REAL TIME -PCR FOR QUANTITATIVE DETECTION OF TOXOPLASMA GONDII
IN ABORTED WOMEN BEFORE AND AFTER TREATMENT

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Abstract:

Background: Human toxoplasmosis is a parasitic disease caused by Toxoplasma gondii. Toxoplasma gondii was the subject of disease burden estimations as a result of the severe clinical symptoms and lifelong implications. Primary infections with T. gondii acquired during pregnancy are usually asymptomatic for the pregnant woman but can lead to serious neonatal complications, isolation of parasite despite it is considered as a golden standard parameter to detect infection, the repetitive B1 gen is the most target gene used in conventional PCR for molecular detection of T. gondii.

Objective: Detection of T. gondii DNA using Real-time PCR seems to be a reliable method in diagnosis and follow up of patients.

Methods: Thirty patients (aborted women), their range age between (20– 46) years, were included in this study. All patients were assessment of Real time Polymerase chain reaction from the same patients was taken before and after spiramycin therapy to confirm the infection of T. gondii and to evaluate the parasite load (copy number).

Result: Data of this study revealed that there is significant difference (P=0.002) in the mean of the copy numbers in aborted women's, the mean of parasite copy number in patients samples before treatment was (4.24E+06) and the mean of copy number after treatment was (7.53E+05).

Conclusion: The diagnosis of toxoplasmosis is mainly based on serological tests detection of T. gondii DNA using Real time- PCR seems to be a reliable method in diagnosis and follow up of patients.

Keywords: Toxoplasma gondii, Real-time PCR, B1-gen

Introduction:

Toxoplasmosis is a worldwide infection that may cause severe disease and is regarded as a serious health problem in world. It is one of the well-studied parasites because of its medical and veterinary importance, and its suitability as a model for cell biology and molecular studies with a unicellular organism. Toxoplasmosis is also a major opportunistic infection in immunocompromised individuals, often resulting in lethal toxoplastic encephalitis. If a woman was infected for the first time during pregnancy the parasite may be transmitted transplacentally to the fetus, this can result in death of the fetus, central nervous system abnormalities, or eye disease and affecting the quality of life of the child throughout its life time. 
Toxoplasma gondii was the subject of disease burden estimations. As a result of the severe clinical symptoms and lifelong implications, the disease burden of T.gondii is high. Primary infections with T.gondii acquired during pregnancy are usually asymptomatic for the pregnant woman but can lead to serious neonatal complications.

Toxoplasma infection stimulates both humoral immune response characterized by antibody production of (IgM and IgG) and cell mediated immunity (CMI) which are essential for the host control of intracellular infections, so the protection against Toxoplasmosis is mediated by cellular defense.

The seroprevalence varies from 5% to 90% depending on geographical location, age, habit of eating raw meat or unwashed fruit and vegetables, and general level of hygiene, the incidence of infections is higher in warmer and humid climate and increases with age.

Serological tests including the detection (and quantification) of T.gondii antibodies in serum are used to establish whether a pregnant woman has been infected and, if so, to determine whether the infection was acquired recently or in the distant past. If serological test results suggest a recently acquired infection, an effort is made to determine whether the infection was likely acquired during gestation or shortly before conception. If so, the fetus is at risk.

In Iraq, many studies were accomplished concerning the sero prevalence of Toxoplasmosis by using different techniques including indirect haemagglutination test (IHA), Indirect Fluorescent Antibody Technique (IFAT), and Enzyme-linked Immunosorbent Assay (ELISA). Real time-PCR can be used as an additional diagnostic tool for the rapid detection of T.gondii in various clinical materials.

The use of the macrolide antibiotic Spiramycin has been reported to decrease the frequency of vertical transmission. However, carefully designed; prospective studies that demonstrate this effect have not been recorded. The protection has been reported to be more distinct in women infected during their first trimester.

