Post-treatment assessment of acute Toxoplasma infection during pregnancy

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Summary
Serological immune profile in cases of Toxoplasma infection is heterogeneous, and responses may be delayed or fail to be represented: this makes it an unreliable method for diagnosis and/or treatment follow-up. Therefore, the present study relied on a sensitive and specific molecular procedure (nested polymerase chain reaction, PCR), using the whole blood sample to establish the diagnosis of acute maternal toxoplasmosis in 27 pregnant women. All of them received the recommended dose of Spiramycin. Only 19 returned for follow-up visits and completed their pregnancies to full term. The achievement of the treatment regimen was evaluated according to the results of PCR amplification of T. gondii DNA at the end of the treatment course. Patients who continued to have positive PCR results were given another treatment course. After treatment with a single course of Spiramycin, 11 (57.9%) patients retained T. gondii DNA in their peripheral blood and in eight (42.1%) patients, T. gondii DNA was absent by PCR: four (21.01%) patients received up to three courses of treatment.

Introduction
Toxoplasma gondii (T. gondii), an obligate intracellular parasite found in many species throughout the world, causes a variety of clinical syndromes in humans (Ertug et al. 2005). Acute infection in pregnancy is usually asymptomatic; nevertheless infection may be transmitted to the fetus and cause severe damage (Pinard et al. 2003). Infections occurring in the first trimester have a 10% chance of transmission to the fetus and have the worst prognosis because of the risk of extensive central nervous system involvement (Morris and Croxson 2004). Therefore, early diagnosis is essential to allow early intervention and prevention of congenital disorders (Remington et al. 2004). Different assays have been developed to detect anti-Toxoplasma antibodies in the sera of pregnant women with a history of repeated abortions, and women suspected of being infected with T. gondii. However, serological tests based on the detection of IgM antibodies cannot differentiate between previous and current infection. Moreover, serological tests to follow-up treatment of toxoplasmosis are not sufficient to evaluate the efficacy of the treatment administered, therefore, it is desirable to detect the pathogen directly (El-Awady et al. 2000; Remington et al. 2004). Accurate detection of T. gondii in the peripheral blood of infected women during pregnancy has been shown to be possible with amplification of a DNA sequence within the 30–35-fold repetitive B1 gene of T. gondii using polymerase chain reaction (PCR) (Burg et al. 1989; Dupouy et al. 1993; El-Awady et al. 2000; Hussein et al. 2002; Nimiri et al. 2004; Iqbal and Khalid 2007).

In this study, a trial was made at testing the utility of a nested PCR assay with four primers targeting Toxoplasma B1 gene to detect and amplify the circulating parasite in blood samples that were obtained from pregnant women suspected to be infected in the first trimester. Moreover, the aim of this work was to evaluate the degree of reliability of nested PCR in the follow-up assessment after treatment by Spiramycin.

Materials and methods
Patients and samples
Cases included in the present study were selected from pregnant women attending the Obstetrics and Gynecology outpatient clinic of Ohod Hospital and the medical unit at Taibah University in Al-Madinah, Saudi Arabia for the first booking visit (during the first trimester); clinical and ultrasonographic examinations were done for all cases. All patients were interviewed and the relevant information was recorded, including the number of pregnancies, number of abortions, if any, previous infections, eating raw or undercooked meat, type of meat, contact with soil, cats, or other domestic animals living nearby. Pregnant women with the following criteria were excluded from this study: residents outside the Madinah area, with a history of other causes of abortion in the first trimester, e.g. chromosomal abnormalities, and maternal systemic diseases, e.g. diabetes mellitus, hyperthyroidism and systemic lupus erythematosus, patients receiving antibiotics, not willing to sign the informed consent and those who did not comply with the regular clinic visiting schedule. Suspected cases for Toxoplasma infection (with positive specific anti-Toxoplasma antibodies IgM and/or IgG, with a history of repeated abortions and those with close contact with cats, even with negative serological results), were submitted to further
investigations, 2 ml blood samples were collected on EDTA from this group to test for Toxoplasma B1 gene. The samples were transferred to the laboratory and stored at −20 °C until processed in the PCR technique. Spiramycin, a specific anti-Toxoplasma agent was given at a dose of 3 MIU, three times daily for 21 days (Stray-Pedersen 1992). Another whole blood sample was collected on EDTA 2 months after treatment with Spiramycin.

