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Research paper

The usefulness of direct agglutination test, enzyme-linked immunosorbent assay and polymerase chain reaction for the detection of *Toxoplasma gondii* in wild animals

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ABSTRACT

The aim of the study was to compare the usefulness of two antibody-based methods, the direct agglutination test (DAT) and enzyme linked immuosorbent assay (ELISA), with that of the polymerase chain reaction (PCR) for detecting anti-*Toxoplasma gondii* in samples derived from naturally-infected wild animals.

Antibodies against *T. gondii* were detected in meat juice samples collected from 129 free- living carnivores and omnivores. *T. gondii* seroprevalence was confirmed in 73,6% of examined samples when DAT and ELISA were used separately, but in only 88,4% samples when both immunological tests were used in parallel.

PCR results confirmed the presence of DNA of the parasite in 24 of all the 129 samples. Sixteen samples were classified as positive when all three tests were used.

A moderate degree of agreement was found between DAT and ELISA ($\kappa = 0.55$). However, no agreement was found between the molecular and serological tests: $\kappa = -1.75$ for DAT versus PCR; $\kappa = -1.67$ ELISA versus PCR.

By using both serological tests, antibodies against *T. gondii* were found in 77.5% of red foxes, 12.5% of badgers, 40% of martens and 8.3% of raccoon dogs. Antibodies against the parasite were detected also in one mink, but not in the sample derived from a polecat. *T.gondii* DNA was found in the brain tissue of 20 red foxes, three badgers and one raccoon dog.

Our studies confirm that ELISA and DAT are suitable and reliable techniques for *T. gondii* antibody detection in meat juice from wild animals when serum samples are unavailable. Positive results obtained by immunological tests do not always reflect that the host was infected by *T. gondii*. They indicate only a contact with parasite. PCR should be used to confirm te presence of DNA from *T. gondii*.

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1. Introduction

Toxoplasma gondii is a parasite found worldwide, with the ability to infect most warm-blooded animals, including humans (Dubey, 2008; Wallander et al., 2015). The parasite has a complex life cycle with felines as a definitive host (Herrmann et al., 2012), although many other animal species can act as potential intermediate hosts. Infection may occur by the ingestion of oocysts excreted to the environment with the feces of the definitive hosts, or by consuming meat containing tissue cysts. Additionally, transplacental transmision of *T. gondii* from mother to fetus has been reported. Most animals infected by *T. gondii* do not show any clinical sympthoms of parasitosis (Śmielewska-Łoś and Turniak, 2004). The parasite affects several organs; however, the predilection sites are the lungs, central nervous system and eyes (Dubey, 2008).

The most commonly-used, and the fastest, way to confirm that an examined animal has been in contact with the parasite is by the detection of antibodies in serum samples. Several different immunological tests for the detection of *T. gondii*-specific immunoglobulin (IgG and IgM) are commercially available, with the most frequently-used methods in seroprevalence studies being DAT and ELISA (Mainar-Jaime and Barberán, 2007; Wallander et al., 2015). ELISA is a serological test used to detect anti-*Toxoplasma*

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antibodies in serum and meat juice in different animal species. It is easy to perform and large number of samples can be tested in a short time. However, studies on the seroprevalence of *T. gondii* antibodies in wild animals suggest that DAT is equally useful. Using this method, Jokelainen et al. (2015) found the seroprevalence of *T. gondii* to be 23.99% in meat juice samples from free-roaming wild boars. Infection and disease can be detected with greater specificity by molecular methods of diagnosis such as the polymerase chain reaction (PCR). This method has been proven to be a valuable method of detecting *T. gondii* DNA in both domestic and wild animals, such as sheep, goats, pigs and mink (Hůrková and Modrý, 2006; Shaapan et al., 2008; Turčeková et al., 2014). Of the numerous DNA sequences which could be used for PCR analysis, the

TGR1E gene has been suggested as the most suitable for detecting toxoplasmosis in wildlife animals (Hill et al., 2005; Luptakova et al., 2010; Turčeková et al., 2014).

The present study compares the usefulness of three methods of detecting *T. gondii* in samples taken from wild animals: commercial DAT and ELISA tests to detect antibodies against *T. gondii,* and PCR to confirm the presence of DNA.

2. Materials and methods

2.1. Sample collection

A total of 129 animals were examined, including 102 red foxes (*Vulpes vulpes*), 12 raccoon dogs (*Nyctereutes procyonoides*), eight badgers (*Meles meles*), five martens (*Martes martes*), one mink (*Neovison vison*) and one polecat (*Mustela putorius*). The animals were taken from the Głeboki Bród Forest District (Fig. 1). Samples of tongue, diaphragm and brain were collected individually and delivered frozen for further examination.

