Title:

Association of ocular toxoplasmosis with type I *Toxoplasma gondii* strains: direct genotyping from peripheral blood samples

Running head: genotyping in ocular toxoplasmosis

Authors:

Karolina Switaj\(^1\)*, Adam Master\(^2\), Piotr Karol Borkowski\(^1\), Magdalena Skrzypczak\(^2\), Jacek Wojciechowicz\(^2\), Piotr Zaborowski\(^1\).

\(^1\) Medical University of Warsaw, Department of Zoonotic and Tropical Diseases, Institute of Infectious and Parasitic Diseases, ul. Wolska 37, Warsaw, Poland

\(^2\) DNA Research Institute, ul. Rakowiecka 36 lok. 36a, Warsaw, Poland

* Corresponding author:

Karolina Świtaj

Department of Zoonoses and Tropical Diseases (IX)

Medical University of Warsaw

Ul. Wolska 37, 01-201 Warsaw, Poland

Tel. +4822 3355288

Fax +4822 6310534

Email: karolinaswitaj@yahoo.co.uk
Abstract

*T. gondii* strains were directly genotyped from blood samples of patients with ocular toxoplasmosis. Analysis of nontranscribed spacer 2 revealed that all detected strains belonged to type I, suggesting an association of ocular toxoplasmosis with this type. The method shows usefulness of blood samples for genotyping in ocular toxoplasmosis.

Key words: *Toxoplasma gondii*, genotyping, ocular toxoplasmosis, PCR, NTS 2.
Ocular toxoplamosis is a recurrent disease caused by an obligatory intracellular parasite *Toxoplasma gondii* and can affect patients with no evident immunodeficiency. The factors promoting development of ocular toxoplamosis are not well established. In animal models, there are recognized highly virulent strains (type I) and avirulent strains (type II and III) (12, 13, 16). It is not clear whether the genotype of *T. gondii* correlates with manifestation of the disease in humans (1, 3, 6, 11) and ocular involvement (10, 14, 17). The aim of the study was to determine the genotypes of *T. gondii* associated with ocular toxoplamosis in immunocompetent patients.

To avoid a possible bias related with the use of animals or tissue cultures prior to *T. gondii* DNA extraction we chose a direct genotyping from clinical samples. In majority of previous works on ocular toxoplamosis samples of ocular fluids were used (10, 15). However, the work of Bou et al. showed that PCR detection of *T. gondii* in aqueous humor and blood samples from patients with ocular toxoplamosis yielded comparable results (4). To determine *T. gondii* genotype we used a repetitive and polymorphic sequence of NTS 2 (nontranscribed spacer 2) located between the 28S and 18S rRNA genes and investigated by Fazaeli et al., who showed the single nucleotide polymorphisms (SNPs) between *T. gondii* strains (9). We chose five SNPs located in the fragment of 288-bp in NTS 2 sequence. To reveal SNPs a novel minisequencing method was designed, which was assessed by analysis of 15 *T. gondii* laboratory isolates of type I, II, III and atypical strains. DNA from tachyzoites was extracted with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). DNA from blood samples (2 ml) collected from 61 patients with active ocular toxoplamosis (of which 26 had severe retinochoroiditis with macula or optic disc involvement) and from 12 patients with old toxoplasmic retinochoroidal scars was extracted with GeneMatrix Blood DNA Purification Kit (Eurx, Gdansk, Poland). None of the patients had signs of
immunodeficiency. As a control group blood samples from 3 healthy volunteers and from 7 patients with eye lesions due to toxocariasis were analyzed.

**PCR.** To amplify the 288-bp long fragment in the NTS 2, two primers were designed:

TgNTS2-U 5′-CGATGTGTTTCCCTAACTGA-3′ and Tg288-L 5′-

GTTGCATGTGTCCCATAACGG-3′. The PCR was performed in 12 µl of mix based on previously described protocol (http://www.fermentas.com/techinfo/pcr/dnaamplprotocol.htm), with 1.75 mM MgCl2, 0.4 µM primers, 0.75 U of polymerase and 2 µl of template DNA.

Thermocycling parameters were as previously described (2), except for annealing temperatures: 10 cycles with touchdown from 68˚C to 58˚C, 15 cycles at 58˚C, and last 15 cycles at 56˚C. The final cycle was followed by an additional 5 min at 72˚C and 30 min at 60˚C. PCR products were purified with alkaline phosphatase from calf intestine (Eurx, Gdansk, Poland) and with exonuclease I *E. coli* (Fermentas, Hannover, USA). According the GenBank (AF158092 for RH, AF 158093 for MAS, AF158094 for C56, AF158095 for Beverly) six minisequencing SNPs primers were designed (Table 1). The reaction was performed according to protocol of the manufacturer (http://docs.appliedbiosystems.com/pebiodocs/04323357.pdf). The concentration of each minisequencing primer in mix of pooled primers is shown in Table 1. Amplicons were analyzed using an ABI PRISM 310 sequence analyzer and GeneScan software (Applied Biosystems, Hannover, USA).

