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RESEARCH

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# *Toxoplasma gondii* genotypes and frequency in domestic cats from Romania

Adriana Györke<sup>1\*†</sup>, Anamaria Balea<sup>1,2†</sup>, Silvia Borșan<sup>1</sup>, Chunlei Su<sup>3</sup>, Tiantian Jiang<sup>3</sup>, Cristian Magdaș<sup>1</sup>, Daniel Mărcuțan<sup>1</sup>, Radu Blaga<sup>4</sup>, Viorica Mircean<sup>1</sup>, Isabelle Villena<sup>5,6</sup>, Furio Spano<sup>7</sup>, Violeta Briciu<sup>8,9</sup> and Vasile Cozma<sup>1,10</sup>

## Abstract

**Background** *Toxoplasma gondii* is a zoonotic protozoan parasite with a heteroxenus life cycle that involves felids as the definitive hosts and any warm-blooded animal, including humans, as intermediate hosts. Cats are key players in parasite transmission as they are capable of shedding high numbers of oocysts in their feces that contaminate the environment.

**Methods** The study was performed on 31 domestic cats (31.23 ± 27.18 months old) originating from rural and urban areas (5.17:1) in the center and north-west Romania. Feces ( $n=31$ ), blood ( $n=28$ ), and heart samples ( $n=27$ ) were collected. Fecal samples were analyzed by flotation technique, and PCR (529 bp repetitive element). Fecal samples with *T. gondii* oocysts were bioassayed in mice. Serum samples were analyzed by modified agglutination test and ImmunoComb for the detection of specific anti-*T. gondii* IgG antibodies. Heart samples were bioassayed in mice, and analyzed by PCR. *Toxoplasma gondii* positive samples were genotyped by nPCR-RFLP targeting eleven genetic loci (SAG1, SAG2, alt-SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico).

**Results** *Toxoplasma gondii* oocysts were found in 2 out of 31 fecal samples collected from a 3-months old stray kitten, and a 4-years old female. In total, 17 out of 27 sera were positive for *T. gondii* IgG antibodies. The antibody titers in MAT ranged from 1:6 to 1:384. *Toxoplasma gondii* DNA was detected in 7 out of 27 heart samples, and four of them were positive also by bioassay. Six *T. gondii* DNA samples from bioassayed mice could be assigned to ToxoDB PCR-RFLP genotype #1 or #3 (Type II) and one *T. gondii* DNA from heart digest to genotype #2 (Type III). Both of these genotypes are common in Europe.

**Conclusions** Our results revealed that the infection with *T. gondii* is still high in cats from Romania. The oocysts shedded by these cats represent an important source of infection for intermediate hosts, including humans. Further studies on a wider range of cases are necessary for a more exhaustive definition of the *T. gondii* genotypes circulating in Romania.

**Keywords** *Toxoplasma*, Cats, Romania, Genotypes

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## Background

The protozoan *Toxoplasma gondii* is an intracellular parasite with a complex life cycle and the ability to infect virtually all warm-blooded animals, including birds, mammals, and even humans [1]. Because felids are the only definitive hosts of *T. gondii*, cats, particularly domestic ones that are both carnivores and have direct contact with humans, are key players in the transmission cycle of this parasite [2]. After infection, cats are capable of shedding an impressive number of oocysts in their feces that contaminate the surrounding environment. Upon sporulation, these oocysts become the main source of infection for the intermediate hosts [3]. After a primary infection cats become immune to oocyst re-excretion [4]. Nonetheless, Zulpo et al. [5] proved that immunity against reinfection decreases over time, and after 24 months from the primary infection, only 25% of cats were protected against oocyst re-excretion. Also, they can excrete almost the same amount of oocysts (less with 30%) as in the primary infection [5]. The infection of intermediate hosts can occur through the consumption of raw or undercooked meat with cysts, exposure to water or food contaminated with oocysts, or transplacentally [1]. The majority of acute cases of toxoplasmosis in humans were caused by oocyst-driven infections, which is thought to be a more severe means of infection than through cysts [2].

Congenital toxoplasmosis in cats is rare [6, 7]. Experimental studies have shown that transplacental infection does not represent an important epidemiological factor in the transmission mechanism of *T. gondii* in cat populations [8]. However, clinical toxoplasmosis is more severe in congenitally infected kittens than in adult cats which usually show unspecific clinical signs [9, 10]. Additionally, transplacental infection usually leads to death due to pulmonary or hepatic forms of the disease [7–9, 11, 12]. The most common clinical signs in cats are pneumonia, fever, anorexia, diarrhea, vomiting, hepatitis, paresis, dermatitis, and ocular inflammation [6, 12, 13].

*Toxoplasma gondii* strains present differences in molecular characteristics. There are three major clonal lines (I, II, and III) and other additional, atypical and recombinant lines [14–16]. In cats, atypical genotypes are predominant, followed by type II clonal strains [17]. The importance of factors such as immunological status, infection dose, co-infection rates, and geographical genetic variant distribution in clinical toxoplasmosis remains unclear [18]. So far, due to limited information, no association can be presumed between a specific genotype and a certain clinical outcome [18].

Since the first *T. gondii* isolation in Romania from a human case in 1956 [19], additional serological and molecular *T. gondii* epidemiological studies, and case reports have been published both in humans and animals

in our country [20–27]. To date, information on *T. gondii* infection in cats in Romania is limited to epidemiological studies by serological or coproparasitological assays [28, 29]. There are few studies in our country reporting *T. gondii* genotypes. The most commonly reported genotype was type II in a case of congenital toxoplasmosis in humans [30], in two goat kids [24], in 3 pigs [27], and in a cat [31].

Therefore given the importance of cats in the epidemiology of *T. gondii* and public health and the lack of molecular data on *T. gondii* in cats, the purpose of this study was to determine the frequency and to genetically characterize the *T. gondii* obtained from domestic cats in Romania.

