



## First Detection of *Toxoplasma gondii* (Nicolle & Manceaux, 1908) (Eucoccidiorida: Sarcocystidae) in River Waters in Serbia

Vladimir Ćirković<sup>1</sup>, Aleksandra Uzelac<sup>1</sup>, Dragana Miličić<sup>2</sup>, Ivana Klun<sup>1</sup> &  
Olgica Djurković-Djaković<sup>1\*</sup>

<sup>1</sup> National Reference Laboratory for Toxoplasmosis, Centre of Excellence for Food- and Vector-borne Zoonoses, Institute for Medical Research, University of Belgrade, Dr. Subotića 4, PO Box 39, 11129 Belgrade, Serbia

<sup>2</sup> Department for Animal Ecology and Zoogeography, Faculty of Biology, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia

**Abstract:** *Toxoplasma gondii* is one of the most successful parasites in the world. Its life form found in the environment, where it may stay viable for a year or more, is the oocyst, excreted by members of the family Felidae as the definite host. During the past decades, several waterborne epidemics of toxoplasmosis worldwide indicated the importance of water as a source of *T. gondii* infection. In Serbia, detection of oocysts in water has not been previously performed and, therefore, there are no data regarding the presence of *T. gondii* in Serbian rivers. To fill this gap, surface water samples (n=20) were collected from four major rivers and their tributaries throughout Serbia. The samples were concentrated by filtration and total DNA was extracted from the pellet using a commercial kit. Next, the samples were screened for *T. gondii* DNA by amplification of the 529 bp repeat fragment by conventional PCR. Three positive samples were detected and all three were confirmed by amplification of GRA6 and SAG2 genes. This first detection of *T. gondii* in river waters in Serbia suggests that the examined rivers are contaminated with this protozoan, which makes them a potential source of infection. In addition, this study has shown that the used PCR methodology can successfully detect *T. gondii* in water samples.

**Key words:** toxoplasmosis, surface water, oocysts, DNA, PCR

### Introduction

*Toxoplasma gondii* (Nicolle & Manceaux, 1908) is one of the most successful parasites in the world, infecting all warm-blooded animals including about one third of the human population (DUBEY & BEATTIE 1988, MONTOYA & LIESENFELD 2004). In the immunocompetent host, toxoplasmosis is self-limiting, usually asymptomatic or mild; however, in immunocompromised individuals, it can be a serious, even life-threatening condition (DJURKOVIĆ-DJAKOVIĆ

1998, MONTOYA & LIESENFELD 2004). Acute infection during pregnancy can result in transplacental transmission to the foetus, causing congenital infection, with potentially serious consequences and late sequelae.

The parasite life cycle includes three distinct life stages: tachyzoites, tissue cysts and oocysts (DUBEY et al. 1998). The latter are excreted into the environment by the definitive hosts, animals of the family Felidae. A single cat can shed between a few and several million oocysts, which can stay viable

\*Corresponding author: [olgicadj@imi.bg.ac.rs](mailto:olgicadj@imi.bg.ac.rs)

in various environmental conditions for a long period of time (DUBEY & BEATTIE 1988, SHAPIRO et al. 2019). It has been documented that *T. gondii* oocysts remain viable for up to 13 months at an average air temperature of 19.5°C without exposure to direct sunlight (YILMAZ & HOPKINS 1972, FRENKEL et al. 1975). Furthermore, experiments have shown that oocysts can stay viable and even infective after 24 months in seawater or 55 months in freshwater at 4°C (DUBEY 1998, LINDSAY & DUBEY 2009). These are important factors for understanding the impact of the aquatic environment as a source of *T. gondii* oocysts. Evidence for the occurrence of *T. gondii* oocysts in the environment is constantly accumulating. Oocysts can be found in freshwater, seawater, soil and vegetables, and depending on their viability and infectivity they are considered a potential source of infection for humans and animals (SHAPIRO et al. 2019).

The aim of this study was to examine the presence of *T. gondii* oocysts in surface waters in Serbia using a newly implemented methodology.

## Materials and Methods

### Sampling locations and method

Water samples were obtained simultaneously with sample collection for the Water Quality Assessment Programme of the Serbian Environmental Protection Agency (SEPA) from rivers and tributaries in Serbia between December 2018 and April 2019, therefore encompassing both winter and spring seasons. The sampling locations were selected based on their significance for human and animal health as well as based on their proximity to state boundaries, cities, raw drinking water sources, farms, etc.