**Nested polymerase chain reaction**

DNA was isolated from blood samples using a commercial purification system (Wizard Genomic DNA Purification Kit; Promega, Madison, WI). Final pellets were resuspended in 30 µl of TE buffer and stored at −70 °C until used. *T. gondii* (P-strain) tachyzoites were collected by peritoneal lavage of infected mice and used as a positive control. The PCR mixture without DNA and with DNase-free water was used as negative control. Nested primer sets were: the outer from bases 171–190 (5'-CCGCCCTCCTTC-3') and from bases 602–583 (5'-GGAAAACAGGGCAAGCTGCT-3') producing an amplified product of 432 bp. Inner primers were from bases 180–196 (5'-CCGCCCTCCTTCGCTCCTGTCCGTCGT-3') and from bases 392–372 (5'-GTGCGGCGGACCTCTCTTG-3') producing an amplified product of 213 bp (according to El-Awady et al. 2000). The amplification products were detected by gel electrophoresis using 3% agarose gel in 1 × tris-borate-EDTA buffer. DNA bands were visualised using 0.5% ethidium bromide in the presence of ultraviolet light.

**Results**

Successful identification of *Toxoplasma* B1 gene was observed in blood samples of 27 cases, suggesting acute *Toxoplasma* infection. None of these cases had apparent symptoms of toxoplasmosis during their pregnancy. These 27 pregnant women were considered to be a high-risk group (abnormal pregnancy outcomes and/or exposure to sources of *Toxoplasma* infection as detected by PCR). All of them received the recommended dose of Spiramycin. Eight of them did not comply with the regular follow-up schedule. Therefore, the study was continued on the 19 pregnant women with regular follow-up visits, to full term. Clinical and fetal sonographic examinations were done for all of them in each visit and no abnormalities were noticed. Out of 19 patients included in this study, seven (36.8%) had a history of repeated abortions, five of them were seronegative in their previous pregnancy. Nine cases (47.36%) had detectable specific anti-*T. gondii* IgM and/or IgG antibodies. From all cases, 10 had a strong history of close contact with cats. Table I represents the results of PCR before and after treatment. The achievement of the treatment regimen was evaluated according to the results of PCR amplification of *T. gondii* DNA at the end of the treatment course. Cases who continued to have positive PCR results were given another treatment course. After treatment with a single course of Spiramycin, 11 (57.9%) patients retained *T. gondii* DNA in their peripheral blood and in eight (42.1%) patients, *T. gondii* DNA was absent by PCR, four (21.01%) patients received up to three courses of treatment and seven (36.8%) cases received two treatment courses. Normal fetal sonographic features were observed for all the cases in each visit until the time of labour.

<table>
<thead>
<tr>
<th>PCR results</th>
<th>Positive</th>
<th>Negative</th>
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<tbody>
<tr>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>Before treatment</td>
<td>19</td>
<td>100</td>
</tr>
<tr>
<td>After treatment</td>
<td></td>
<td></td>
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<tr>
<td>First dose</td>
<td>11</td>
<td>57.9</td>
</tr>
<tr>
<td>Second dose</td>
<td>4</td>
<td>21.01</td>
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<tr>
<td>Third dose</td>
<td>0</td>
<td>4</td>
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</tbody>
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**Discussion**