## Methods

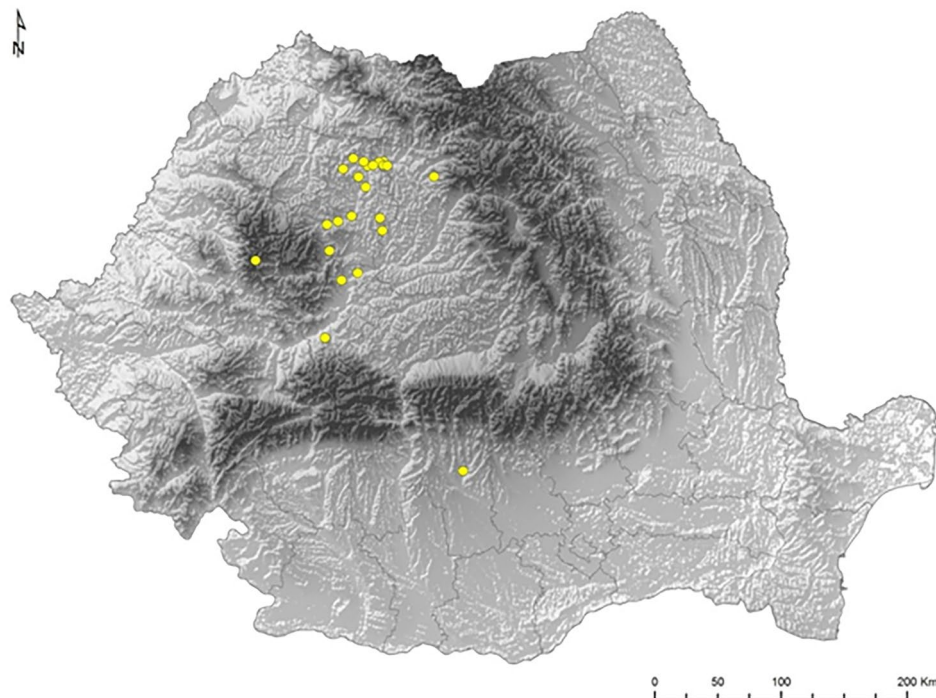
### Animals and samples

The study was performed between 2013 and 2015 on a total of 31 domestic cats originating from rural and urban areas in three counties in the center and north-west Romania (Cluj, Alba, and Bistrița Năsăud) (Fig. 1). Twenty-seven out of 31 cats were donated by the owners for a parallel study carried out within a project, with their informed consent for euthanasia. The remaining four cats were stray cats presented to our service for general clinical examination. Euthanasia was performed in compliance with the legislation in force following the approval of the Commission of Bioethics of USAMV CN (18099/23.11.2012). The cats were anesthetized with xylazine (Narcoxy, 1 mg/kg, IM) followed after 20 min by administration of ketamine (Ketamidol, 25 mg/kg, IM). Thereafter, when cats became unconscious, a combination of embutramide/mebezonium and iodide/tetracaine hydrochloride (T61) was administered by intra-pulmonary injection in a volume of 3–10 ml according to the cat size.

Feces, blood, and heart samples were collected from each cat. Blood samples were collected from the jugular vein after anesthesia, and serum samples were obtained after blood clotting. Sera were stored at -20 °C until processing. Fecal and heart samples were stored at 4 °C for 24–48 h until further processing. Direct (fecal flotation technique, bioassay, and PCR) and indirect (serological techniques for IgG antibodies) methods were used for *T. gondii* identification. Moreover, *T. gondii* DNA positive sample were genotyped by PCR-RFLP [32].

### Coproparasitological examination

Fecal samples were analyzed for the presence of *T. gondii*-like oocysts by sodium chloride (specific gravity 1.18) flotation followed by microscopy [33]. *Toxoplasma gondii*-like oocysts (10–14 μm) were distinguished from other parasitic eggs based on their morphology. *Toxoplasma gondii* oocysts are similar from a morphological



**Fig. 1** Spatial distribution of the domestic cats ( $n=31$ ) included in the study (center and northwest Romania)

point of view to *Hammondia hammondi* oocysts and were differentiated by PCR from sporulated oocysts.

*Toxoplasma gondii*-like oocysts from positive fecal samples were sporulated in 2.5% potassium dichromate solution ( $K_2Cr_2O_7$ ) and bioassayed in mice. Briefly, the fecal sample was mixed with 2.5%  $K_2Cr_2O_7$ , filtered, and incubated at laboratory temperature (21 °C) with constant aeration and homogenization until complete sporulation of oocysts (approximately 48–72 h). Following  $K_2Cr_2O_7$  removal from the sporulated oocyst suspension by repeated centrifugation (3x, 3000 rpm for 5 min), the sediment was resuspended in distilled water and stored at 4 °C.

Irrespective of the flotation results, *T. gondii*-like oocysts were concentrated from 5 g fecal sample with the flotation technique, followed by sedimentation (3000 rpm for 5 min) with distilled water to remove the salt solution, three times. The obtained fecal pellets were kept at -20 °C until PCR analysis.

#### Serological analysis

Serum samples were analyzed by an in-house modified agglutination test (MAT) and a commercially available enzyme-linked immunosorbent assay (ImmunoComb® Feline Toxoplasma & Chlamydophila Antibody Test Kit; Biogal – Galed Labs., Israel) for the detection of specific anti-*T. gondii* IgG antibodies. A serum sample was considered positive if at least one of the two methods was positive.

**MAT.** *Toxoplasma gondii* formalin-fixed tachyzoites (RH strain), supplied by National Reference Centre on toxoplasmosis/Toxoplasma Biological Resource Center from Reims (France), were used as antigens. The protocol was carried out according to the procedure described by Villena et al. [34]. Each serum sample was serially diluted twofold until the end of seropositivity. Positive and negative controls from a previous study [29] were used in each set of reactions. Samples with titers  $\geq 1:6$  were considered positive.

**ImmunoComb.** The Feline Toxoplasma&Chlamydophila antibody test kit (Biogal Galed Laboratories AcS Ltd., Israel) was used and applied according to the manufacturer's instructions as described [29]. Titers  $\geq 1:32$  were considered positive.

