



Molecular confirmation of the systemic toxoplasmosis in cat

Jacek Sroka^{1,2,A-F}✉, Angelina Wójcik-Fatla^{2,B,C,E}, Ewa Bilaska-Zajac^{1,C-D}, Jacek Karamon^{1,C-E},
Jolanta Małgorzata Zdybel^{1,C}, Weronika Piotrowska^{1,C}, Joanna Dąbrowska^{1,C},
Małgorzata Samorek-Pieróg^{1,C}, Weronika Korpysa-Dzirba^{1,C}, Tomasz Cencek^{1,A,E-F}

¹ National Veterinary Research Institute, Puławy, Poland

² Institute of Rural Health, Lublin, Poland

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Abstract

Introduction and Objective. Systemic toxoplasmosis with tissue-spread parasites occurring in intermediate hosts may also occur in immunocompromised cats (e.g., infected with FLV or FIV). To the best of our knowledge, no reports have been published on the detection and genotyping of *T. gondii* DNA in cats with extraintestinal toxoplasmosis in Poland. The article describes the case of the sudden death of 3 out of 4 cats in a cattery, and the detection and molecular characterization of *T. gondii* DNA detected in the tissues of one of the dead cats.

Materials and method. Samples of brain, lungs, heart, and liver of the cat that died suddenly were examined for the presence of *T. gondii* DNA (B1 gene) by nested PCR and real-time PCR. DNA positive samples were also genotyped at 12 genetic markers using multiplex multilocus nested PCR-RFLP (Mn-PCR-RFLP) and multilocus sequence typing (MLST).

Results. A total of 9 out of the 20 DNA samples were successfully amplified with nested and/or Real-time PCR. DNA from 3 out of 5 types of tested samples were genotyped (brain, heart and muscle). Mn-PCR-RFLP and MLST results revealed type II (and II/III at SAG1) alleles at almost all loci, except a clonal type I allele at the APICO locus. This profile corresponds to the ToxoDB#3 genotype, commonly identified amongst cats in Central Europe.

Conclusions. To the best of our knowledge, this is the first study describing the genetic characteristics of *T. gondii* population determined in a cat in Poland. These data confirm the importance of this host as a reservoir for this pathogen, and demonstrate the genotypic variation of this parasite. Veterinarians should take into account that cats may develop disseminated toxoplasmosis, and that it is a systemic disease which may lead to the death of the cat, and to transmission of the pathogen to other domestic animals and to humans.

Key words

Toxoplasma gondii, genotyping, cats, systemic toxoplasmosis

INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite that can infect many mammalian species, including humans, worldwide. The danger of clinical forms of human toxoplasmosis occur in women in pregnancy and in immunocompromised persons (e.g. AIDS patients). Humans usually become infected by *T. gondii* through consumption of undercooked meat containing parasite cysts, or by food and water contaminated with the parasite [1]. The ingestion of vegetables, fruit or water contaminated with sporulated oocysts has been considered the common means for the acquisition of toxoplasmosis [2]. Felids are the definitive hosts of this parasite which pass oocysts in the faeces to environment. Cats often acquire the infection by ingestion of tissue cysts present in prey species of animals (e.g. the carcasses of small mammals, including mice, rats and birds), or by ingestion of oocysts. Infection may also occur through the placenta in the case of parasitaemia in the pregnant female. In terms of epidemiology and public health risk, outdoor cats especially represent the most significant group.

In immunocompetent cats, the infection is asymptomatic and has the form of typical coccidian enteroepithelial cycle in the intestinal epithelium, with the excretion of oocysts in the faeces [1]. Sporulated, infective oocysts (1–5 days after excretion) are resistant to unfavourable conditions in the environment, where they may survive for months, or even years [2].

The systemic toxoplasmosis with the dissemination of parasites in tissues, as occurs in the intermediate hosts, can also occur in the cats, usually simultaneously with the entero-epithelial cycle. Acute systemic toxoplasmosis has been described in neonates, young immunologically-immature kittens and immunocompromised cats. In adult cats with immunosuppression caused by concurrent feline leukaemia virus (FLV) or feline immunodeficiency virus (FIV) infection reactivation of dormant cysts, has been reported. This form of the disease, however, is considered rare [3].