**Results.** Genotyping of laboratory isolates showed usefulness of the method for differentiation of *T. gondii* strains (Table 2). We noted patterns of SNPs characteristic for type I, type III and atypical isolates and three different genotypes among seven isolates of type II. We noticed also double nucleotides variants in strains ELG, S1K, C56 and GANGI. In each of these positions one nucleotide of double variant was characteristic for type I genotype.
T. gondii genotyping in clinical samples. Genetic material of T. gondii was detected in 86.9% (53 out of 61) of samples taken from patients with active toxoplasmic retinochoroiditis and in 50% (6 out of 12) of samples taken from patients with old toxoplasmic scars on the fundus. All detected T. gondii strains belonged to type I according to our SNPs analysis of NTS 2 region.

Genotyping in samples from control clinical groups revealed that all of them except for one were negative for T. gondii DNA (specificity 90%). One false positive result indicating the presence of type I isolate was in a sample taken from a patient with toxocariasis. It was likely due to cross-contamination during SNPs genotyping, since a repeat of genotyping with this isolate gave a negative result.

Diagnostics of ocular toxoplasmosis based on peripheral blood samples seems to be safer and more convenient procedure in comparison to collection of aqueous humor. T. gondii as an intracellular parasite persists within circulating leukocytes (4, 5). We assume that the same strain causing potent ocular inflammation is detected in white blood cells. We can not exclude that T. gondii genotyped in peripheral blood samples could also derive from possible asymptomatic reactivation in the brain or muscles concomitant to reactivation in the eye.

However, our results identifying only type I strains in patients with ocular toxoplasmosis are coherent with previous studies in which DNA was isolated from retinochoroidal specimens or ocular fluids (10, 17). The study from Brazil revealed that all 11 strains causing toxoplasmic retinochoroiditis examined in SAG2 locus were of type I (17). Also the study conducted in USA by Grigg et al., in which vitreous fluids from patients with severe or atypical ocular toxoplasmosis were analyzed, showed the predominance of type I and recombinant genotypes with type I allele (10). Recombinant strains were identified by multilocus analysis of five loci (SAG1, SAG2, SAG3, SAG4 and B1) - analysis of single locus would identify these strains as type I or III. Therefore, it is possible that analysis of our isolates in other loci would also
reveal recombinant strains. Our and previous studies (10, 17) showed that human ocular toxoplasmosis is associated with *T. gondii* strains possessing alleles characteristic for highly mouse-virulent strains of type I. There are also epidemiological data coherent with these results. The strain assigned as type I was identified in the outbreak in Victoria, Canada (14), where ocular toxoplasmosis was diagnosed in 20.6% of infected persons (in comparison to 2-3% of persons chronically infected with *T. gondii*). Also mouse-virulent strains were isolated in prevalence from chickens (7) and cats (8) in regions of Brazil endemic for ocular toxoplasmosis.

We conclude that in ocular toxoplasmosis peripheral blood samples, analyzed for NTS 2 region, can be used for direct *T. gondii* genotyping, and that human ocular toxoplasmosis may be associated predominantly with type I strains. The following features of type I strain – faster multiplication, the ability of extracellular migration and a decreased conversion to the bradyzoite form, in comparison to types II and III - render the final conclusion very likely, however further studies are needed to confirm this association of *T. gondii* virulence with ocular involvement.

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**REFERENCES**


<table>
<thead>
<tr>
<th>Primer</th>
<th>Position of SNP in NTS2 sequence</th>
<th>Sequence of the SNP primer</th>
<th>Concentration in primers’ mix for minisequencing reaction</th>
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<tr>
<td>TgNTS300-109U</td>
<td>723</td>
<td>5’GCTGACTGACTGAGAGTGCTT</td>
<td>34.29 µM</td>
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<tr>
<td>TgNTS300-147U</td>
<td>761</td>
<td>ACGAGACCATGAGAAAA3’</td>
<td>5.14 µM</td>
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<td>TgNTS300-177L</td>
<td>765</td>
<td>5’GCACTGACTGACTGTCGCAC</td>
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<tr>
<td>TgNTS300-255L</td>
<td>843</td>
<td>GATGGAATATATGATAAGG3’</td>
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<tr>
<td>TgNTS300-268L</td>
<td>856</td>
<td>5’GACGCATGCAGGTGTTCCC</td>
<td>2.64 µM</td>
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**TABLE 1. Primers and their concentration in the mix for minisequencing reaction**
<table>
<thead>
<tr>
<th>Strain</th>
<th>Position of SNP</th>
<th>Type I:</th>
<th>Type II:</th>
<th>Type III:</th>
<th>Atypical:</th>
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<td></td>
<td>723 761 765 843 856</td>
<td>RH, P, ENT,</td>
<td>Jones, CAL, PRU</td>
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<td>P80, GANGI</td>
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