#### Bioassay in mice

Sporulated *T. gondii*-like oocysts from positive fecal samples and cat hearts were bioassayed in mice. All bioassays were performed in 6–8 weeks old females CD1 mice. The mice were purchased from the Biobase of the University of Medicine and Pharmacy “Iuliu Hațieganu” Cluj-Napoca, housed in the Parasitology and Parasitic Disease's Biobase (USAMV CN, FMV) and maintained according to animal welfare and protection regulations. Considering the 3R concept (*replacement, reduction, refinement*) [35], two mice per sample were used. All biological residues were incinerated at the Department of Pathological

Anatomy, Necropsy Diagnosis and Forensic Medicine (USAMV CN, FMV).

**Fecal bioassay.** Sporulated *T. gondii*-like oocysts were given to the mice by oral route in a volume of 100 µl/mouse using a micropipette.

**Heart bioassay.** Entire heart was used for each cat when available. Before bioassay, each heart was prepared by artificial digestion as elsewhere described [23, 24, 27]. Briefly, the heart was weighed, grinded (Grindomix GM 200 Knife Mill, Retsch, Germany), and homogenized with 0.25% trypsin digestion solution (50 ml solution for 20 g tissue). The weight of hearts ranged from 3.07 to 11 g. Hearts digestion was performed at 37 °C for 90 min. After digestion, the samples were filtered through a double-layered gauze, and the obtained suspension was centrifuged at 3000 rpm for 10 min. After centrifugation, the supernatant was removed, and the sediment (digest) was washed three times with PBS. After the last centrifugation, the supernatant was removed and the digest was mixed with 2 ml PBS. Two hundred µl of each digest were transferred in Eppendorf tubes and stored at -20 °C until PCR analysis. The remaining digest was treated with 200 µl antibiotic solution (Sigma Aldrich, Penicillin-Streptomycin, code P0781), and inoculated intraperitoneally (1 ml/mouse).

**Bioassay interpretation.** After inoculation, mice were monitored twice daily. At 4 weeks postinfection (pi), the mice were euthanised by cervical dislocation, and brains were harvested. Mouse brains were examined for *T. gondii* cysts by microscopy and PCR. A mouse bioassay was considered positive if at least one of the two mice was positive by either of the two methods.

## PCR

**DNA extraction.** Genomic DNA (gDNA) extraction was performed with Isolate Fecal DNA Kit (Bioline) from 200 µl fecal pellets, and with Isolate II Genomic DNA Kit (Bioline) from either 200 µl heart digests or 250 mg mouse brains according to the manufacturer's instructions. The extracted gDNA was quantified with NanoDrop 1000 Spectrophotometer (Thermo Scientific) and stored at -20 °C until further use.

**PCR.** DNA samples were analyzed by PCR targeting the 529 bp repetitive element of *T. gondii*, using primers Tox4 (5'-CGCTGCAGGGAGGAAGACGAAAGT TG-3') and Tox5 (5'-CGCTGCAGACACAGTGCATC TGGATT-3') [36]. PCR reactions were performed in a final volume of 25 µl: 12.5 µl PCR Master Mix (2x Green PCR Master Mix, Rovalab), 1 µl Tox4 primer (10 µM/µl), 1 µl Tox5 primer (10 µM/µl), 4 µl of gDNA and 6.5 µl of ultrapure water. Positive (*T. gondii* RH strain gDNA) and negative (ultrapure water) controls were used in each set of reactions. The amplification program consisted of one initial denaturation cycle at 95 °C for 1 min, followed by

35 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72 °C for 10 s, followed by a final extension at 72 °C for 5 min.

**Gel electrophoresis.** Genomic amplicons were analyzed by electrophoresis in 1.5% agarose gels stained with SYBR Safe DNA gel stain (Invitrogen). Gel Doc XR+Gel Documentation System (Bio-Rad) was used to visualize the presence of specific products. The amplified DNA fragments were compared to the positive control amplicon using as size marker the O'GeneRuler 100 bp DNA Ladder (Thermo Scientific).

## PCR-RFLP

Positive gDNA samples treated with RNase I (Thermo Scientific) were sent to the University of Tennessee (Knoxville, USA) for genotyping and analyzed by PCR-RFLP method according to the protocol described by Su et al. [32]. Eleven genetic loci (SAG1, SAG2, alt-SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico) were used to identify *T. gondii* genotypes.

The first PCR amplification was done in a final volume of 25 µl consisting of 1.5 µl of gDNA sample and 23.5 µl of PCR mix (16.5 µl H<sub>2</sub>O, 2.5 µl PCR buffer 10x (Mg<sup>-</sup>), 2 µl of dNTPs at 2.5 mM, 2 µl of MgCl<sub>2</sub> at 25 mM, 0.15 µl of each primer (25 µM), FastStart Taq (5U/µl). Positive and negative controls were used in each set of reactions. The amplification program was comprised of one initial denaturation cycle at 95 °C for 4 min, 30 cycles of amplification (94 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min).

The second amplification (nested-PCR) was done in a final volume of 25 µl consisting of 23.5 µl PCR mix (16.5 µl H<sub>2</sub>O, 2.5 µl PCR buffer 10x (Mg<sup>-</sup>), 2 µl of dNTPs at 2.5 mM, 2 µl of MgCl<sub>2</sub> at 25 mM, 0.15 µl of each primer (50 µM), FastStart Taq 5U/µl) and 1.5 µl of the first PCR reaction. Positive and negative controls were used in each set of reactions. The amplification program for each marker consisted of one initial denaturation cycle at 95 °C for 4 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min and extension at 72 °C for 1.5 min. For the Apico marker, the annealing was done at 55 °C and the extension protracted up to 2 min.

The obtained nested PCR products were digested with restriction enzymes specific for each molecular marker (Table 3). Enzyme digestion of nested PCR products was performed in a final volume of 20 µl consisting of 17 µl of digestion mixture (14.6 µl of H<sub>2</sub>O, 2 µl 10x NEB buffer, 0.2 µl of 100x BSA, 0.1 µl of enzyme/enzymes), and 3 µl of nested PCR product. Digestion was performed according to the protocol described by the manufacturer (New England BioLabs). Obtained digested products (4 µl of digested product mixed with 5x loading dye) were analyzed by electrophoresis using 2.5-3% agarose gels. Genotype calling for each marker was performed

by comparing fragment numbers and sizes with those of the positive controls. The genotype of each strain was then determined by referring to the database of existing genotypes.