Genotyping of *T. gondii* is widely used to determine the potential impact of a particular genotype on the course of the disease. In Europe, the most frequently isolated *T. gondii* strains are categorized into three major clonal lineages (I, II, III) which exhibit a different level of virulence in mice [4]. A fourth clonal genotype has been discovered in the USA [5]. Recent genotyping studies in South America and Europe have found virulent *T. gondii* hybrid isolates or atypical

✉ Address for correspondence: Jacek Sroka, National Veterinary Research Institute, Partyzanów Av. 57, 24-100 Puławy, Poland
Email: jacek.sroka@piwet.pulawy.pl

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strains [6, 7]. In European studies, *T. gondii* type II isolates appeared to be most common in animals [8, 9]; however, data concerning the molecular prevalence of *T. gondii* infection in cats in Poland are scarce, and mostly represented by the results of own studies.

To the best of our knowledge, no reports have been published in Poland on *T. gondii* DNA detecting and genotyping from cat with extraintestinal toxoplasmosis. The presented findings will extend knowledge about the epidemiology and genetic profiles of *T. gondii* existing in Poland.

MATERIALS AND METHOD

Case description. According to the note described by veterinary inspection, during one week, 3 of 4 cats bred by their owner died suddenly. The cats breeding had outdoor access, but their welfare was inefficient, they were neither vaccinated nor dewormed, and the presence of fleas was also detected.

The first cat died with the symptoms of dyspnoea, convulsions and bloody / stringy exudate from the nose. There is no data on the treatment of this cat before the decline, and the vaccination status was unknown. Another one of the breeding cats, a one-year-old female, was delivered to the veterinary clinic during childbirth, which had started 12 hours earlier, and the cat had already given birth to two dead kittens. The female was found with weakness and respiratory distress, had mucous, stringy exudate flowing from the nose and mouth. Serologic testing for Feline leukemia virus (FeLV) and Feline immunodeficiency virus (FIV) showed negative results. The red blood cell count was within normal range, although with slightly lower values of haematocrit (20%) and haemoglobin (7.5 g/dl). Hematological analysis showed a marked decrease of leukocytes (0.13 G/l), neutrophils (0.06 xG/l) and platelets (59 x1,000/ μ l). Serum biochemistry revealed a marked increase in total bilirubin (59.2 mmol/l), urea (23.8 mmol/l), and medium increase in glucose (7.7 mmol/l), with reference interval aspartate aminotransferase (AST) and alanine aminotransferase (ALT). The blood test showed severe leukopenia, disturbances in the white blood cell system, elevated eosinophils (15.4%), monocytes (15.4%), and icterus. At this stage, marked bilirubinuria and severe leukopenia indicated an overwhelming hepatic injury or hepatitis (Tab. 1). Ultrasound examination revealed the presence of two live foetuses. While preparing for a caesarean section, the cat gave birth to another stillborn foetus, and then died. Unfortunately, a necropsy was not performed.

The next day, the owner informed the vet that another cat from the cattery, a 2-year-old male, was having severe convulsions and respiratory distress. The cat showed no other symptoms (including diarrhea). After the owner arrived at the vet clinic, the cat was found to be dead. Shortly after the cat's death (0.5 h), unusually high rigor mortis was observed. A necropsy was performed and tissue samples of brain, liver, lungs and heart of this cat were sent to the laboratory of Department of Parasitology and Invasive Diseases in National Veterinary Research Institute in Pulawy (Poland) for testing for the presence of *Toxoplasma gondii* DNA. Unfortunately, there was no additional data on the results of the necropsy performed by the vet.