Material and methods:

Thirty women with spontaneous miscarriage were included in the study all have had curettage operation at the Obstetrics and Gynecology Department of Al-Kadhimyia Teaching in Baghdad and private clinic during the period from November 2012 to April 2013. Their age ranged from (20-45) years old. Ten healthy pregnant women with a history of a normal pregnancy attending the outpatient clinics for routine gynecologic checking enrolled in this study as control group, their age were ranging between (21-39) years old. From each patient two blood samples were collected pre and post treatment with oral spiramycin, 4 capsules (at 500mg) two times a day, three ml. were placed in EDTA tubes for DNA extraction, and all samples were stored at -20 °C DNA was extracted from the whole blood samples using a commercial purification system (ExiPrepTM DNA/ RNA Prep Kit).
The DNA concentration was determined by using the spectrophotometer (Bioneer), 10 μL of each DNA sample were added to 990 μL of D.W. and mixed well. The spectrophotometer was used to measure the optical density (O.D) at wave length of 260 nm and 280 nm. An O.D of one corresponds to approximately 50 μg / ml for double stranded DNA. The concentration of DNA was calculated according to the following formula: DNA concentration (μg/ml) = O.D 260 nm x 50 x Dilution factor

The spectrophotometer was used also to estimate the DNA purity ratio according to this following formula: DNA purity ratio = O.D 260 nm / O.D 280 nm.

Amplification of B1-gene by Real-Time Polymerase chain reaction

Principle

TaqMan probes are hydrolysis probes that are designed to increase the specificity of quantitative PCR. The principle of this technique relies on the 5´–3´ exonuclease activity of Taq polymerase to cleave a dual-labeled probe during hybridization to the complementary target sequence and fluorophores-based detection. 

Toxoplasma gondii Real-TM PCR kit uses “hot-start”, (Anatolia geneworks- Turkey) which greatly reduces the frequency of nonspecifically primed reactions. The forward and reverse sequences were (GCATTCCCGTCCAAACT and AGACTGTACGGAATGGAGACGAA) respectively.

The control positive DNA copy number was determined as follows: copy number (copies /ml) = (C x Avogadro constant) / (660 x control positive DNA in bp 10^9) Where C (copy) is given in ng/ml, Avogadro constant (N_A) = 6.022 140 857(74) ×10^23 /mole.

Preparing the PCR:

Real-time PCR assays were carried out in duplicate and performed following manufactured protocol in a total volume of (41μl) for one reactions as follow PCR Mix (20μl), (1.6μl) from detection Mix 1 which contains forward and reverse primers and dual-labeled probe, (1.2μl) from detection mix 2 which contains internal control-specific forward and reverse primers and dual-labeled probe, Internal control (0.2μl). Twenty three microliter of this master mix was added in the PCR tube with 18μl of DNA (sample, positive or negative control). Tubes were closed well and spin, The PCRs were performed with the Agilent Real-time PCR (Techne-UK). After initial activation of hotStar Taq DNA polymerase at 94°C for 2 min, 40 PCR cycles of 93°C for 10 s, 60°C for 10 s and 72 for 30s were performed.

Calculation of results:

Data were analyzed by smart-cycler software using arithmetic baseline adjustment, T.gondii DNA copy number was estimated from the cross point threshold relative to positive
The standard curve correlates each copy number with a particular Ct, the copy number value of the unknown samples are driven from the standard curve (According to this study).

Statistical Analysis:
Experimental data were presented in terms of observed numbers and percentage frequencies, and then analyzed by using Chi-square ($\chi^2$) test. P value $\leq 0.05$ was considered statistically significant.

Results:
The detection of B1 gene of *T. gondii* in whole blood specimens from aborted women's and control group by Real-time PCR:
In this study, the absolute quantification method was used for data analysis to determine the actual copy number before and after treatment, the standard curve was generated, in which concentration is fixed to a specific Ct value as the Ct value is inversely related to the amount of starting template.

Standard curve generation:
A standard curve was generated using a 10-fold dilution of a template amplified on the Cycler - real-time system (Figure 1), the copy number and CT value correlation was 0.9987.