The positive controls included the *T. gondii* typical strains GT1 (type I), PTG (type II), and CTG (type III), and the atypical TgCgCa1, MAS, TgCatBr5, TgCatBr64, and TgRsCr1.

### Statistical analysis

Data processing was performed with Microsoft Excel and Epi Info version 3.5.1 [37]. The frequency, prevalence, and its 95% confidence interval (95% CI) were calculated for each diagnostic method, and by age (youth:  $\leq$  one year; adults:  $>$  one year). The differences in prevalences between age groups were analyzed by Chi-square test with Yates correction. A  $p$ -value  $< 0.05$  was considered statistically significant.

## Results

### Animals and samples

The age of investigated cats ranged from 3 months to 11 years with an average of  $31.23 \pm 27.18$  months (95% CI: 21.26–41.20) (median 24 months; 95% CI: 12–36). Twelve cats were less than or equal to one year old (age median 10.5 months; 95% CI: 9.2–12.0), and 19 cats were older than one year (age median 3 years; 95% CI: 2–4.2 years). The ratio between females ( $n=25$ ) and males ( $n=6$ ) was 4.2. Six cats were from the urban area, and 25 were from the rural area and all of them had outdoor access. Fecal samples were available from all cats, serum samples from 28 cats, and heart samples from 27 cats.

### Fecal samples analysis by flotation, PCR, and bioassay

*Toxoplasma gondii*-like oocysts (Fig. 2) were found in 5 out of 31 fecal samples, two of which were confirmed to be positive for *T. gondii* by PCR (Fig. 3) and mouse bioassay (Fig. 4). None of the other fecal samples were positive by PCR (Table 1). The *T. gondii*-positive fecal samples were obtained from one 3-month-old (stray kitten) female from an urban area and one 4-year-old female from a rural area. Both cats presented clinical signs. The stray kitten showed bilateral conjunctivitis, respiratory distress, and diarrhea, and the adult cat had fever, conjunctivitis, and diarrhea.

### Toxoplasma Gondii serology

Sixteen (57.1%) out of 28 sera were positive by MAT, and 14 (56.0%) out of 25 sera were positive by ImmunoComb® (Table 1). The antibody titers in MAT ranged from 1:6 to 1:384. Overall, 17 (60.7%) out of 28 cats had *T. gondii* antibodies. One out of 25 sera tested by both serological methods had disagreement results; it was collected from

a 4 years old female from a rural area, and was negative in MAT and positive in ImmunoComb®.

### Heart samples analysis by PCR and bioassay

*T. gondii* DNA was detected in seven out of 27 heart samples (Table 1), and four of them were positive also by bioassay (TgCatRo2-5). Six out of seven positive cats were from rural areas, and five were adults (average age  $5 \pm 1.6$  years) (Table 2).

