

## Association of Ocular Toxoplasmosis with Type I *Toxoplasma gondii* Strains: Direct Genotyping from Peripheral Blood Samples<sup>∇</sup>

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***Toxoplasma gondii* strains were genotyped directly 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 the usefulness of blood samples for genotyping in ocular toxoplasmosis.**

Ocular toxoplasmosis 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 toxoplasmosis are not well established. In animal models, there are recognized highly virulent strains (type I) and avirulent strains (types 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 toxoplasmosis in immunocompetent patients.

To avoid a possible bias related to the use of animals or tissue cultures prior to *T. gondii* DNA extraction, we chose the method of direct genotyping from clinical samples. In the majority of previous works on ocular toxoplasmosis, 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 toxoplasmosis yielded comparable results (4).

To determine *T. gondii* genotype, we used a repetitive and polymorphic sequence of nontranscribed spacer 2 (NTS 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 the NTS 2 sequence. To reveal SNPs, a novel minisequencing method was designed and assessed by analysis of 15 *T. gondii* laboratory isolates of types I, II, and III and atypical strains. DNA from tachyzoites was extracted with a QIAamp DNA mini kit (QIAGEN, Hilden, Germany). DNA from blood samples (2 ml) collected from 61 patients with active ocular toxoplasmosis (of which 26 had severe retinochoroiditis with macula or optic disc involvement) and from 12 patients with old toxoplasmic retinochoroidal scars was extracted with a GeneMatrix blood DNA purification kit (Eurz, Gdansk, Poland). None of the patients had signs of immunodeficiency. As a control

group, blood samples from three healthy volunteers and from seven patients with eye lesions due to toxocariasis were analyzed.

**PCR.** To amplify the 288-bp-long fragment in NTS 2, two primers were designed: TgNTS2-U (5'-CGATGTGTTCCCTAACTGA-3') and Tg288-L (5'-GTTGCATGTGTTCCCATACGG-3'). The PCR was performed in 12 µl of mix based on a previously described protocol (<http://www.fermentas.com/techinfo/pcr/dnaamplprotocol.htm>), with 1.75 mM MgCl<sub>2</sub>, 0.4 µM primers, 0.75 U of polymerase, and 2 µl of template DNA. Thermocycling parameters were maintained as previously described (2), except for the following 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 (Eurz, Gdansk, Poland) and with *Escherichia coli* exonuclease I (Fermentas, Hanover, Md.). According to GenBank sequences (GenBank accession no. AF158092 for strain RH, AF158093 for strain MAS, AF158094 for strain C56, and AF158095 for strain Beverly), minisequencing SNP primers were designed (Table 1). The reaction was performed according to the protocol of the manufacturer (<http://docs.appliedbiosystems.com/pebi/docs/04323357.pdf>). The concentration of each minisequencing primer in the mix of pooled primers is shown in Table 1. Amplicons were analyzed using an ABI PRISM 310 sequence analyzer and GeneScan software (Applied Biosystems, Foster City, Calif.).

**Results.** Genotyping of laboratory isolates showed the usefulness of the method for the differentiation of *T. gondii* strains (Table 2). We noted patterns of SNPs characteristic of type I, type III, and atypical isolates and three different genotypes among seven isolates of type II. We noticed also double nucleotide variants in strains ELG, SIK, C56, and GANGI. In each of these positions, one nucleotide of double variant was characteristic for the type I genotype.

***T. gondii* genotyping in clinical samples.** Genetic material of *T. gondii* was detected in 86.9% (53 of 61) of samples taken from patients with active toxoplasmic retinochoroiditis and in 50% (6 of 12) of samples taken from patients with old toxoplasmic scars on the fundus. All detected *T. gondii* strains

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TABLE 1. Primers and their concentrations in the mix for minisequencing reaction

Primer	SNP position in NTS 2 sequence	SNP primer sequence	Concn (μM) in primer mix for minisequencing reaction
TgNTS300-109U	723	5'GCTGACTGACTGAGAGTGTCTTACGAGACCATGAGAAA3'	34.29
TgNTS300-147U	761	5'GCACTGACTGACTGTTCGCACGATGGAATATATGATAAGG3'	5.14
TgNTS300-177L	765	5'CGACTGACCGACACAATACAGAGCTAGTTCTTCTCC3'	1.11
TgNTS300-255L	843	5'CTATACTGCACAATGAGCGCCACGCG3'	2.22
TgNTS300-268L	856	5'GACGACATGCAGGTGTTCCCATACGGCACAA3'	2.64

belonged to type I according to our SNP analysis of the NTS 2 region.

Genotyping of samples from control clinical groups revealed that all of them except one were negative for *T. gondii* DNA (specificity, 90%). One false-positive result indicating the presence of a type I isolate occurred with a sample taken from a patient with toxocariasis. It was likely due to cross-contamination during SNP 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 a safer and more convenient procedure than 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 cannot exclude that *T. gondii* genotyped with 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 consistent with previous studies in which DNA was isolated from retinochoroidal specimens or ocular fluids (10, 17). A study from Brazil revealed that all 11 strains causing toxoplasmic retinochoroiditis examined in the SAG2 locus were of type I (17). Also, a study conducted in the United States 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 a type I allele (10). Recombinant strains were identified by multilocus analysis of five loci (SAG1, SAG2, SAG3, SAG4, and B1); analysis of a 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. This study and previous studies (10, 17) have shown 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 consistent with these results. The strain assigned as type I was identified in an outbreak in Victoria, British Columbia, Canada (14), where ocular toxoplasmosis was diagnosed in 20.6% of infected persons (in comparison to 2 to 3% of persons chronically infected with *T. gondii*). Also, mouse-virulent strains isolated from chickens (7) and cats (8) were prevalent in regions of Brazil where ocular toxoplasmosis is endemic.

We conclude that, with ocular toxoplasmosis, peripheral blood samples analyzed for the NTS 2 region can be used for direct *T. gondii* genotyping and that human ocular toxoplasmosis may be associated predominantly with type I strains. Certain features of the type I strain—faster multiplication, ability of extracellular migration, and 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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TABLE 2. Genotyping of *T. gondii* control isolates

Strain type and designation(s)	SNP or double nucleotide variant at position				
	723	761	765	843	856
Type I RH, P, ENT, ATIH, and GIL	C	T	A	G	A
Type II Jones, CAL, and PRU	T	G	T	A	G
BOU and S3	T	G	T	G	G
S1K and ELG	T	G	T	A/G	G
Type III C56	C/T	T/G	A/T	G	A/G
Atypical P80	T	G	A	G	A
GANGI	C	T/G	A/T	G	A/G

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