![Standard curve](image)

**Figure (1):** Standard curve for quantitation of *T. gondii* tachyzoites. (Serial dilution ranging from (100– 108) copies used as template for real time PCR analysis)
Parasite load in abortive women’s as determined by Real-time PCR:

The range of *T. gondii* load in abortive women’s under study before and after treatment with oral spiramycin, 4 capsules (at 500mg) two times a day in table 1 which is found to be (0.0192X10^8 – 0.110X10^4) before treatment and (0.156X10^7 – 0.036X10^6) after treatment, (P value=0.002). Data of this study revealed that there is significant difference (P=0.002) in the mean of the copy numbers in aborted women’s, results in our study revealed that the mean of parasite copy number in patients samples before treatment was (4.24E+06) and the mean of copy number after treatment was (7.53E+05) figure(2)

**Figure (2):** Mean of copy number parasite /ml of patients’ blood samples before and after treatment
Table (1): *Toxoplasma gondii* copy numbers screening in abortive women's as determined by Real- time PCR

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>CT-pre treatment</th>
<th>Copy no. pre treatment</th>
<th>CT-post treatment</th>
<th>Copy no. post Treatment</th>
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<tr>
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<tr>
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<td>32.9</td>
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<td>Negative control</td>
<td>NTC</td>
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</table>
Regarding response to treatments toxoplasmosis infected women in this study were divided to the following groups:

**Group I** include patients had high parasite load \((0.062 \times 10^5 - 0.112 \times 10^7)\) which decrease completely after completion the course of treatment \((0.036 \times 10^0 - 0.223 \times 10^0)\).

**Group II** include patients with high parasite load \((0.225 \times 10^4 - 0.036 \times 10^7)\) at time of diagnosis and decreases after completion the course of treatment \((0.055 \times 10^1 - 0.223 \times 10^1)\).

**Group III** includes patients with high parasite load at diagnosis \((0.225 \times 10^4 - 0.036 \times 10^7)\) which still with high parasite load or slightly lower \((0.002 \times 10^3 - 0.201 \times 10^6)\).

**Group IV** includes patients with high parasite load \((0.158 \times 10^4 - 0.129 \times 10^5)\) at time of diagnosis which showed elevated parasite load after treatment \((0.014 \times 10^4 - 0.156 \times 10^5)\).

**Discussion:**

Firstly DNA was successfully extracted from whole blood samples for all subjects, Menotti, et al \(^1\) showed that the parasites are as likely to be found in the plasma or red cell fractions, which might indicate that the most suitable sample is a whole blood extract. However, in patient's blood *T. gondii* present might be intracellular in leukocytes \(^2\); in that case the white cell fraction would be as appropriate as whole blood.

Parasitemia in pregnant women may be due to the toxoplasmosis reactivations occur during immunosuppressive states in pregnancy. Tachyzoites or *T. gondii* DNA release from the cyst in the body tissues may be the source of parasitemia or *T. gondii* DNA in blood \(^3\). Quantitative detection of pathogen by real time PCR has been successfully applied to a variety of specimens and the finding of *T. gondii* DNA in peripheral blood by PCR might help in the early diagnosis of invasive diseases \(^4\).

The qRT-PCR that have been applied in this study to diagnose and for the determination of copy number of *T. gondii* DNA in whole blood, it gives reproducible diagnostic and quantitative results over conventional PCR with the risk of false positive due to contamination with previously amplified products since in qRT-PCR there is no risk of hand manipulation and the use of internal control will exclude the false negative which due to inhibitors in amplified mixture, reproducibility of qRT-PCR quantification was tested by the testing of the 10 fold dilution.

In the present study a qRT-PCR based B1 gene – specific Taq man assay have been developed for quantitative detection of *T. gondii* which extremely sensitive to \((0.168 \text{ parasite} / \text{reaction})\) and its ability to quantify infection load of clinical specimen and its long linear range of 7 logs of DNA concentration with CT value as shown in figure (1) where \(R^2=0.998\) which is indicative of a significant correlation between logarithmic value of each PCR product of copy number amplified and CT value. These results were in agreement with Mei-hui-lin, \(^5\). In this assay an increase of the fluorescent signal above the preset threshold within 40 PCR cycle was considered positive \((\text{CT} < 40)\), Table (1) where CT for negative control was 40. Quantification of
parasite load in pregnant women under study has been used to assess disease severity and the treatment outcome.

The relative quantity of tachyzoites in each DNA sample was determined using the standard curve presented in Figure (2) which showed a range between (0.110X10^4 to 0.0192 x10^8) before treatment and (0.036x10^0 to 0.156x10^7) after treatment. In addition, figure (2) revealed that there is significance difference (P<0.05) of in the mean of the copy number before and after treatment.