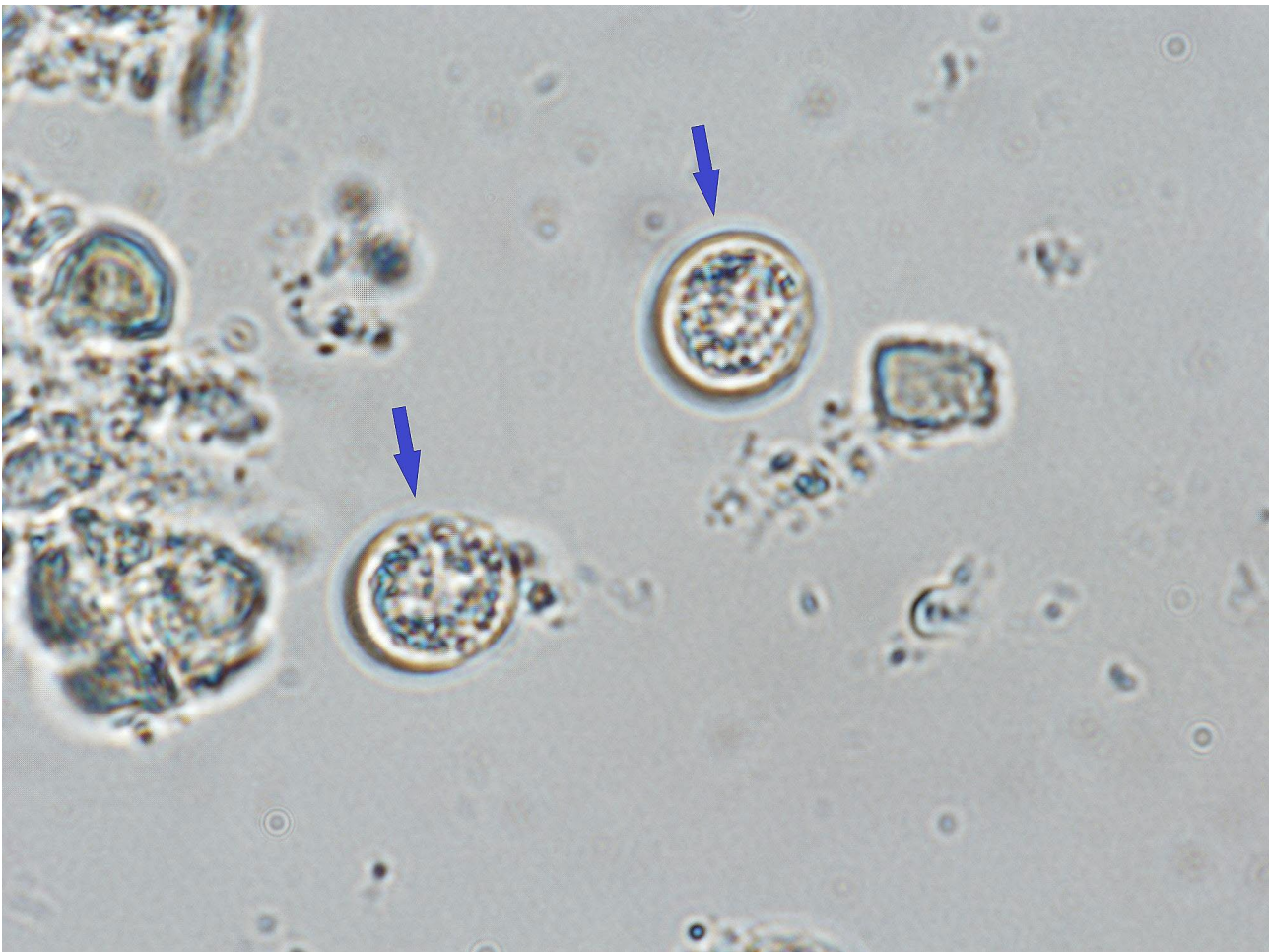
### Toxoplasma gondii genotypes

*T. gondii* DNA samples ( $n=9$ ) (two fecal bioassays, four heart bioassays, and three heart digests) were genotyped by PCR-RFLP (Tables 2 and 3) (Fig. 5). Two *T. gondii* DNA samples (TgCatRo4 and TgCatRo6) were genotyped at 10 of the 11 markers and matched ToxoDB PCR-RFLP genotype #1 or #3 (Type II) [38]; one *T. gondii* DNA sample (TgCatRo2) genotyped at 8 of the markers and matched #1 or #3; one *T. gondii* DNA sample (TgCatRo1) at 7 of the markers and matched #1 or #3; and one *T. gondii* DNA sample (TgCatRo3) at 3 of the markers and matched #1 or #3. One *T. gondii* DNA sample (TgCatRo5) was typed at 6 of the 11 markers and matched genotype #2 (Type III). Only one of the three PCR positive heart digests was genotyped at 6 of the 11 markers and matched #1 or #3 (Type II) (Table 3).

## Discussions

The main purpose of this study was to genetically characterize the *T. gondii* in domestic cats from Romania and to determine its frequency. *Toxoplasma gondii*-like oocysts were found in 5 out of 31 (16.1%) examined cats by fecal flotation, and *T. gondii* was confirmed by PCR and bioassay in two (6.5%) of these cats. The two *T. gondii* oocyst-shedding cats showed clinical signs. These values are higher compared with previous data reported in Romania, which showed the presence of *T. gondii*-like oocysts in 0.0 to 1.2% of investigated cats [28, 39, 40], and of *T. gondii* DNA in fecal samples of 4.7% of cats [41]. In Europe, *T. gondii*-like oocyst prevalence in feces varies widely from 0.0 to 20.5% [12], with most values below 1.0%. In Germany and other European countries, 0.23–0.31% of cats were found to shed *T. gondii*-like oocyst, but parasite DNA was confirmed by PCR in only 0.11–0.14% of cats [42, 43]. In another study, *T. gondii* DNA was found in 0.4% of cat fecal samples from Switzerland [44]. Higher values were reported in Israel (9.01%) [45], and Italy (16%) [46].

The shedding of oocysts by cats represents an element of great importance in the epidemiology of *T. gondii*, and it also has a great impact on public health because a single oocyst can cause the disease [47]. Oocyst shedding is influenced by various factors that depend on both the



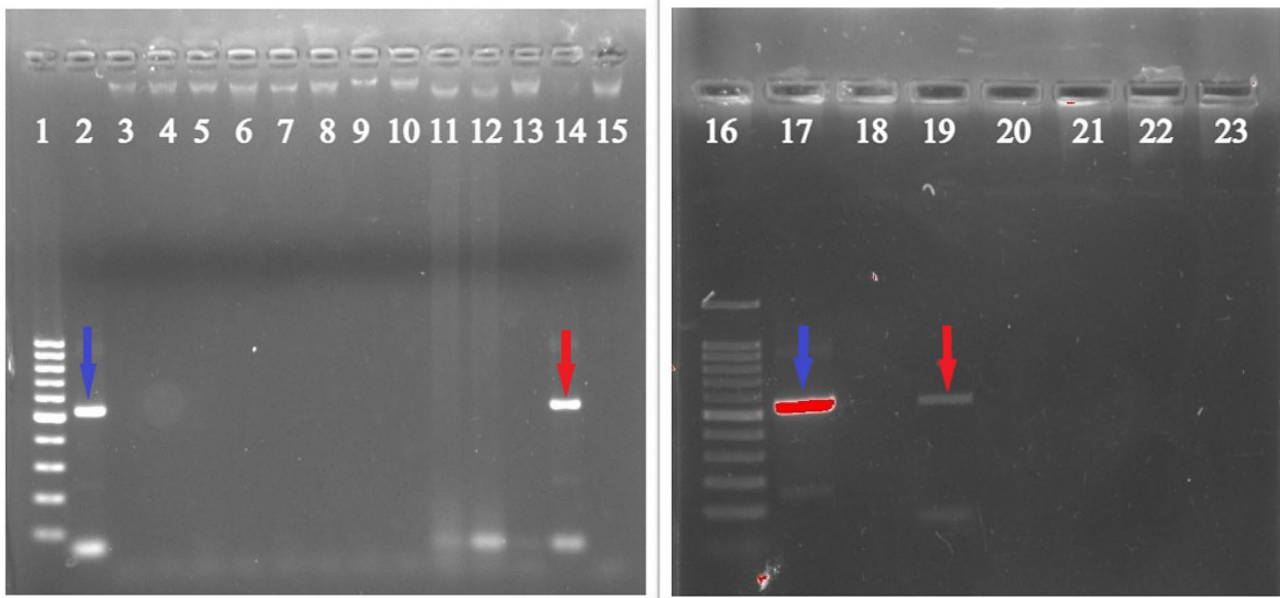
**Fig. 2** *Toxoplasma gondii*-like oocysts (blue arrows) (x400)

outdoor environment and the individual. Most primary *T. gondii* infections in cats are asymptomatic, oocyst shedding occurs in cats for about a week only a few days after exposure, and antibodies appear at two weeks postinfection [3, 48], therefore, detection and isolation of the parasite from feces in cats is difficult.

