Prevalent Genotypes of *Toxoplasma gondii* in Pregnant Women and Patients from Crete and Cyprus

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**Abstract.** Molecular genotyping has been used to characterize *Toxoplasma gondii* strains into the three clonal lineages known as types I, II, and III. To characterize *T. gondii* strains from Greece and Cyprus, polymerase chain reaction–restriction fragment length polymorphism analysis on the GRA6 gene was performed directly on 20 clinical samples from 18 humans (11 pregnant women, six patients with lymphadenopathy, and one patient positive for human immunodeficiency virus) and two rats. Characterization of *T. gondii* types was performed after digestion of amplified products with Mse I. The 20 strains were characterized as type II (20%) and type III (80%). Of these strains, 19 originated from the island of Crete (4 strains type II and 15 strains type III), and 1 from the island of Cyprus (type III). Although both type II and type III strains were found, type III was the most prevalent in Crete.

**INTRODUCTION**

*Toxoplasma gondii* is an intracellular protozoan that infects humans and other warm-blooded animals. It is considered one of the most widespread parasites in the world. The severity of *T. gondii* infection in humans depends on host factors, such as immunity and genetic background, and parasite factors, such as strain virulence, inoculum size, and parasite stage in the life cycle. Although toxoplasmosis is asymptomatic in most immunocompetent persons, *T. gondii* causes significant morbidity and mortality in congenitally infected infants, immunocompromised persons, and transplant recipients.

The first evidence for the existence of different strains of *T. gondii* came from isoenzyme analysis of isolates mainly of French origin. Parasite strains can be divided into three clonal lineages: types I, II, and III, according to their pathogenicity in mice. Type I is highly virulent in murine infections and shows a 100% lethal dose (LD100) of just one parasite, whereas type II and III strains are relatively less virulent, showing an LD100 > 10^4 parasites. However, there is no evidence that the difference in virulence observed in mice correlates with respective infections in humans. Congenital toxoplasmosis associated mainly with type I and II strains. Most strains from patients with acquired immunodeficiency syndrome are type II, and animal strains are predominantly type III.

Molecular genotyping is now being used for the categorization of *T. gondii* strains into the three clonal lineages and serotyping and pyrosequencing have been introduced as methods for obtaining results quickly. Although up to one-third of the population worldwide is infected by this parasite, information on genotyping is limited to the United States and some European countries. Clinical samples are used directly in genotyping studies because there is evidence that differences in methods of parasite isolation (culture method, number of passages) may introduce a risk of bias into genotyping results.

The aim of this study was to characterize *T. gondii* from clinical samples of pregnant women, patients, and rats using molecular tools and to correlate the genotype derived with the clinical manifestation of the infection. Our study is the first attempt to characterize *T. gondii* in Greece and Cyprus.

**MATERIALS AND METHODS**

**Serologic analysis.** A total of 9,285 human serum samples (pregnant women and patients with suspected toxoplasmosis) were tested for IgG (cut-off value = 11 IU), IgM, and IgA antibodies (qualitative test) by an enzyme-linked immunosorbent assay (Alphadia, Wavre, Belgium) with the Triturus automatic enzyme-linked immunosorbent assay analyzer (Gri- fols, Barcelona, Spain). Samples positive for any of these three antibodies were further tested by an indirect immunofluorescence antibody test (IFAT) (Biosna, Athens, Greece) for IgM and IgG antibodies (cut-off values = 1/50 and 1/1,350, respectively) and an immunosorbent agglutination assay for IgM antibodies (bioMerieux, Marcy l’Etoile, France). All samples were tested after informed consent was obtained from the persons involved. The study was reviewed and approved by the Scientific Board of the University Hospital of Heraklion, Crete. Results and clinical and epidemiologic data were stored in an automated database for statistical analysis and for monitoring each case. Parallel to human samples, 112 rat sera were tested for *T. gondii* by an IgG IFAT (Biosna) using rat anti-IgG antibodies starting at an initial dilution of 1:30 (positive titers were those > 320).

**Clinical samples and controls.** Of the 9,285 samples tested by serologic analysis, 290 (232 from females and 58 from males) showed evidence of acute toxoplasmosis and were further tested by polymerase chain reaction (PCR) for amplification of *T. gondii*. These included 206 asymptomatic pregnant women, 72 patients with lymphadenopathy and 12 patients positive for human immunodeficiency virus (HIV) with high IgG titers. Two seropositive rats were also tested. Three *T. gondii* strains served as lineage controls: RH (type I), LGE 94-2 (type II), and VEG (type III). These strains were obtained from O. Djurkovic-Djakovic (Department of Parasitology, Institute of Medical Research, Belgrade, Serbia), S. L. Croft (London School of Hygiene and Tropical Medicine, London, United Kingdom), and J. P. Dubey (Animal Para-
Table 1

