DETECTION OF TOXOPLASMA GONDII DNA AND SPECIFIC ANTIBODIES IN HIGH-RISK PREGNANT WOMEN

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Abstract. Primary maternal infection with toxoplasmosis during pregnancy is frequently associated with transplacental transmission to the fetus. This study was conducted to test the utility of a polymerase chain reaction (PCR) assay to detect recent infections with Toxoplasma in pregnant women. One hundred forty-eight women with high-risk pregnancies who had abnormal pregnancy outcomes (cases) and 100 with normal pregnancies (controls) were tested for the presence of Toxoplasma DNA in their blood by a nested PCR and specific antibodies to Toxoplasma by an enzyme-linked immunosorbent assay. The IgG results of the cases differed significantly from those of the controls (54% and 12%, respectively; P < 0.02). Four (2.7%) of the cases were IgM positive, but none of the controls were positive. Detection of Toxoplasma DNA in 20 (8.1%) of the IgG-positive cases suggests a recent infection. The risk factors associated with the infection were eating raw meat and contact with soil. The diagnostic serology of recent infection in early pregnancy could be confirmed by a positive Toxoplasma-specific PCR result in blood samples collected in the first half of pregnancy, even in the presence of serologic results difficult to interpret due to the lack of sequential follow-up during pregnancy.

INTRODUCTION

Toxoplasmosis caused by the intracellular parasite Toxoplasma gondii is a zoonotic infection of cosmopolitan distribution. Serologic evidence indicates that human infections are common in many parts of the world. Although adult-acquired toxoplasmosis is usually mild to asymptomatic, the disease can be severe in the immunocompromised, leading to encephalitis. In addition, the influence of sex-and pregnancy-associated hormones on the severity of T. gondii infection is of particular public health interest due to the ability of this parasite to cause congenital disease if infection occurs during pregnancy. In a study of non-homosexual, human immunodeficiency virus (HIV)–positive Europeans, toxoplasmic encephalitis was found to be a more frequent acquired immunodeficiency syndrome–defining disease in females than in males. These observations suggest a detrimental role for female hormones during the course of T. gondii infection.

Primary maternal infection during pregnancy is frequently associated with transmission of T. gondii to the fetus. Transplacental transmission of Toxoplasma from an infected, pregnant woman to the unborn results in fetal damage to a degree depending on the gestational age. Early first-trimester maternal infections are less likely to result in congenital infection, but the sequelae are more severe. Transplacental passage is more common when maternal infection occurs in the latter half of pregnancy, but fetal injury is usually much less severe. It may lead to miscarriage, stillbirth, or congenital defects depending on the stage of gestation when the infection occurs. Several studies have suggested its role in the causation of abortions.

Effective prevention of congenital toxoplasmosis depends mainly on avoidance of infection during pregnancy. Infection is acquired by ingestion of viable tissue cysts in meat or oocytes excreted by cats that contaminate the environment. Uncertainty about how most women acquire infection results in advice to avoid numerous risk factors, making compliance difficult.

Current diagnosis of toxoplasmosis relies either on serologic detection of specific anti-Toxoplasma immunoglobulin, on culture of amniotic fluid or fetal blood, or on other non-specific indicators of infection. In countries where no systematic screening of pregnant women is performed, diagnosis of acute toxoplasmosis is mainly by antibody detection and generally only undertaken in pregnant patients with risk factors for transplacental transmission. All positive screening test results in pregnant women must be confirmed at a Toxoplasma reference laboratory. Recent studies have shown that polymerase chain reaction (PCR) testing of amniotic fluid is useful for identification or exclusion of fetal T. gondii infection. Ultrasound can be used to allow the detection of fetal lesions due to congenital toxoplasmosis. A PCR was also used to detect the parasite in blood samples from patients presented with acute toxoplasmic lymphadenopathy, or to complement culture and serologic testing for the diagnosis of active toxoplasmosis. The proportion of women at risk of acquiring the infection during pregnancy in Jordan is not well known and maternal screening is not mandatory.

In this study, we tested the utility of a PCR assay to detect recent Toxoplasma infections in pregnant women and whether it can be used as a confirmatory test at a reference laboratory to detect recent infections in pregnant women with positive IgM or IgG enzyme-linked immunosorbent assay (ELISA) result.

MATERIALS AND METHODS

This study was reviewed and approved by the University Ethical Committees, and informed consent was obtained from all the study groups.

Study groups. Cases were selected among women delivering at one of the obstetric and gynecology clinics at the University of Jordan and Al Bashir hospitals, two of the major hospitals in Amman, the capital of Jordan. One blood sample was collected from 248 eligible cases selected based on their history of pregnancy. The cases included 148 pregnant women considered to be a high-risk group (abnormal pregnancy outcomes and possible exposure to sources of Toxoplasma). The 100 controls were healthy women with a history of a normal pregnancy, matched for age (with an SD of two years), and were selected from a consecutive sample of women attending the outpatient clinics for routine gynecologic checking. Ad-
ditional samples were not feasible from all women because not all the participating women were willing to provide another sample. In addition, some women did not comply with a regular visitations schedule. Relevant information about the study groups and the possible risk factors are shown in Table 1.