A screening programme targeting pregnant women in their first trimester would ideally detect only very recent infection. However, screening for toxoplasmosis is made difficult by the unusual persistence of the IgM antibody response. Therefore, it seems difficult to distinguish recent and past infections, and it is better to detect the pathogen itself (Morris and Croxson 2004). PCR has been used to detect *Toxoplasma* gene in different body fluid (El-Awady et al. 2000). The most common use of PCR in toxoplasmosis, is for prenatal diagnosis of the congenital infection using amniotic fluid, which necessitates the performance of amniocentesis. The latter does, however, pose a risk to the pregnancy and requires considerable medical resources (Romand et al. 2001). Moreover, such a procedure is refused by most of our patients. On the other hand, it has been reported that PCR of the amniotic fluid is of limited diagnostic value if done before 18 weeks’ gestation (Guy et al. 1996; Remington et al. 2004). 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. Infections with this parasite were suspected, all of them received the treatment, but only 19 of them completed the follow-up schedule to full term. In this study, the sensitivity of PCR detection of *T. gondii* DNA was high enough to detect approximately the DNA of one trophozoite depending on four primers that target the B1 gene which contains 30–35 copies of repetitive sequences in every *T. gondii* trophozoite and this coincides with the results of El-Awady et al. (2000). The clearance time for *Toxoplasma* DNA from the blood of patients with acute *Toxoplasmosis* was estimated to be 5.5–13 weeks (Nimiri et al. 2004). Based on this, the presence of *Toxoplasma* DNA in the maternal blood most probably indicates a recent infection, or an indicator of apparent parasitaemia, which is likely to be clinically significant (Nimiri et al. 2004). This suggests that the 19 positive cases by PCR are recent, acute infections (recent primary or remote infection resulting from activation of an old lesion or even from repeated infection). The cause of the previous abortions in five out of seven of these women was not clear, and it cannot be directly related to previous maternal *Toxoplasma* infection, since these women were seronegative in their previous pregnancy.

In the present study, administration of only one course of Spiramycin helped eradicate *T. gondii* infection in eight (42.1%) out of 19 patients who had *Toxoplasma* DNA in their peripheral blood before treatment. A total of 11 patients (57.9%) retained *T. gondii* DNA in their peripheral blood. Seven (36.8%) cases received two courses, while four (21.01%) patients received three courses of treatment.
Clearance of maternal blood of Toxoplasma B1 gene and normal sonographic pictures were good signs, suggesting normal outcomes. The variability regarding treatment responses in the present work could be explained by individual differences in several pharmacokinetic parameters: intestinal absorption, tissue distribution, cellular uptake, metabolism, transfer across the placenta and maternal drug elimination. The heterogeneity of the data could also be related to differences in patient compliance with the medication prescribed (Gratzl et al. 2002). On the other hand, DNA stability represents a possible source of false-positive results since PCR can amplify DNA from dead organisms. After successful treatment, the genetic component of the organism in some cases, is not immediately destroyed. In such a situation, a false PCR-positive finding in the absence of a viable parasite cannot be excluded. For this reason, PCR-positive findings are inferred to be an indicator of apparent parasitaemia only. Therefore, amplification of more labile RNA is confirmatory (Vaneechoutte and Van Eldere 1997).

On the other hand, in a study to seek evidence from randomised controlled trials on the effects of treatments on women who showed signs of toxoplasmosis infection during pregnancy, Peyron et al. (1999) prepared a report after reviewing 3,332 related papers. No randomised controlled trials were identified. Therefore, there is no sound evidence on which to base screening and treatment programmes. The previous authors concluded that screening is expensive and it is important to evaluate the effects of treatment and the impact of screening programmes in countries where screening or treatment is not routine.

Relatively high numbers of positive Toxoplasma infection among Saudi pregnant women, previously reported by El-Hady (1991) and Ghazi et al. (2002), may be attributed to the fact that in Saudi Arabia, consumption of lamb is greater than that of beef. These animals are usually reared outdoors, which put them at greater risk of environmental exposure than animals reared indoors, such as beef or poultry, thus increasing their risk of acquiring T. gondii infection (Cook et al. 2000). Unfortunately, until now, the data about toxoplasmosis are not clear. First, the incidence of congenital toxoplasmosis is not well known, so the risks/benefits of a screening programme cannot be assessed. Second, there are not enough data on the benefits of treatment during pregnancy. Third, current serological tests cannot distinguish between recently acquired and remote infections (Morris and Croxson 2004).

In conclusion, it is recommended that reliance be on molecular, rather than serological methods in confirming and post-treatment monitoring of acute T. gondii infection. Nested PCR using whole blood is a rapid, sensitive and specific molecular diagnostic procedure and of value in the diagnosis and post treatment assessment of T. gondii infection in adult females before and during pregnancy. Further studies are recommended to differentiate between live and dead trophozoites either by culture, mice inoculation or detection of T. gondii RNA.

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References