<table>
<thead>
<tr>
<th>Patients</th>
<th>No. (%) seropositive samples</th>
<th>No. (%) PCR-positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>58 (0.62)</td>
<td>5 (8.62)</td>
</tr>
<tr>
<td>Females</td>
<td>232 (2.50)</td>
<td>13 (5.60)</td>
</tr>
<tr>
<td>Pregnant women</td>
<td>206 (2.22)</td>
<td>11 (5.34)</td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>72 (0.78)</td>
<td>6 (8.33)</td>
</tr>
<tr>
<td>HIV positive</td>
<td>12 (0.13)</td>
<td>1 (8.33)</td>
</tr>
<tr>
<td>Total no. tested</td>
<td>290 (3.12)</td>
<td>18 (6.20)</td>
</tr>
</tbody>
</table>

*PCR = polymerase chain reaction; HIV = human immunodeficiency virus.*

RESULTS

Of 9,285 persons tested for toxoplasmosis at the University Hospital of Heraklion, 290 (3.12%) had serologic evidence of acute toxoplasmosis.12 PCR analysis showed positive results in samples from 18 patients (16 in peripheral blood, 1 in a placenta, and 1 in a lymph node aspirate), all of whom had specific IgG. Specific IgM and IgA antibodies were detected in 80% and 40%, respectively. Clinically, these 18 patients included 11 cases in pregnant women, 6 cases in patients with lymphadenopathy, and 1 HIV-positive patient (Table 1).

The PCR results for GRA6 gene were positive for two rat brain samples. The two positive rats had specific IgG antibodies with a titer of 640 by IFAT. The PCR–RFLP analysis of the 20 positive cases (18 in humans and 2 in rats) and the controls were classified into one of the three types according to their restriction pattern as shown in Figure 1. None of the clinical samples was type I, 4 (20%) were type II, and 16 (80%) were type III (Table 2).

Table 2

<table>
<thead>
<tr>
<th>Sample origin</th>
<th>Genotype, no (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type I</td>
</tr>
<tr>
<td>Pregnant women</td>
<td>0</td>
</tr>
<tr>
<td>HIV-positive patients</td>
<td>0</td>
</tr>
<tr>
<td>Patients with lymphadenopathy</td>
<td>0</td>
</tr>
<tr>
<td>Rats</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
</tr>
</tbody>
</table>

*HIV = human immunodeficiency virus.*

PCR amplification of the GRA6 gene. DNA was extracted from peripheral blood, umbilical blood, placenta, cerebrospinal fluid, lymph nodes, amniotic fluid, ocular fluid, and pleuritic fluid samples of patients and from brains of rats using the QiAamp DNA Blood Mini kit (Qiagen, Valencia, CA) and the DNeasy Tissue kit (Qiagen) accordingly. "Toxoplasma gondii" GRA6 gene PCR amplification was carried out using GRA6 gene–specific nucleotide primers: forward primer 5′-GTAGCTGGTCGTGCCCAGGAC-3′ and reverse primer 5′-TACAAGACATAGAGTCCC-3′ (positions 372–391 and 1162–1143, respectively).13 The amplification reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 0.2 mM dATP, dCTP, dGTP, and dTTP, 1 μM of each primer, 2.5 U of Taq DNA polymerase (Qiagen), and 1–15 μL of DNA template in a reaction mixture of 50 μL. After 3 minutes of initial denaturation at 95°C, 30 cycles of amplification (each cycle: 1 minute at 95°C, 1 minute at 60°C, and 2 seconds at 72°C) and a final extension step for 10 minutes at 72°C were performed in an automated thermocycler (Peltier Thermal Cycler, PTC 200; MJ Research Inc., Waltham, MA). Amplicons were visualized by electrophoresis on a 1.6% agarose gel, and the DNA was detected by staining with GelStar Nucleic Acid Gel Stain (Cambrex Bio Science, Walkersville, MD). After electrophoresis, the gel was placed in a polypropylene container with 1× staining solution and incubated with gentle agitation for 30 minutes. The container was covered with silver foil to ensure gel protection from light during staining. A 312-nm ultraviolet transilluminator was used for dye excitation and visualizing bands. DNA-free water was used as a negative control and three T. gondii strains (RH, LGE 94-2, and VEG) were used as positive controls.

PCR–restriction fragment length polymorphism (PCR-RFLP) analysis. To determine the GRA6 gene type of T. gondii, the amplified product was digested with 1 U/μL of Mse I (Invitrogen, Carlsbad, CA). After incubation at 37°C for 4 hours, the restriction fragments were separated by electrophoresis on a 1.6% agarose gel, stained with GelStar Nucleic Acid Gel Stain, and visualized with a 312-nm ultraviolet transilluminator.