Forty (27%) of the cases and five (5%) of the controls were seropositive for Toxoplasma during the previous pregnancy. The two groups were included to gain insight into the predictive value of a PCR in the diagnosis of active Toxoplasma infection in both groups. None of the mothers had apparent symptoms of toxoplasmosis during their pregnancy.

All women were interviewed using a structured questionnaire, and demographic and other relevant information were recorded for each woman. Information included the number of pregnancies, number of abortions if any, previous infections, eating raw or undercooked meat, drinking unpasteurized milk, contact with soil (e.g., backyard gardening activity), cats, or other domestic animals living nearby.

Serologic tests. The sera were tested for the presence of specific IgM and IgG antibodies to Toxoplasma using an ELISA kit (Biokit, Barcelona, Spain) according to the manufacturer’s instructions.

Isolation of DNA. DNA was isolated from blood samples using a commercial purification system (Wizard Genomic DNA Purification Kit; Promega, Madison, WI) following the manufacturer’s instructions for DNA purification from blood. Final pellets were resuspended in 30 μL of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.2) and stored at −70°C until used.

Nested PCR assay. The nested PCR was performed on all DNA samples to amplify a fragment from the B1 gene, which is present in 35 copies and is conserved in the T. gondii genome, as described by Burg and others.16 The primers used in the first round of the PCR (inner primers) were (5’-GGAACTGCATCGTCCATAG-3’, and (5’-TCTTTAAACGTTCGTTGCTGTC-3’), which correspond to nucleotides 694-714 and 887-868, respectively. The primers used in the second round (outer primers) were (5’-TGCATAG GTTGCAAGTCACTG-3’ and (5’-GGCCACATGTGCGAATAGACC-3’), which correspond to nucleotides 757-776 and 853-831, respectively.

Three microliters of template DNA were added to a final volume of 50 μL of PCR mixture consisting of 5 μL of 10× PCR buffer (50 mM Tris-HCl, pH 9.1, 3.5 mM MgCl2), 8 μL of 1.25 mM deoxynucleoside triphosphates, 0.5 μL of Tag DNA polymerase [5 units/μL], and 1.5 μL (20 pmol) of each of the outer primers. The amplification was performed in the GenAmp 9700 PCR System (Applied Biosystems, Foster City, CA). The cycling conditions for both PCRs were 95°C for five minutes, followed by 30 cycles at 94°C for 30 seconds, 55°C for 90 seconds, and 72°C for one minute, and a final extension at 72°C for 10 minutes.

Three microliters of the first-round product were used as template for the second-round PCR in a total volume of 50 μL under the same conditions as in the first round, using the inner primers. DNA extracted from RH strain of T. gondii from the collection of the Service de Parasitologie-Mycologie (Grenoble, France) was used as a positive control. The PCR mixture without DNA and with DNase-free water were used as negative controls to monitor for cross-contaminations.

Two negative controls and one positive control were included for every 24 samples in each PCR. The positive controls were loaded last to avoid contamination of the sample. To guaranty the reliability of the results and detect any possible contamination, all samples were processed in duplicate. The test result was considered positive if the amplified DNA fragment was clearly visible in both samples.

Sample cross-contamination problems were avoided following a number of precautions including the use of aerosol-guarded tips, performing DNA extraction in a laminar flow hood with subsequent irradiation by UV light and the use of three separate areas for the DNA extraction, preparation of PCR mixture and a separate area for PCR amplification and running gels. Five microliters of the PCR product were subjected to electrophoresis on a 1.2% agarose gel stained with ethidium bromide.

Statistical analysis. Data were analyzed with the chi-square and Fisher exact tests to evaluate the possible differences between the study groups. A P value < 0.02 was considered significant.

RESULTS

The mean age of women in both groups was 28 years (range = 16–40 years). Results of the ELISA and PCR are summarized in Table 2.

IgM ELISA. Only 4 (2.7%) of the 148 high-risk cases tested positive for specific IgM antibodies with high titers compared with none in the control group (titers < 10 IU/mL).

