii* were found in 5 sera samples out of 56 analysed (seroprevalence = 8.9% [4.4–16.8]). The results confirmed that rodents participate in the life cycle of *T. gondii* as reservoirs of this parasite in the sylvatic environment. However, for effective bio-monitoring of *T. gondii* in small mammals, the results suggest a preference for utilizing ELISA tests to detect *T. gondii* antigens, as opposed to relying solely on molecular methods.

Key words

biomonitoring, Toxoplasma gondii, rodents, wildlife, rodent-borne diseases, environment contamination.

INTRODUCTION

Toxoplasmosis is known to be one of the most prevalent protozoan zoonoses caused by an intracellular Apicomplexan parasite *Toxoplasma gondii* [1–3]. *T. gondii* is distributed globally with a cosmopolitan host range [4]. Felid species, including domestic cats and their relatives, are the definitive hosts and the only hosts that can shed oocysts into the environment [5]. Environmental contamination is an important health issue, therefore monitoring of the occurrence of this parasite is crucial for the One Health approach [6, 7]. Rodents pose a particular threat to public health because they are intermediate hosts of *T. gondii* and are also the reservoirs of infection for other animals [8]. They play a significant role in the food chain of many carnivore species and consequently, in the transmission of the pathogen to their predators, contributing to the spread of toxoplasmosis also in human communities [9]. Furthermore, small mammals are considered to be markers for estimating contamination of the environment, and also for assessment of the infection risk for definitive hosts of *T. gondii* [10]. Toxoplasmosis occurs mostly in regions with a subtropical climate [11]. The spread of *T. gondii* depends on many factors, including both extrinsic factors (e.g. geographic location, habitat) and intrinsic factors (e.g. host gender, host age). Wild rodents become synanthropic as humans encroach on their natural habitats [12].

The parasite's life cycle is complex, with several infective forms and different transmission pathways [13]. Rodents and other warm-blooded animals act as intermediate hosts after ingesting food or water contaminated with oocysts [14, 15]. After ingestion, oocysts change into the tachyzoite stage, and later directly into bradyzoites that form tissue cysts. The parasitic forms *T. gondii* can pass from host to host via the food chain or the placenta as congenital toxoplasmosis. Animals can become infected after eating oocysts from the environment or infected animals, e.g. rodents (reservoirs of infection), but only felids can shed *T. gondii* oocysts into the environment [16]. Rodents, being a frequent prey of felids, as well as other carnivores and omnivores, are considered to play a key role in the maintenance of the *T. gondii* life cycle [17].

Laboratory detection of *T. gondii* is necessary to establish the number of infected hosts [18]. *T. gondii* detection can be conducted using serological tests, bioassay, histologic demonstration of the parasite, and by amplification of parasite-specific nucleic acid sequences using polymerase chain reaction (PCR) [19, 20].

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Considering the impact of rodents in the transmission of *T. gondii* to felid species, for this study biomonitoring of *T. gondii* was conducted in north-eastern Poland. The main aims were to assess the role of small free-living rodents in maintaining the *T. gondii* life cycle, and to monitor the prevalence and seroprevalence of the parasite in the seven wild rodent species endemic in the region (*Microtus arvalis*, *Microtus agrestis*, *Apodemus sylvaticus*, *Apodemus agrarius*, *Apodemus flavicollis*, *Myodes glareolus*, *Mus musculus*). Different diagnostic approaches were applied, both molecular (PCR assay, nested-PCR assay) and serological techniques (ELISA method, agglutination test) to identify the best method for application in large-scale bio-monitoring of small mammals [21].

MATERIALS AND METHOD

The grassland study site is located near the University of Warsaw's research station in Urwitałt, in north-eastern Poland. The location and methods of rodent trapping, sampling, and processing of trapped animals have been described previously [22–25]. Briefly, rodents were trapped in 2021 and 2022 using wooden live traps with seeds, peanut butter and fruits as a lure [26–28].

Genomic DNA from rodents' brains, spleens and blood samples was extracted using commercial kits ('Genomic Mini AX Tissue' and 'Blood Mini', A&A Biotechnology, Gdynia, Poland), following the manufacturer's instructions. The PCR assay provided by Schwab and Mc Devitt (2003) was applied [29]. Primers TOXO1 (5'GGAACTGCATCCGTTCATGAG3') and TOXO2 (5'TCT TTA AAG CGT TCG TGG TC 3') were used for amplification of the 194 bp fragment of the 35-fold-repetitive B1 gene [30]. The reaction mixture volume was 25 µl. To confirm and compare our results, nested PCRs targeting the B1 gene were performed [31]. Inner primers: B1 up (5'-CGTCCGTCGTAATATCAG-3') and B1 down (5'-GACTTCATGGGACGATATG-3') were used for amplification of the 178 bp fragment of the B1 gene. Outer primers: B1 nested forward (5'-GGGAATGAAAGAGACGCTAATGTG-3') and B1 nested reverse (5'-CTTTTCGCCAGCAGAGGG-3') were used for amplification of the 94 bp fragment of the B1 gene (BMR Genomics, Padua, Italy). The reaction mixture volume was 25 µl.