Table 1. Blood parameters of the cat

Blood count	Measured values	Reference range
Erythrocytes ($\times 10^{12}/l$)	5.6	5.0-10.0
Haematocrit (%)	20.0	30.0-44.0
Haemoglobin (g/dl) (mmol/l)	7.5 / 4.7	9.0-15.0/5.6-9.3
MCV (Mean Corpuscular Volume) (fl)	36.0	40.0-55.0
MCH (Mean Corpuscular Haemoglobin) (pg)	13.5	13.0-17.0
MCHC (Mean Corpuscular Hemoglobin Concentration) (g/dl)	37.5	30.0-36.0
Leukocytes (G/l)	0.13	6.0-11.0
Neutrophils quantitatively (xG/l)	0.06	3.0-11.0
Neutrophils (%)	46.0	60.0-78.0
Lymphocytes (%)	23.1	15.0-38.0
Monocytes (%)	15.4	0.1-4.0
Monocytes quantitatively (G/l)	0.02	0.04-0.5
Eosinophils (%)	15.4	0.1-6.0
Eosinophils quantitatively (G/l)	0.02	0.1-0.6
Basophils (%)	0.1	0.1-1.0
Basophils quantitatively (G/l)	0.0	0.01-0.1
Platelets	59.0	180.0-560.0
Remark: leukopenia		
Biochemistry profile		
ALT (U/l)	45.7	1.0-91.0
AST (U/l)	53.5	1.0-59.0
Bilirubin (mmol/l)	59.2	0.01-5.5
Creatinine (mmol/l)	150.7	1.0-168.0
Urea (mmol/l)	23.8	5.0-11.3
Glucose (mmol/l)	7.7	3.05-6.1
Remark: icterus		

Digesting of tissues samples and DNA extraction. In general, 20 samples of brain, lungs, heart, and liver (50 g each) of the 2-year-old male cat were digested by pepsin solution, according to the method described by Sroka et al. (2019) [10].

One hundred μ l of each pellets solutions obtained after pepsin digestion, were used for DNA extraction using a commercial kit (QIAmp DNA Mini Kit, Qiagen), according to the manufacturer's instructions, except for an overnight incubation with proteinase K. DNA was also extracted from 100 mg of each homogenized tissue samples without digestion step. All DNA samples were stored at -20°C until examination.

Nested and Real-time PCR. First, the amplification of 35-fold-repetitive B1 fragment gene in nested polymerase chain reaction (PCR) was performed, according to the method by Grigg and Boothroyd (2001) [11]. PCR were carried out in a C1000 Thermal Cycler (Bio-Rad). Simultaneously, for each DNA sample, Real-time PCR (B1 gene) was performed, according to the method by Lin et al. (2000) [12], using the commercial master mix QI Supermix (Bio-Rad, Hercules, CA).

Multiplex multilocus nested PCR-RFLP (Mn-PCR-RFLP) and multilocus sequence typing (MLST). To estimate the clonal type for the selected B1 gene positive samples, Mn-PCR-RFLP and MLST methods were performed using following markers: SAG1, SAG2 (5' and 3'), altSAG2, SAG3, GRA6, BTUB, C22-8, C29-2, L358, PK1 and APICO, according to the method described by Su et al. (2010) [13].

GT1 Type 1 ToxoDB#10, PTG Type 2 ToxoDB#1, and CTG Type 3 ToxoDB#2 DNA isolates of *T. gondii* strains were used as positive controls, and nuclease-free water as a negative control. Nested and Mn-PCR-RFLP amplification products after electrophoresis were identified on an agarose gel under ultraviolet light. Mn-PCR-RFLP products (5 µl) were digested with restriction endonucleases in a 20 µl volume, and DNA banding patterns were resolved on a 2.5% agarose gel. The DNA bands from each sample were compared with the patterns of positive controls. The Multi-Locus Sequence Typing (MLST) amplification products of the positive samples were sequenced by an external company (Genomed S.A., Poland) and sequences were analyzed using Geneious v. 11.1.4. software (Geneious Co., Wellington, New Zealand), with the sequences deposited in the National Centre for Biotechnology Information (NCBI) database, using Blast. The MLST genotype pattern was compared with genotypes deposited in ToxoDB (<http://toxodb.org/toxo/>).

Phylogenetic analysis of GRA6 loci. The sequence of GRA6 loci with a length of 329 positions from the cat sample was aligned with other available sequences of this loci deposited in Genbank, and originated in Europe, and clonal reference strains GT1, PTG and CTG. Altogether, sequences from 26 *Toxoplasma gondii* strains (loci GRA6) were applied to phylogenetic analysis. The Neighbour-joining Method with Maximum Composite Likelihood method was used to calculate distances and generate unrooted phylogram with bootstrap values of 1,000 replicates.

RESULTS

A total of 9 out of the 20 DNA samples were successfully amplified (B1 gene) with nested and/or Real-time PCR (Tab. 2). All B1 PCR-positive samples were positive in RT PCR.