Water was collected from a depth of 30 cm below the surface, avoiding the river bottom and thus reducing the amount of contaminants such as pollen and plant debris. Ten litres of water per sample were collected in clean plastic containers and transported to the laboratory for further analysis.

### Filtration for oocyst concentration

To capture and concentrate any oocysts present, water samples were passed through a 1.2 µm pore size membrane filter (Millipore, Burlington, Massachusetts, USA) using a peristaltic pump (AxFlow, Dublin, Ireland). The filter was rinsed with distilled water and wash buffer periodically during the filtration process. As a final step the filter membrane was scrubbed manually for 3 minutes, and all the eluate was collected in 50 ml centrifugation tubes. The filter and the water-intake hose on the pump were

changed after each sample to prevent cross contamination. The eluate was centrifuged at 3000 x g for 10 minutes in a swinging bucket desktop centrifuge (Heraeus Megafuge 1.0R, Kendro, Langensfeld, Germany) and the supernatant discarded. All pellets of each sample were combined and a 200µl aliquot was taken for genomic DNA extraction.

### Extraction of gDNA and detection by PCR

DNA was extracted using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, 200 µl of sample was placed in PowerBead tubes and vortexed at maximum speed 3 times for 5 minutes in the BeadRuptor 4 instrument (Omni international, Kennesaw, GA, USA) with a 5-min pause in-between.

Samples were tested for the presence of *T. gondii* DNA by amplification of the 529 bp repeat element (AF146527) as previously described (VUJANIĆ et al. 2011, LÉLU et al. 2012). Briefly, each PCR reaction contained 10 µl of 2X PCR MasterMix (Thermo Fischer Scientific, Waltham, MA, USA), 0.25 mM of each forward (HO1: 3'-AGA GAC ACC GGA

**Table 1.** PCR results of the surface water sources tested

Rivers	PCR		
	529bp	AltSAG2	GRA6
Sava 1	-	-	-
Sava 2	-	-	-
Sava 3	-	-	-
Sava 4	-	-	-
Danube 1	+	+	+
Danube 2	-	-	-
Danube 3	-	-	-
Drina 1	+	+	+
Drina 2	+	+	+
V. Morava 1	-	-	-
V. Morava 2	-	-	-
Pek	-	-	-
Mlava	-	-	-
Lugomir	-	-	-
Lepenica	-	-	-
Bosut	-	-	-
Jadar	-	-	-
Lešnica	-	-	-
Kudoš	-	-	-
Rača	-	-	-



**Fig. 1.** Geographical distribution of *Toxoplasma gondii* at sampling points from rivers in Serbia (river, location): 1 – Sava 4, Jamena; 2 – Bosut, Batrovci; 3 – Jadar, Lešnica; 4 – Lešnica, Lešnica; 5 – Drina 1, Badovinci; 6 – Drina 2, Bajina Bašta; 7 – Sava 1, Šabac; 8 – Sava 2, Šabac; 9 – Kudoš, Jarak; 10 – Sava 3, Ostružnica; 11 – Danube 1, Zemun; 12 – Danube 3, Vinča; 13 – Danube 2, Smederevo; 14 – Lepenica, Lapovo; 15 – Rača, Svilajnac; 16 – Velika Morava 1, Ljubičevski Most; 17 – Velika Morava 2, Bagrdan; 18 – Lugomir, Ribare; 19 – Mlava, Bratinac; 20 – Pek, Kusiće.

ATG CGA TCT-5') and reverse (HO2: 3'-CCC TCT TCT CCA CTC TTC AAT TCT-5') primers (Invitrogen, Life Technologies, Carlsbad, CA, USA) and 3  $\mu$ l of extracted gDNA as template in a final reaction volume of 20  $\mu$ l. The thermal cycling program consisted of initial denaturation for 5 min at 95°C, followed by 45 cycles of denaturation for 15 s at 95°C, annealing for 30 s at 56°C and extension for 45 s at 72°C. For confirmation, AltSAG2 and GRA6 genes were amplified by multiplex nested RFLP-PCR according to the protocol described by Su et al. (2010). Briefly, the first (multiplex) PCR reaction contained 10  $\mu$ l of 2X PCR MasterMix (Thermo Fischer Scientific, Waltham, MA, USA), 0.15  $\mu$ M of the external forward and reverse primers and 2  $\mu$ l of gDNA template. The second (nested) PCR reaction was performed using 0.3  $\mu$ M of each internal forward and reverse primer and 2-4  $\mu$ l of the multiplex reaction as template. The thermal cycling program was as follows: for the multiplex reaction 3 min at 95°C for initial denaturation, followed by 30 cycles of 95°C for 30 s, 55°C for 60 s and 72°C for 60 s