Amplifications were performed in a ProFlex PCR System Thermocycler (Applied Biosystems, USA). All PCR reactions were performed including *T. gondii* positive control (genomic DNA from *T. gondii* RH strain) to ensure the correct functioning of the reaction and negative control (water template) to ensure lack of contamination of the PCR components. PCR products were analysed using the Essential V6 Imaging Systems (UVITEC, Cambridge, UK) after electrophoresis on a 2% agarose gel (Sigma, St. Louis, Missouri, USA) stained with Midori Green Advance DNA Stain (Nippon Genetics Europe GmbH, Germany).

Rodents which had been previously tested by molecular methods, were screened by a serological approach. Of 68 individuals, 56 individuals were tested because it was not possible to collect serum samples from 12 individuals. All serum samples were analysed according to the manufacturer's instructions for application of the diagnostic commercial kit: an agglutination test (Toxo-Screen DA, BioMérieux, Marcy-l'Étoile, France). In addition, a bespoke ELISA described

Figure 1. Sampling site (red symbol) in north-eastern Poland (Google Maps, 2023). Study site located in Mazurian Lake District (53°48'21.0"N 21°39'36.7"E). Black bar – 100 km

by Krupinska et al. (2023) was used to detect antibodies to *T. gondii* using a commercially available *T. gondii* antigen (CD90 Creative Diagnostics, New York, USA) prepared from *T. gondii* RH strain [27]. Horse-radish peroxidase-labelled anti-mouse immunoglobulin (Anti-mouse polyvalent immunoglobulins IgG, IgA, IgM; Sigma-Aldrich, Steinheim, Germany) was used as conjugate. Since no positive control sera from *T. gondii* infected rodents trapped in the study were available, the cut-off value was determined according to Naguleswaran et al. (2004). The first cut-off value was determined by the mean of all sera on the microtitre plate plus three standard deviations (SD). Sera with OD above this value were excluded and the remaining sera were used for calculation of mean absorption of negative samples (M_{neg}) and SD_{neg}. Sera with OD values above M_{neg} + 4 SD_{neg} were considered to be positive [32].

Prevalence / seroprevalence is given with 95% confidence limits in parenthesis (95% CI). The values were calculated using a bespoke software as shown in previous reports by the authors [8, 26, 33].

RESULTS

Firstly, 68 rodents from seven different species were tested. These included *A. agrarius* (n=18), *A. flavicollis* (n=13), *A. sylvaticus* (n=8), *M. glareolus* (n=12), *M. arvalis* (n=11), *M. agrestis* (n=4), and *M. muscullus* (n=2). PCR screening was performed using blood, spleen, and brain tissues of these animals. No *T. gondii* positive samples were by found using molecular detection.

Next, nested-PCR was performed to confirm PCR results. However, no positive samples were detected.

After the molecular approach, serological tests – agglutination and ELISA tests – were performed with sera the samples of 56 individuals. The agglutination test did not detect any seropositive animals; however, using the ELISA test antibodies against *T. gondii* were found in the sera of three rodent species (*A. flavicollis, M. agrestis* and *M. glareolus*), with an overall seroprevalence of 8.9% [4.4–16.8] (Tab. 1).

Table 1. Seroprevalence of *T. gondii* within investigated rodent species

Rodent species	No. of seropositive individuals	Total	Seroprevalence (%) +/- 95%CI
Apodemus agrarius	0	13	0.0 (0.0-22.5)
Apodemus flavicollis	3	21	14.3 (4.0-35.4)
Microtus agrestis	1	6	16.7 (0.9-58.9)
Microtus arvalis	0	2	0.0 (0.0-77.6)
Myodes glareolus	1	14	7.1 (0.4-31.7)
Overall	5	56	8.9 (4.4-16.8)

DISCUSSION

The results confirmed that wild rodents trapped in the Mazurian Lake District are exposed to *T. gondii* and may pose a particular threat for carnivore pets, such as domestic cats, and thus also to humans. The study reports an overall seroprevalence of *T. gondii* in small rodents of 8.9 %, based on the ELISA test. These results are consistent with a previous study by the authors [8] in which 32 samples (5.5%) were found out of 545 seropositive to *T. gondii*. No seropositivity was detected by the agglutination test.