DNA from 3 out of 5 types of tested samples were genotyped (brain, heart and muscle); one sample (muscle) was able to genotype with 10 markers (SAG1, 5'+3' SAG2, SAG3, GRA6, C22-8, C29-2, L358, PK1 and APICO), one sample (heart) with 5 markers (SAG1, SAG3, GRA6, L358 and APICO), one sample (muscle) with 4 markers (5'+3' SAG2, SAG3 and C22-8), and one sample (brain) with 3 markers (BTUB, Alt.SAG2 and APICO). By using 12 markers, a total of 20 amplicons were obtained. In total, type II/III, type II and type I *T. gondii* lineages were determined for 2 (10%), 15 (75%) and 3 (15%) amplicons, respectively. Mn-PCR-RFLP and MLST results revealed type II (or II/III at SAG1) alleles at almost all loci, with the exception of a clonal type I allele at the APICO locus (Tab. 3). This profile correspond to the ToxoDB#3 genotype, the most commonly identified among cats in Central Europe [14].

Neighbour-joining phylogram of 26 *Toxoplasma gondii* strains derived from polymerase chain reaction – restriction fragment length polymorphism typing at loci GRA6.

Table 2. Results of nested and Real-time PCR (B1 gene).

No.	Tissue		Real time	Nested PCR	
1	Brain	1	Not digested*	+ (Ct =33.13)	+
2					
3		Digested	n/a-	n/a	
4					+
5	Muscle	1	Not digested	+ (Ct =35.86)	
6					+
7		3	Digested	+ (Ct =30.02)	
8					4
9	Lungs	1	Not digested	n/a	
10					2
11		3	Digested	n/a	
12					4
13	Heart	1	Not digested	+ (Ct =34.06)	
14					2
15		3	Digested	n/a	
16					4
17	Liver	1	Not digested	n/a	
18					2
19		3	Digested	n/a	
20					4

* digestion tissue samples with pepsin solution, according to the method described by Dubey and Beattie (1988)

Distances were calculated according to the Maximum Composite Likelihood method [15], and the distance matrix analyzed using the phylogenetic analysis programme MEGA X to generate an unrooted phylogram [16]. The numbers on the branches indicate the bootstrap values (1,000 replicates).

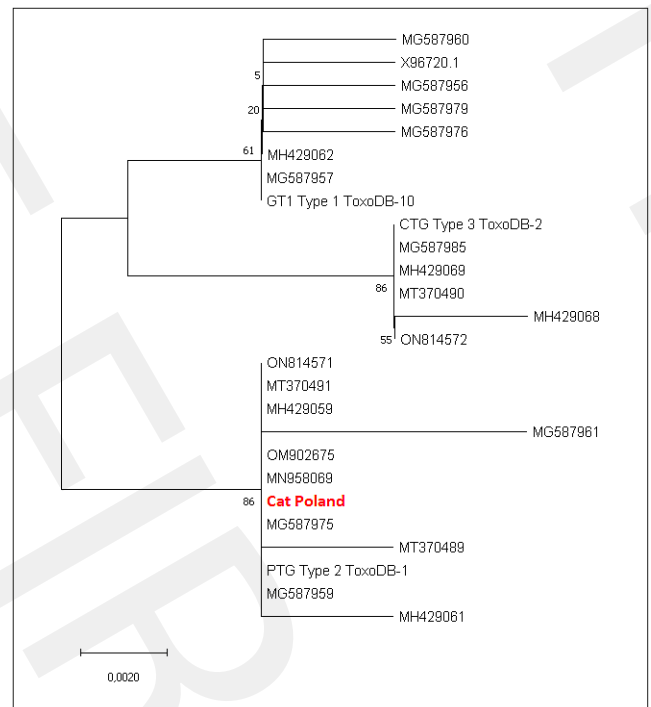


Figure 1. Results of phylogenetic analysis at GRA6 loci

Table 3. Results of genotyping B1 positive samples using additional markers

Samples	SAG1	5'+3' SAG2	SAG3	GRA6	BTUB	Alt. SAG2	C22-8	C29-2	L358	PK1	APICO
1 Brain not digested (100 µg)	n/a	n/a	n/a	n/a	II	II	n/a	n/a	n/a	n/a	I
2 Heart not digested (100 µg)	II/III	n/a	II	II	n/a	n/a	n/a	n/a	II	n/a	I
3 Muscle not digested (100 µg)	II/III	II	II	II	n/a	n/a	II	II	II	II	I
4 Muscle digested (50 g)	n/a	II	II	n/a	n/a	n/a	II	n/a	n/a	n/a	n/a
Total	II/III	II	II	II	II	II	II	II	II	II	I