and final extension at 72°C for 5 min. For the nested reaction, initial denaturation and final extension remained the same, but with 35 cycles of 95°C for 30 s, 60°C for 60 s and 72°C for 60 s. PCR products were visualized by electrophoresis in 2.0% agarose gels stained with ethidium bromide. Amplification was performed in a Veriti thermal cycler (Applied Biosystems, Foster City, CA, USA). Genomic DNA isolated from tachyzoites of the RH strain of *T. gondii* was used as the positive control for each batch of PCR reactions while nuclease (DNase and RNase) free water was used as a negative control.

## Results

During the study period, a total of 20 samples were collected from the Danube, Sava, Drina, and Velika Morava rivers and their tributaries (Fig. 1). *T. gondii* DNA was detected in three samples (15%), namely Danube 1, Drina 1 and Drina 2 (Table 1), indicating the presence of oocysts. These included a sample collected in the Danube at the Belgrade

(Serbia's capital) municipality of Zemun, a highly urban area (Fig. 1, point 11). The other two were collected from rural areas in Western Serbia, of which one near the mouth of the Drina, close to the village of Badovinci (Fig. 1, pt. 5), and the other 90 km upstream, near the township of Bajina Bašta (Fig. 1, pt. 6).

## Discussion

This study demonstrates the importance of surface water as a reservoir of *T. gondii* oocysts, which was shown for the first time in Serbia. Several methodologies have been used in previous studies for the detection of *T. gondii* oocysts in water (VILLENA et al. 2004, AUBERT & VILLENA 2009, HARITO et al. 2017, KOURENTI & KARANIS 2004). In contrast to *Cryptosporidium* and *Giardia* (oo)cysts, *T. gondii* oocysts are difficult to isolate by immunomagnetic separation due to the lack of a good monoclonal antibody specific for the oocyst wall (DUMÈTRE & DARDE 2005). Lectin magnetic separation has been proposed as an alternative but it is a difficult procedure and has therefore not been widely used (HARITO et al. 2017). Most studies, including this one, have thus relied on a combination of filtration and centrifugation for the collection of oocysts, while the detection is performed by molecular methods to demonstrate the presence of parasite DNA, which confirms the presence of oocysts in the filtrate (DUMÈTRE & DARDE 2003, VILLENA et al. 2004).

Waterborne transmission of *T. gondii* has been associated with several large-scale outbreaks (BENENSON et al. 1982, BOWIE et al. 1997, PALANISAMY et al. 2006). For instance, in the epidemic described in the Canadian province of British Columbia (BOWIE et al. 1997), a total of 100 cases of acute toxoplasmosis have been recorded among local residents, of which even 19 with symptoms of ocular involvement, which in itself is a rare presentation of acute acquired infection in adults. Epidemiological investigations showed that the cases were preceded by extensive rainfall and water runoff, suggesting contamination of water reservoirs with *T. gondii* oocysts originating from *domestic, feral and wild* cats (including cougars *Felis concolor*).

Positive findings in this study may have been facilitated by the collection period during the colder seasons, since oocyst viability is prolonged in cold water (LINDSAY & DUBEY 2009); similarly, a higher proportion of *T. gondii* DNA-positive samples was noted during the autumn period as opposed to the summer in a study in Scotland (WELLS et al. 2015). Our finding of *T. gondii*-positive samples in the

Danube in a highly urban area, suggests that cats, whether stray / feral or household animals which occasionally leave their homes are the most likely source of oocysts. This area of the Danube is densely populated, with a large number of houses on the riverbank. The presence of oocysts here may be significant for human health, as this part of the Danube is used recreationally. The other two positive locations were on the Drina River. The Drina flows through a rural area of Serbia characterized by mountainous and forested terrain which represents the foothills of the Dinaric Alps. Serbian forests are home to 19 species of carnivores, two of which belong to the family Felidae, the lynx (*Lynx lynx*) and the wildcat (*Felis silvestris*) (PAUNOVIĆ 2008). In fact, the range of the Eurasian lynx in Serbia possibly includes the area (PAUNOVIĆ 2002), from which the Drina River water samples have been collected. Like the Danube at the Zemun location, the Drina River is often used recreationally, so the presence of oocysts may be of public health concern. The findings of this study indicate that Serbian rivers are a reservoir of *T. gondii* oocysts, and may represent a potential source of infection for humans and animals.

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