IgG ELISA. For IgG antibodies, 80 (54%) of 148 cases tested positive; these included the four cases that were IgM positive, and the 40 who were positive in their previous pregnancy. Of the total positive, 31 (38.9%) had relatively high titers compared with the positive control (10–200 IU/mL).

| TABLE 1 |
| Relevant information for the 248 pregnant women included in the study |

<table>
<thead>
<tr>
<th>Factor</th>
<th>Cases (n = 148)</th>
<th>Controls (n = 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age range (years)</td>
<td>No. (%)</td>
<td>No. (%)</td>
</tr>
<tr>
<td>≤ 20</td>
<td>24 (16.2)</td>
<td>15 (15)</td>
</tr>
<tr>
<td>21–30</td>
<td>80 (54.1)</td>
<td>50 (50)</td>
</tr>
<tr>
<td>31–40</td>
<td>44 (29.7)</td>
<td>35 (35)</td>
</tr>
<tr>
<td>Trimester</td>
<td></td>
<td></td>
</tr>
<tr>
<td>First</td>
<td>33 (22.3)</td>
<td>25 (25)</td>
</tr>
<tr>
<td>Second</td>
<td>57 (38.5)</td>
<td>35 (35)</td>
</tr>
<tr>
<td>Third</td>
<td>58 (39.2)</td>
<td>40 (40)</td>
</tr>
<tr>
<td>No. of pregnancies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–4</td>
<td>100 (67.6)</td>
<td>65 (65)</td>
</tr>
<tr>
<td>5–10</td>
<td>48 (32.4)</td>
<td>30 (30)</td>
</tr>
<tr>
<td>No. of abortions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0 (0.0)</td>
<td>100</td>
</tr>
<tr>
<td>1–2</td>
<td>143 (96.6)</td>
<td>None</td>
</tr>
<tr>
<td>≥ 3</td>
<td>5 (3.4)</td>
<td>None</td>
</tr>
<tr>
<td>Residency</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urban</td>
<td>110 (74.3)</td>
<td>80 (80)</td>
</tr>
<tr>
<td>Rural</td>
<td>38 (25.7)</td>
<td>20 (20)</td>
</tr>
<tr>
<td>Eating raw meat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>43 (29)</td>
<td>20 (20)</td>
</tr>
<tr>
<td>No</td>
<td>105 (71)</td>
<td>80 (80)</td>
</tr>
<tr>
<td>Contact with cats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>38 (25.7)</td>
<td>22 (22)</td>
</tr>
<tr>
<td>No</td>
<td>110 (74.3)</td>
<td>78 (78)</td>
</tr>
<tr>
<td>Contact with soil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>120 (81.1)</td>
<td>28 (28)</td>
</tr>
<tr>
<td>No</td>
<td>28 (18.9)</td>
<td>72 (72)</td>
</tr>
</tbody>
</table>

* Women with abnormal pregnancy outcomes (high-risk group).
† Women with normal pregnancies.
Positive results for IgG antibodies and risk factors for the DNA indicate the possibility that *T. gondii* the five women who were positive in their previous pregnancy. Significant risk factors were both IgM and IgG antibodies. None of the PCR-positive cases were seronegative in their previous pregnancy. A negative IgM result and a negative PCR result excluded recent infection in the other women. Direct contact with soil during gardening activity (16, 20%) (Table 3). They were both significantly higher (*P* < 0.02) and by the PCR polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay. *PCR* = polymerase chain reaction.

**Polymerase chain reaction assay.** Twenty (13.5%) of the cases were positive (Table 2), compared with none in the control group, giving a total of 20 PCR-positive individuals in both groups (8.1%). All the PCR positive cases were seropositive: these included 16 (25%) that were positive only for IgG antibodies and the four samples that were positive for both IgM and IgG antibodies. None of the PCR-positive cases was seropositive in their previous pregnancy. A negative IgM result and a negative PCR result excluded recent *Toxoplasma* infection in the other women.

The results showed a significance difference between the two groups obtained by the ELISA (*P* < 0.02) and by the PCR assay (*P* < 0.05). The presence of both specific antibodies and DNA indicate the possibility that *T. gondii* infection occurred during the current pregnancy if the serum sample was collected within the first 20 weeks of gestation. The infection rate in cases was significantly higher (*P* < 0.02) than in controls. No abortions or evidence of fetal infections were reported at delivery in these cases, and follow-up of fetuses was recommended. Risk factors strongly associated with acute infection in the IgG-positive cases were eating raw meat (18, 22.5%) and contact with soil during gardening activity (16, 20%) (Table 3). They were both significantly higher (*P* < 0.02) in the seropositive group compared with the seronegative group. Direct contact with cats or the residence of the subject were not significant risk factors.

**DISCUSSION**

Precise knowledge of acute *Toxoplasma* infections during pregnancy is needed for risk assessment of vertical transmission of infections as basis for counseling, prevention, and treatment. The proportion of women at risk of acquiring the infection during pregnancy in many countries, including Jordan, is not well known. Primary infection with *Toxoplasma* during pregnancy may lead to severe complications, if not fatal infection of the fetus. Therefore, emphasis is placed on preventive measures and early diagnosis of the infection to prevent these severe complications.