According to their response to treatments, Group 1 patients which represent 5(16.66%)% of patients having high parasite load at time of diagnosis with a range of (0.062x10^5-0.112X10^7) copies /ml, they show complete response to treatment with decline in parasite load to undetectable value, these results were in accordance with Costa, et al. who recorded that the parasite count decreased sharply in patients having cerebral toxoplasmosis symptoms, when treated with anti-toxoplasma therapy which was efficient and reducing the parasite load and they conclude that Light Cycler PCR provide precise evaluation of parasite load in immunocompromised patients and PCR is useful in monitoring of treatments.

In addition, Kupferschmidt, concluded that decreasing in parasite load in patient's blood sample demonstrated that treatment used was effective. Contini, et al. recorded that patient under specific treatment did not show detectable B1 DNA levels in samples collected after many days of therapy. So that T.gondii DNA load is a valuable tool for monitoring of toxoplasmosis.

Group II patients which represent 10(33.33 %) of patients having high parasite load at time of diagnosis with a range of (0.225x10^4-0.036x10^7) copies /ml, they show lower parasite load with a range of (0.055x10^1-0.223x10^1) copies /ml after treatment. These results observed by Menotti, et al. They consider that prolonged persistent detection of T. gondii DNA in blood justifies the maintenance of anti T.gondii treatment and close follow up by PCR. These results were in agreement with Romand, et al. who used quantitative Real time PCR to evaluate T. gondii concentration in amniotic fluid and conclude that quantitative PCR may help the clinician to form prognosis of congenital infections with regard to fetal and neonatal outcome. Other study by Christianne, et al. reported that high parasite load in amniotic fluid was found in cases of severe congenital toxoplasmosis as determined by quantitative PCR.

Group III which represent 11(36.66 %) include patients with high parasite load at time of diagnosis with a range of (0.038x10^7-0.019x10^8) copies/ml, they did not show any response to treatment, parasite load was remained with undetectable decrease. After treatment it's found to be (0.002x10^3 – 0.201 x10^6) copies/ ml. These results were in agreement with Kupferschmidt, et al who used Tag man PCR to monitor parasite load in the blood of an immunocompromised patients after allogeneic bone marrow transplantation. They found that at day 199 after therapy with pyrimethamine the detected parasite load remained unchanged and then decreased after prolonged therapy.
Regarding group IV of patients which represent 4(13.33 %) having high parasite load at time of diagnosis with a range of (0.158x10^4 – 0.129x10^5) copies /ml , they showed elevated parasite load after treatment with a range of (0.014x10^4 -0.156x10^7) copies /ml .This increase of parasite load seems to be correlated to progression of disease or to poor response to treatment. These results were in agreement with Menotti, et al\textsuperscript{12} who recorded evolution in parasite load in patients under treatments and those with slow decrease in parasite load after initiation of treatments with pyrimethamine and sulfadiazine on day 43 post transplantation. They give explanation to the prolonged presence of \textit{T.gondii} DNA that could be due to disseminated form of disease with a peak of parasitemia (29000 tachyzoites/ml) of blood buffy coat.

Real time-PCR can be used as an additional diagnostic tool for the rapid detection of \textit{T.gondii} in various clinical materials. Studies have documented that Real time- PCR can actually detect \textit{T.gondii} in blood specimens of women before or during pregnancy. Nimri, et al\textsuperscript{22} and Khan et al\textsuperscript{23} showed that a small numbers of parasites may be detected in circulating blood from some patients with acute or recurrent toxoplasmosis.

Several studies have reported that, PCR could detect parasitemia a few weeks prior to the appearance of any clinical signs or symptoms .PCR is highly sensitive and specific because a single tachyzoite can be detected in a clinical sample\textsuperscript{24}.

The most important records in this study is that the parasite load could be determined and correlated with treatment. The parasite quantification in some treated subjects become lower and in others, \textit{T.gondii} load disappeared when spiramycin was given, this exhibit that the anti-toxoplasma therapy was efficient in reducing the parasite load.

The quantitative real-time PCR assay developed in this study can be used not only to detect the presence of \textit{T.gondii} DNA but also to provide precise evaluations of the parasite load in pregnant women’s. This PCR test should be useful for the monitoring of treatment efficacy and should help provide an understanding of the pathogenesis of \textit{T.gondii} reactivation.

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