*At SAG1 locus, types II and III are indistinguishable; n/a - product not amplified

DISCUSSION

In the tissues of a cat that suddenly died, *Toxoplasma gondii* DNA was detected and the molecular characteristics of this parasite were determined. The weakness of the presented study is the lack of certainty that *Toxoplasma gondii* actually caused the disease symptoms and death of the examined cat, and 2 others from the cattery. Due to technical reasons (frozen material was delivered to the laboratory for testing), no attempt was made to isolate a live strain of the parasite. Histopathological tests were also not performed by a veterinary service during an epidemiological investigation.

The clinical form of feline toxoplasmosis has been described, i.e. in Spain [17], UK [18] and USA [19]. To the development of the disseminated form of toxoplasmosis may be predisposing of an immunocompromised state of the cat, caused by feline immunodeficiency virus (FIV), feline infectious peritonitis (FIP), or cyclosporin therapy [20]. The FIV and FIP viruses may also activate latent infections. Non-specific clinical signs may include lack of appetite, persistent pyrexia, depression and lethargy. The severe phase of the disease can manifest with the development of pneumonia, hepatitis, pancreatic necrosis, myositis, myocarditis, uveitis, dermatitis, and encephalitis [21]. During the acute phase of clinical toxoplasmosis haematological and biochemistry parameters may be non-specific, such as leukocytosis, eosinophilia, neutrophilia, lymphocytosis, monocytosis, anaemia and hypoalbuminemia [22]. In a study in Finland in cats with systemic toxoplasmosis, marked elevations were found in the liver enzyme alanine aminotransferase (ALT) [23]. However, in the current study, the ALT value of the cat did not increase. An undiagnosed disease causing an immunocompromised state could be responsible for reactivation of a latent *T. gondii* tissue cyst [22].

There is a scarcity of knowledge about the genetic diversity of the *T. gondii* population in Poland. Previous own studies revealed *T. gondii* genotype III as the most prevalent in retail raw meat products and goat milk [24, 25], and type II ToxoDB #3 genotype in the diaphragm and heart of pigs [26]. On the basis of the analysis carried out in ToxoDB, samples from cat tissues with type II alleles at all successfully amplified loci, except one – Apico – which displayed the type I allele, may correspond to the ToxoDB #3 genotype. The same genotyping results were reported in Europe in cats [14, 27], sheep [28] and arctic foxes [29]. The predominance of genotype II *T. gondii* in cats in Germany was reported by Schares et al. (2008) [30], and in Switzerland [14], which is in accord with findings this type in other European countries, e.g. Portugal [31] and Spain [32].

ToxoDB#3 genotype, with the exception of causing diarrhea, is usually avirulent in immunocompetent cats and mice infected experimentally, but clinical form of the disease can be provoked if the cats are immunocompromised. Natural infection with *T. gondii* type II, APICO I, led to fatal systemic toxoplasmosis in a cat, as reported Spycher et al. 2011 [14], where serologic testing of cat for FeLV and FIV showed negative results, similar to the current study. The literature indicates that host factors can play an important role in the outcome of *T. gondii* infection. A recent study by Pardo Gil et al. (2023) [33] revealed *T. gondii* ToxoDB #3, a Type II variant circulating in small wild mammals (cat prey) in Switzerland. In Serbia, however, the lineage II archetype (ToxoDB#1) was the dominant genotype in intermediate hosts in 33% of the total strain population, followed by lineage II variant strains, with 21% [7].

CONCLUSIONS

Taking into account the results of serological studies in Poland, *T. gondii* infection in domestic cats is highly prevalent [34]. To the best of our knowledge, this is the first study aiming to describe the genetic characteristic population of *T. gondii* determined in cats in Poland. These data confirm the importance of this host as a reservoir for this pathogen, and shows the genotypic variation of this parasite. Veterinarians should take into account that cats (especially immunocompromised) may develop disseminated toxoplasmosis, and that it is a systemic disease which may lead to death of the cat, and to transmission of the pathogen to other domestic animals and to humans.

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