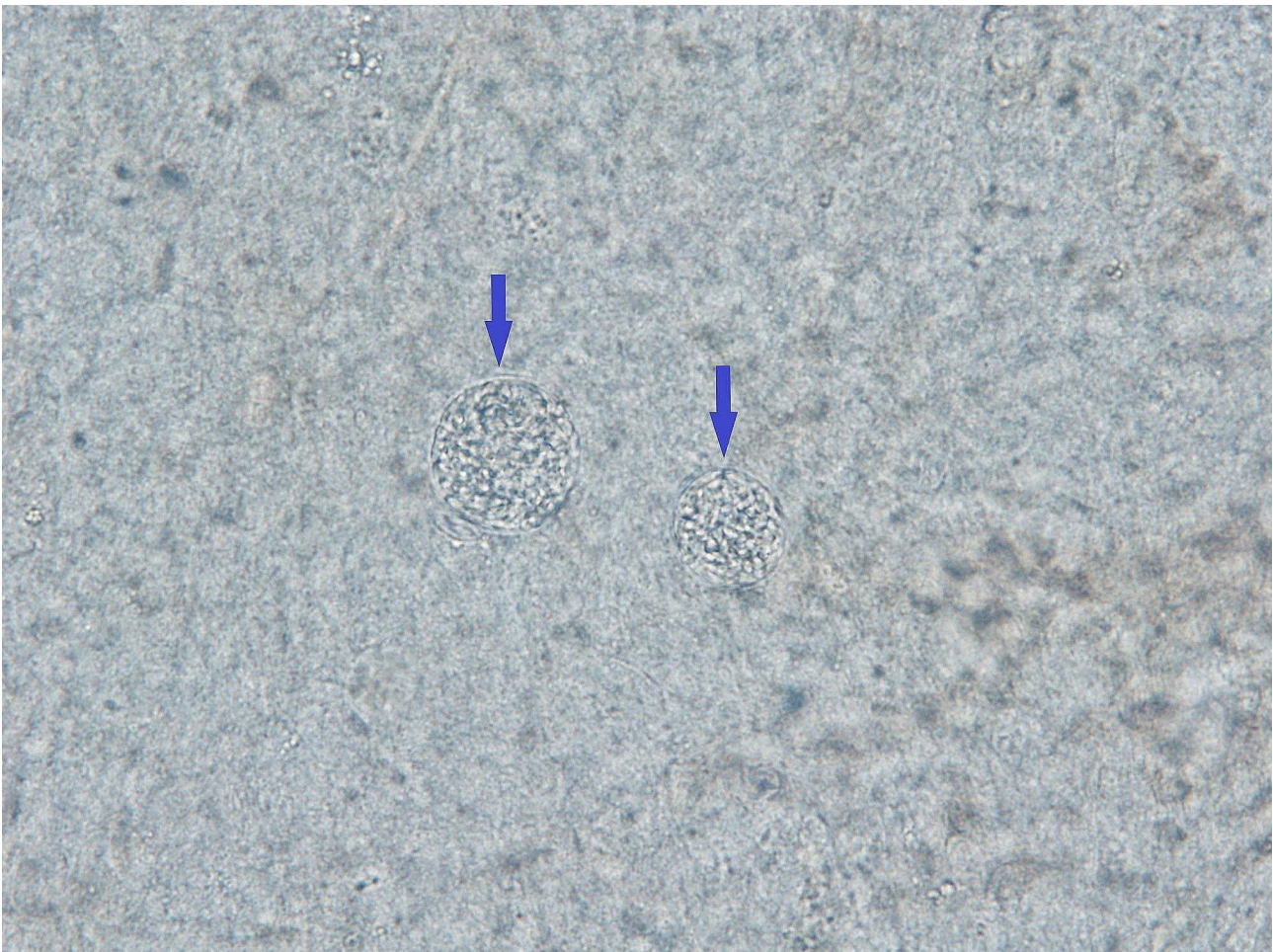
In cats, the seroprevalence of *T. gondii* infection varies according to region, climatic conditions, the origin of the cats, the analyzed cat population, and the methods used [7, 29, 41, 43, 49–51]. The data obtained in this study (17/28; 60.7%) were similar to the values reported elsewhere in our country (30.7–80.5%) [20], in Europe, or the world [12]. However the seroprevalence was high, *T. gondii* was identified by PCR or bioassay in nine cats (29.0%). Two of these cats (6.5%) had *T. gondii* oocysts in the feces, they were serologically positive, and the parasite was not identified in the heart. One of these two cats was three months old, while the other one was two years

old. For the remaining seven cats (23.3%) (46.14±16.3 months old), the parasite was not detected in the feces, they were serologically positive and the parasite was identified by bioassay in four of them (13.3%) (positive also by PCR), and only by PCR in three of them. Most of the bioassay-positive cats (3/4) had antibody titer of 1:384. The rate of *T. gondii* identification by direct methods (bioassay or PCR) in the present study is quite similar to most of the studies published in the last 14 years [12]. Based on a study performed in Egypt, *T. gondii* was more frequently identified in the heart than in the tongue or brain [12].