The ideal situation for the diagnosis of *T. gondii* infection in pregnancy of having an antibody-negative serum sample collected at the very beginning of pregnancy or preferably before conception is usually not possible. In Jordan, as in some other countries such as the United States, testing for antibodies to *Toxoplasma* in pregnancy is performed only in suspected cases. The first serum sample is usually taken at the first antenatal health care visit confirming pregnancy, which is usually between the eighth and thirteenth weeks of gestation. The test or the combination of tests to be used in such an antenatal screening should ideally be able to determine if the infection occurred after conception. If seroconversion is the only accepted criterion for the diagnosis of *T. gondii* infection, then the first gestational weeks accounting for 25–30% of the total duration of the pregnancy will not be covered by the serologic test aimed at the detection of congenital toxoplasmosis.

Routine serologic diagnosis of toxoplasmosis provides high sensitivity, but specificity varies depending on the test used, and false-positive IgM antibody test results have been reported by other investigators. In addition, even true positive results must be interpreted with caution because IgM antibodies may persist for several years after acute infection. Diagnosis of primary infection with *T. gondii* in early pregnancy can be improved by determination of *anti*-toxoplasma IgG avidity, which has the ability to discriminate between recent and prior infections.

The high sensitivity, combined with the high specificity, offered by the PCR led us to investigate the presence of the *T. gondii* genome in maternal blood in cases of pregnancy where infections with this parasite were suspected. Twenty (13.5%) women with abnormal pregnancy outcomes compared with none in the control group were identified to be possibly infected during the current pregnancy (e.g., recent infection) based on the combined results of a positive IgG ELISA result (four were also positive for IgM), and the de-
tection of *Toxoplasma* DNA by a PCR assay. They were seronegative in the previous pregnancy. The negative results obtained by both the PCR and serology rule out an infection in the rest of women in both groups.

Previous studies confirmed that the PCR could actually detect *T. gondii* in blood specimens of women before or during pregnancy. The clearance time for *Toxoplasma* DNA from the blood of patients with acute toxoplasmosis lymphadenopathy was estimated to be 5.5–13 weeks. Based on this, the presence of *Toxoplasma* DNA in the maternal blood most probably indicates a recent infection or an indicator of apparent parasitemia, which is likely to be clinically significant. This suggests that the 20 (8.1%) positive cases by both the ELISA and PCR are recent infections. The cause of the previous abortions in these women was not clear, and it cannot be directly related to previous maternal *Toxoplasma* infections since these women were seronegative in their previous pregnancy. However, a negative PCR result does not exclude recent infections because the sensitivity of the PCR, in which a single trophozoite can be detected in a clinical sample, has potential problems for some types of specimens, and because the exact kinetics of parasitemia in infected people are not well known. The short duration of parasitemia, or if the numbers of trophozoites circulating in peripheral blood are low, could lead to a sampling error that will produce false-negative results in such cases. The sensitivity of the PCR was found to be significantly higher for maternal infections that occurred between 17 and 21 weeks gestation (*P* < 0.02) when the amniotic fluid was tested. If the same applies to blood samples, further studies are needed.

The percentage of women seropositive for IgG women with abnormal pregnancy outcomes was 54% compared with 12% in those with normal pregnancies. Although, the percentage (20.3%) of IgG positive women in the third trimester of their pregnancy was the highest, the difference compared with other trimesters was not statistically significant. These results are consistent with those in previous studies conducted in northern Jordan and Mexico.

The gestational age during which the maternal infection occurs is important. Although, the frequency of fetal infection is higher when maternal infection occurs later in the pregnancy (e.g., third trimester), the sequelae are more severe when maternal infections occur early in the first trimester.

A previous study of serial blood samples from acutely infected pregnant women indicated that in the presence of *Toxoplasma*-specific IgG and IgM antibodies, and the additional presence of a high dye test titer were insufficient criteria for identifying *Toxoplasma* infection in early pregnancy because some acute infections will not be detected. Conversely, some women will be falsely identified as being infected, and undergo unnecessary diagnostic amniocentesis and antiparasitic treatment. In a study of uterine *Toxoplasma* infections in women with repeated abortions, five (71.4%) of seven *Toxoplasma*-positive endometrium patients were serologically negative. These reports emphasize the importance of confirmatory testing.

In conclusion, this study highlights the need for a confirmatory test to detect primary acute toxoplasmosis in pregnant women. It demonstrates the possibility of defining and selecting the high-risk cases for mother-to-child transmission of infection by combining specific serology and PCR tests to formulate a specific approach. A single PCR-positive sample collected in the first half of pregnancy in conjunction with an IgG-positive test result could confirm a recent infection, even in the presence of serologic results that are difficult to interpret due to the lack of sequential follow-up during pregnancy. It reduces the number of samples required for testing in suspected cases and the assay can be done at any reference microbiology laboratory after standardization of the method.

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