Modified agglutination test (MAT) and ELISA are highly specific methods widely used for the detection of antibodies to *T. gondii*, and applicable for serological diagnosis of infection as well as in epidemiological studies. Several authors compared ELISA and agglutination test for the detection of *T. gondii* and declared lower specificity and sensitivity of MAT than that of ELISA [34, 35]. Moreover, the agglutination test can detect only IgG antibodies, while goat Anti-Mouse Polyvalent Immunoglobulins (G, A, M) used for ELISA in the presented study, is capable to detect three classes of antibodies, IgG, IgM and IgA. While IgG antibodies indicate previous or chronic infection, IgM and IgA signalize a recent or acute phase. Thus, the difference observed between the results of the agglutination test and ELISA may be also associated with the detected different stages of the infection. In addition, the ELISA test is better at identifying different classes of antibodies than the agglutination test, thus this method has a higher sensitivity and specificity [36, 37].

Laboratory tests based on serological methods appear to be the best option for detecting *T. gondii* [38–40]. Unfortunately, due to the small size of rodents, in some cases it is not possible to obtain sufficient amount of blood (serum) to enable serological testing. In a previous study [8], rodent sera was also tested using the serological method (a bespoke ELISA test), and therefore in this case, the results of serological tests were compared with molecular tests. In small rodents, *T. gondii* tissue cysts (bradyzoites) are mainly located in the brain [41, 42]. Based on this knowledge, the brains, spleens and blood of seven rodent species were used in this study to detect *T. gondii* DNA. However, no *T. gondii* DNA was detected in any of these tissue samples from the rodents in the current study.

The results obtained in this study compare well with other studies. For example, Ivovic et al. (2019), found that on the Istrian peninsula on the Adriatic Sea, *T. gondii* was rarely detectable among species of small rodents, such as *A. sylvaticus*, *A. flavicollis* and *A. agrarius* [36]. Recent studies by Rizwan et al. (2023) based on the molecular detection of *T. gondii* via amplification of the B1 and the SAG3 genes by PCR and nested-PCR assays, showed that only 14 (5.9%) of 236 rodents trapped in Pakistan showed signs of the presence of *T. gondii* DNA [42]. This is in line with results from rats tested on a mink farm in China, where Zou et al. (2022) found 18 samples (7.93%) out of 227 samples positive for *T. gondii* [43]. However, a much higher prevalence of *T. gondii* was found among wild rats in Nigeria – 64 (76.2%) of the 84 rodent individuals were positive [44], while on the other hand, none of 78 rodents (*A. agrarius* and *M. arvalis*) tested by Herrmann et al. (2011) using both serological and molecular methods were seropositive or PCR positive [45].

Urbanization, global warming, and globalization affect the maintenance and transmission of many diseases caused by parasites, including those caused by *T. gondii* species [46]. The life cycle of *T. gondii* is based on contamination of the environment with pathogen oocysts and on the prey-predator relationship [36, 47]. Anthropogenic and environmental factors affect the survival time and infectivity of oocysts [12]. The environmental contamination by this parasite also depends on the size of the population of the intermediate and definitive hosts [48]. Despite many studies on the impact of factors affecting the spread of *T. gondii*, the mechanisms related to the promotion of the pathogen in the environment are still not fully known [49]. Wild rodents are frequent prey of predators, including felid species. By introducing resources into natural environments, humans influence the rodent population structure, which affects the circulation

of *T. gondii* in the wild environment [11, 50, 51]. However, the role of wild rodents in the transmission of *T. gondii* is still underestimated, therefore further studies are necessary to reveal their impact on the emergence, transmission, and distribution of the parasite.

In summary, no *T. gondii* were detected in brain, spleen and blood samples using a molecular approach; however, serological evidence for the presence of *T. gondii* was found in four vole species from wild rodent populations in north-eastern Poland. The results of this study clearly indicate that the serological approach is the best option for bio-monitoring *T. gondii* in small mammals.

CONCLUSIONS

The study suggests that rodents living in Urwitałt in the Mazurian Lake District in north-eastern Poland are exposed to *T. gondii*, posing a potential public health risk to residents and visitors to the region. Considering other recent reports [44, 45, 52] based on molecular and serological techniques, further monitoring is needed to fully understand the status of *T. gondii* in the wild, and to comprehensively assess the risk of transmission of this pathogen to the human communities in the region.

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Institutional Review Board Statement: This study was carried out in strict accordance with the recommendations in the Guidelines for the Care and Use of Laboratory Animals of the Polish National Ethics Committee for Animal Experimentation, and according to the Polish national law for field work involving the trapping and culling of wild unprotected vertebrates for scientific purposes (Resolution No. 12/2022 of the Polish National Ethics Committee for Animal Experiments, 11 March 2022). The study was performed according to the ARRIVE guidelines 2.0.

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