Results

<table>
<thead>
<tr>
<th>Sample origin</th>
<th>Type I</th>
<th>Type II</th>
<th>Type III</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnant women</td>
<td>0</td>
<td>3</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>HIV-positive patients</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Patients with lymphadenopathy</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Rats</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
<td>4</td>
<td>16</td>
<td>20</td>
</tr>
</tbody>
</table>

*HIV = human immunodeficiency virus.*

FIGURE 1. Polymerase chain reaction–restriction fragment length polymorphism analysis of the GRA6 gene coding region of Toxoplasma gondii with Mse I. Lane M, 100-basepair DNA size marker; lanes 1, 2, and 3, reference strains: type I (RH), type III (VEG), and type II (LGE 94-2) respectively; Lanes A and B, type II and type III strains respectively.
DISCUSSION

Crete and Cyprus do not have a national screening program for toxoplasmosis during pregnancy. In a study conducted on the island of Crete during 1998–2003, 0.23% of the women examined had their first serologic screening for T. gondii before pregnancy: 54.44% during the first trimester, 30.4% during the second trimester, and 14.91% during the third trimester. Because most cases have not had a previous test for toxoplasmosis, the blood sample from a pregnant woman or patient is kept until initial serologic test results are available. If acute toxoplasmosis is suspected, this blood sample is used in a PCR. The PCR-positive pregnant women in this study were 2–6 months pregnant at their first serologic examination. They had a second serum sample tested to confirm the laboratory diagnosis before given treatment and the fetus was monitored monthly by ultrasound. All pregnant women were asymptomatic and delivered healthy children, as shown by clinical examination after birth, serologic analysis for one year, and Western blotting of blood samples at birth and when the infant was three months of age. All patients with toxoplasmic lymphadenitis were immunocompetent and swollen cervical or occipital lymph nodes returned to normal sizes after 4–7 weeks without treatment.

By using PCR–RFLP analysis, one can assign T. gondii strains to one of three clonal types (I, II, or III). This method uses the polymorphism associated with the presence or absence of restriction sites for specific restriction enzymes. In this study, PCR–RFLP analysis was performed on the DNA of 3 reference strains and 20 other strains derived directly from clinical samples. The GRA6 gene was used as a marker because it can clearly differentiate between the three T. gondii genotypes, as well as between some atypical genotypes. However, typing of T. gondii using one marker may not identify non-clonal strains. To discriminate closely related isolates and achieve high resolution within a clonal lineage, multilocus PCR-RFLP and microsatellite analysis should be used.

Type I was not found in any of the clinical samples tested. This genotype has been associated with ocular toxoplasmosis in humans and it would have been informative if samples were available for testing from such patients in Crete. The most prevalent genotype detected was type III (80%; 72.7% of the cases in pregnant women, 80% of the patients with lymphadenopathy, the one sample from the island of Cyprus, a child with lymphadenopathy, and the HIV-positive patient). The two PCR-positive rats were also infected with a type III strain. These rats were trapped in a garden in the outskirts of Heraklion, Crete, where 16 of the 18 human cases originated. An epidemiologic link between chronic infections in some food animals and rodents may underlie the prevalence of T. gondii genotypes that cause human disease. Genotype analysis can identify the source of an infection. This analysis will help in understanding the pathogenesis of the disease and its epidemiology, and in planning preventive strategies.

Although chronic infections in domestic and wild animals are equally divided between type II and III strains, most reports suggest that the high prevalence of type II strains in humans is caused by the high prevalence of that strain in animals. Strains from animals and humans in different European countries show a predominance of genotype II, but types I and III have also been reported in Portugal and type III in Denmark (Figure 2). Strains from infected animals in Egypt and Iran were typed as II and III. Multiple infections and atypical genotypes have been described, and new virulent strains of T. gondii adapted to wild animals.
are reported to produce severe symptoms in immunocompe-
tent persons. It appears that countries near Crete have ei-
ther type III strains (Egypt, typed in animals, one human case in Cyprus [this report]) or type II strains (Italy and Serbia, typed in humans and animals) (Figure 2).

Currently, there is insufficient information to relate T. gon-
dii genotypes to geographic regions or sources of infection. The absence of the type I strain in the countries in the eastern Mediterranean region could be explained by similarities in geographic and ecologic factors, human habits, and diet, which may influence distribution of genotypes.

There are few reports of toxoplasmosis in Greece. Seropositivity for T. gondii infection ranges from 11.1% to 51% in specific groups and from 29% to 45% in the general population. We found that most human strains belonged to type III (Table 2), a type reported mainly in animals. Although the route of T. gondii transmission in the population cannot be established, infection by bradyzoites and sporozoites is possible in Greece and Cyprus because many stray cats are found in rural and urban areas and the warm environment favors survival of oocysts. The diet of the people in Greece and Cyprus includes consumption of large amounts of raw, wild vegetables and salads that could easily be contaminated with parasite oocysts. In addition, lamb, sheep, goat, and pork (occasionally undercooked) and smoked pork and sausages are also consumed.

The agricultural and livestock-raising nature of these countries favors sustaining large rodent populations. In a study conducted in Cyprus during 2000–2003, 625 rodents were tested for antibodies to T. gondii and 22% of the animals were seropositive (Antoniou and others, unpublished data). It will be noteworthy to see whether reduced consumption of home-grown fruit and vegetables, consumption of pasteurized milk, use of freezers in the home, urbanization, and education will affect the seroprevalence and genotype distribution of T. gondii in different countries.

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