Seven out of nine *T. gondii* DNA samples were partially genotyped by eleven PCR-RFLP markers. The reason we were not able to genotype all 11 markers for these samples is due probably to low DNA concentration for these samples. Six *T. gondii* DNA samples were extracted from brains of bioassayed mice, which likely



**Fig. 3** Gel electrophoresis of the PCR products. Lines 1 and 16–100 bp molecular marker; lines 2 and 17–529 pb positive control (blue arrows); lines 3–15 and 18–23 – analyzed samples; red arrows – positive samples



**Fig. 4** *Toxoplasma gondii* cysts (blue arrows) in the brain of the bioassayed mouse (x100)



**Table 1** Frequency and prevalence of *Toxoplasma gondii* infection in domestic cats by indirect and direct detection methods

	Frequency	Prevalence	95% Confidence interval
<b>Serology</b>			
MAT (n=28)	16	57.1	37.2–75.5
ImmunoComb (n=25)	14	56.0	37.1–73.3
Total serology (n=28)	17	60.7	42.4–76.4
Youth (n=11)	5	45.5	21.3–72.0
Adults (n=17)	12	70.6	46.9–86.7
	$\chi^2$ , P	0.872, 0.35	
	OR (95% CI)	0.35 (0.07–1.69)	
<b>Fecal analysis</b>			
Flotation (n=31)	5	16.1	7.1–32.6
Youth (n=12)	2	16.7	4.7–44.8
Adults (n=19)	3	15.8	5.5–37.6
	$\chi^2$ , P	0.19, 0.66	
	OR (95% CI)	1.07 (0.15–7.54)	
PCR (n=31)	2	6.5	1.8–20.72
<b>Heart analysis</b>			
PCR (n=27)	7	25.9	13.2–44.7
Youth (n=10)	3	30.0	10.8–60.3
Adults (n=17)	4	23.5	9.6–47.3
	$\chi^2$ , P	0.007, 0.9	
	OR (95% CI)	1.39 (0.2–8.1)	
Bioassay (n=27)	4	14.8	5.9–32.5
Youth (n=10)	2	20.0	5.7–51.0
Adults (n=17)	2	11.8	3.3–34.3
	$\chi^2$ , P	0.0004, 0.98	
	OR (95% CI)	1.88 (0.2–15.9)	

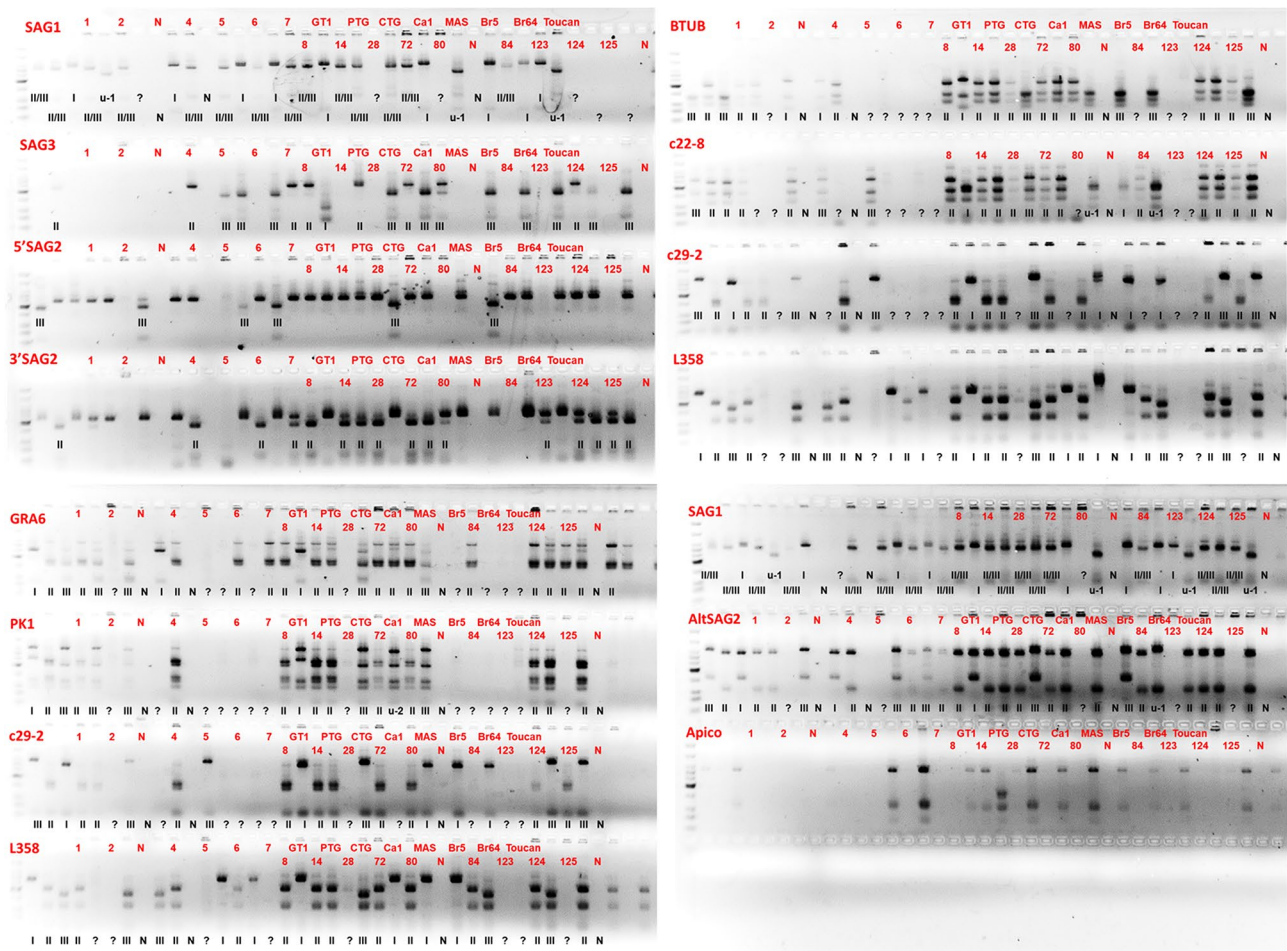
have low number of *T. gondii* tissue cysts. This issue may be solved by expanding the parasite in cell culture, or inoculating these parasites to INF- $\gamma$  knockout mice that leads to acute toxoplasmosis to reach a high tissue burden of parasites for DNA extraction. Based on partial genotyping results, we identified ToxoDB PCR-RFLP genotype #1 or #3 (Type II) in six *T. gondii* DNA samples and genotype #2 (Type III) in one *T. gondii* DNA sample. Both of these genotypes are common in Europe [38, 52]. To our best knowledge, this is the first study reporting on circulating *T. gondii* genotypes in domestic cats in Romania.