The meat juice samples were collected from tongue and diaphragm, and pooled due to small amount of fluid.

2.2. Immunological test

2.2.1. DAT

The presence of IgG antibodies against *T. gondii* was confirmed in meat juice using commercially available Toxo-Screen DA (bioMerieux, France) according to the manufacturer's instructions. Before analysis the meat juice samples were centrifuged, and supernatant was used until further examination. Each sample was diluted to 1:40 in duplicate wells. The plates with diluted samples were covered with a self-adhesive sheet, mixed thoroughly and incubated at room temperature overnight. Sedimentation of the antigen-antibody complex at the bottom of the well were recorded as a negative result while clear agglutination above half of the well was positive, at either dilution.

2.2.2. ELISA

All samples were analyzed for the presence of antibodies to *T. gondii* using the multi-species ID Screen Toxoplasmosis Indirect kit (IDvet, Montpellier) according to the manufacturer's instructions. Supernatant from meat juice samples was diluted to 1:2 and tested in duplicate. The optical density was measured at 450 nm using an EL*800 ELISA automated plate reader (Bio-tek). For each sample, the S/P percentage was calculated according to the following formula:

S/P% = ODvalue of the sample/ODvalue of the positive control × 100.

Samples with an S/P ratio less than or equal to 40% were considered as negative for *Toxoplasma* antibodies. Samples with S/P ratio between 40% and 50% were considered doubtful. If the S/P ratio was greater than or equal to 50%, the sample was considered positive for *T. gondii.*

Table 1
Prevalence of *T. gondii* obtained by three tests.

Species	No. of positive			No. of tested
	DAT	ELISA	PCR	
Fox (*Vulpes vulpes*)	84	87	20	102
Raccoon dog (*Nyctereutes procyonoides*)	3	1	1	12
Badger (*Meles meles*)	5	3	3	8
Marten (*Martes martes*)	2	3	0	5
Polecat (*Mustela putorius*)	0	0	0	1
Mink (Neovison vison)	1	1	0	1

3. Molecular methods

3.1. DNA isolation

Brain samples were prepared as described by Dubey (1998), with some modification. Briefly, tissue was digested for one hour with HCl/pepsin/H_2O solution (2.10 ml/3 g/300 ml), homogenate was centrifuged and the pellet was used for further DNA isolation. Three samples (300 μl each) were taken from each pellet for genomic DNA extraction by a commercially available NucleoSpin Tissue kit (Machery Nagel, Germany) according to the manufacturer's instructions.

3.2. PCR

Amplification of the isolated DNA was carried out using standard PCR from the *T. gondii* TGR1E gene region, which is repeated in the genome 30–35 times. The following specific primers were used: TGR1E1 (sense): 5′-ATGGTCCGGCCGGTGTATGATATGCGAT and TGR1E2 (antisense): 5′-TCCCTACGTGGTGCCGCATTGCCT (Lamoril et al., 1996). The final positive PCR product is 191 bp in size. The reaction was performed in accordance with the protocols described by Lamoril et al. (1996) with some modifications. Briefly, the PCR solution was prepared in a final volume of 25 μl containing 1 μl of DNA template, 2 mM of each primer, 10 mM dNTPmix (Fermentas, USA), 25 mM $MgCl_2$ (Fermentas, USA), PCR Taq buffer 10× (Fermentas, USA), 1U Taq polymerase (Fermentas, USA) and ddH_2O. The reactions were performed in a T 100 thermocycler (BioRad, USA) as follows: an initial denaturation step of 94 °C for three minutes, followed by 35 cycles of denaturation at 94 °C for one minute, anneling at 64.5 °C for one minute, extention at 72 °C for one minute, and a final extention of 72 °C for seven minutes. Negative controls (DNA from PolB1 *Neospora caninum* tachyzoites) and positive controls (DNA from *T. gondii* tachyzoites) were included in each experiment. PCR products were analyzed on a 2% agarose gel stained with GelRed (Biotum, US). Gels were visualised under UV light and analyzed using the KODAK1D^{TM} Electrophoresis Documentation and Analysis System (Table 1).

4. Statistical analysis

Statistical analyses were performed using Microsoft Excel 2008 (Microsoft Corporation, Redmond, USA). The positive and negative results obtained by DAT, ELISA and PCR were classified into two-by-two contingency tables. Sensitivity, specificity, positive predictive value and negative predictive value were calculated using ELISA as a reference method. Additionally, the results were analyzed based on binominal analysis, by calculating the proportion of positive (P_A), negative (N_A) and overal (p_o) agreement (Cicchetti and Feinstein, 1990). The Kappa coefficient (κ) was calculated to evaluate the agreement between methods. Kappa is a measurement on a −1

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