The predominance of type II *T. gondii* in cats described in the present study is in agreement with the results of previous surveys conducted in Finland [53], Germany [42, 54], and Switzerland [55]. Other studies reported the presence of genotypes I [56, 57], III [56, 58, 59], or atypical ones [58]. The presence of genotype II was also reported in Romania in humans, in a case of congenital toxoplasmosis [30], and in domestic animals [24, 27, 31].

**Table 2** Results of different diagnostic methods in positive cats by bioassay or PCR

Isolate	County	Area	Age (months)	Sex	Fecal samples		Serum samples		Heart samples	
					PCR	Bioassay	IgG Ab	Ab titer <sup>1</sup>	Bioassay	PCR
TgCatRo1 (P2874)	CJ	U	3	F	+	+	+	1:384	ND	ND
TgCatRo6 (P1625)	CJ	R	48	F	+	+	+ <sup>2</sup>	-	-	-
TgCatRo2 (P0026)	CJ	R	11	F	-	ND	+	1:384	+	+
TgCatRo3 (P1088)	CJ	R	36	F	-	ND	+	1:96	+	+
TgCatRo4 (P1089)	CJ	R	132	F	-	ND	+	1:384	+	+
TgCatRo5 (P1099)	CJ	U	12	F	-	ND	+	1:384	+	+
P0039	AB	R	36	M	-	ND	+	1:96	-	+
P1623	CJ	R	72	F	-	ND	+	1:96	-	+
P2911	BN	R	24	F	-	ND	+	1:12	-	+

<sup>1</sup>Antibody titers obtained in MAT; CJ – Cluj; AB – Alba; BN – Bistrița Năsăud; U – urban area; R – rural area; F – female; M – male; “+” positive; “-” negative; Ab – antibodies; ND – not done. <sup>2</sup>These sample was positive only in ImmunoComb



**Fig. 5** Gel electrophoresis of the eleven genetic loci (SAG1, SAG2, alt-SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico) used for genotyping *T. gondii* in positive samples (N is negative control; GT1, PTG, CTG, Ca1 - TgCgCa1, MAS, Br5 - TgCatBr5, Br64 - TgCatBr64, Toucan - TgRsCr1 are references, and 1, 2, 4, 5, 6, 7, 8, 14, 28, 72, 80, 84, 123, 124, 125 are analyzed samples (1–28 cat samples; 72–125 other samples))

**Conclusions**

Our results showed that the infection with *T. gondii* is still high in cats from Romania, and that genotype II is predominant like in other European countries. The oocysts

shed by these cats represent an important source of infection for intermediate hosts, including humans. Further studies on a wider range of cases are necessary to explore the presence of other circulating *T. gondii* genotypes.

**Table 3** Genotypes of *Toxoplasma Gondii* obtained in investigated domestic cats from Romania

	Genetic markers										ToxoDB PCR-RFLP Genotype	
	SAG1	SAG2	alt-SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1		Apico
<b>Isolated strains</b>												
TgCatRo1 (P2874)	II or III	II	II	II	nd	II	II	nd	II	nd	nd	#1 or #3 (Type I)
TgCatRo2 (P0026)	II or III	nd	II	nd	II	II	II	II	II	II	nd	#1 or #3
TgCatRo3 (P1088)	II or III	nd	II	nd	nd	na	nd	na	II	na	na	#1 or #3
TgCatRo4 (P1089)	II or III	II	II	II	II	II	II	II	II	II	nd	#1 or #3
TgCatRo5 (P1099)	II or III	nd	III	III	nd	nd	III	III	III	nd	nd	#2 (Type III)
TgCatRo6 (P1625)	II or III	II	II	II	II	II	II	II	II	II	nd	#1 or #3
P1623	II or III	II	II	nd	nd	II	II	nd	II	nd	nd	#1 or #3
<b>Reference strains</b>												
GT1	I	I	I	I	I	I	I	I	I	I	I	#10 (Type I)
PTG	II or III	II	II	II	II	II	II	II	II	II	II	#1 (Type I)
CTG	II or III	III	III	III	III	III	III	III	III	III	III	#2 (Type III)
TgCgCa1	I	II	II	III	II	II	II	u-1	I	u-2	I	#66
MAS	u-1	I	II	III	III	III	u-1	I	I	III	I	#17
TgCatBr5	I	III	III	III	III	III	I	I	I	u-1	I	#19
TgCatBr64	I	I	u-1	III	III	III	u-1	I	III	III	I	#111
TgRsCr1	u-1	I	II	III	I	III	u-2	I	I	III	I	#52

nd – not detected; na – not analyzed

Tables

## Abbreviations

USAMV CN	University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca
IM	intramuscular
MAT	modified agglutination test
PCR	polymerase chain reaction
nPCR-RFLP	nested polymerase reaction - restriction fragment length polymorphism
BSA	bovine serum albumin
DNA	deoxyribonucleic acid

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## Author contributions

AG designed the study, made the statistical analysis, critically revised and prepared the manuscript; AB performed serology, bioassays and PCRs, wrote the manuscript in Romanian; SB wrote the manuscript in English, and updated the references list; CS and TJ performed PCR-RFLP for genotyping, analyzed the obtained data and revised the manuscript; CM and DM collected the samples and performed artificial digestion; VM performed copro-parasitological examination; RB: performed PCRs and revised the manuscript; IV provided the antigen for MAT, and revised the manuscript; FS maintained the *T. gondii* isolates on cell culture, and revised the manuscript; VB: made the statistical analysis, written the background section and revised the manuscript; VC designed the study, updated the reference list and revised the manuscript.

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## Data availability

Data obtained during this study will be available to scientist request for non-commercial purposes from authors.

## Declarations

### Ethics approval and consent to participate

For this study, we obtained approval from the Commission of Bioethics of USAMV CN (18099/23.11.2012). Also, cat owners gave their informed consent for euthanasia. All methods were performed in accordance with the relevant guidelines and regulations.

### Consent for publication

Not applicable.

### Competing interests

The authors declare no competing interests.

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