Comparison between *Toxoplasma gondii* DNA and specific immunoglobulins during pregnancy

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المقارنة بين دنا المقوسات القندية وبين الغلوبلينات المناعية النوعية أثناء الحمل

مصطفى كامل العوضي ولمياء أحمد الحسيني وسمية إسماعيل ومحمد طلعت عبد العزيز ومها عادل الدملاوي

خلاصة: إن التشخيص المبكر لداء المقوسات في السيدات الحوامل يمكن أن يكون عظيم النفع في التدخل المبكر والوقاية من الاضطرابات الخِلقية التي تودي عادة إلى موت الأجنة. وكان الغرض من هذه الدراسة تقييم عملية تضخيم الجين B1 للمقوسة القندية بطريقة nested PCR، قبل وبعد المعالجة، وبالمقارنة بالمتابعة المصلية أثناء المعالجة. وتبيَّن أن نجاعة المعالجة على أساس اكتشاف دنا المقوسة القندية باختبار PCR كانت ذات دلالة إحصائية، بينما لم تكن كذلك عند استعمال الأضداد المكوَّنة من الغلوبلينات المناعية الميمية Igm والجيمية IgG النوعية ضد المقوسات. إن اكتشاف دنا المقوسة القندية باختبار PCR عند تطبيق هذه الطريقة على الدم الكامل هو إجراء تشخيصي سريع وحساس ونوعي، ويعتبر وسيلة عظيمة الأهمية لإثبات تشخيص عدوى المقوسة القندية في النساء قبل الحمل أو أثناءه.

ABSTRACT Early diagnosis of toxoplasmosis in pregnant women can be of great help in early intervention and prevention of congenital disorders that usually lead to fetal death. The purpose of the present study was to evaluate nested PCR amplification of the B1 gene of *Toxoplasma gondii* before and after treatment and in comparison to serological follow-up during treatment. The efficiency of treatment on the bases of PCR detection of *T. gondii* DNA was statistically significant, while it was insignificant when anti-toxoplasma specific IgM and IgG antibodies were used. PCR detection of *T. gondii* DNA when performed on whole blood is a rapid, sensitive and specific diagnostic procedure and is a valuable tool for establishing the diagnosis of *T. gondii* infection in women before or during pregnancy.

Comparaison entre l'ADN de *Toxoplasma gondii* et des immunoglobulines spécifiques pendant la grossesse

RESUME Le diagnostic précoce de la toxoplasmose chez la femme enceinte peut être d'une grande utilité pour l'intervention rapide et la prévention des troubles congénitaux qui entraînent habituellement la mort fœtale. La présente étude avait pour objectif d'évaluer l'amplification par PCR nichée du gène B1 de *Toxoplasma gondii* avant et après le traitement et par comparaison avec le suivi sérologique pendant le traitement. L'efficacité du traitement sur la base de la détection par PCR de l'ADN de *T. gondii* était statistiquement significative, alors qu'elle n'était pas significative en cas d'utilisation des IgM et IgG antitoxoplasmiques spécifiques. La détection par PCR de l'ADN de *T. gondii* lorsqu'elle est réalisée sur du sang total est une procédure diagnostique rapide, sensible et spécifique et elle est considérée comme un instrument précieux pour poser le diagnostic de l'infection à *T. gondii* chez les femmes avant ou pendant la grossesse.

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Introduction

Toxoplasmosis is caused by infection with *Toxoplasma gondii*, a single-cell protozoan that belongs to the family Coccidia. *T. gondii* is an obligatory intracellular protozoan with a heterogeneous life cycle in humans and other vertebrates [*1*]. *T. gondii* infection is found in 30%–50% of the human population worldwide, most of the infected people being clinically asymptomatic [*2*]. Infection with this protozoan usually occurs by ingestion of food or water contaminated with its oocytes. Infection with *T. gondii* is a major cause of fetal death since *T. gondii* can be transmitted to the fetus through the placenta (transplacental) from an infected mother or at vaginal delivery [*3*]. Furthermore, toxoplasmosis has been implicated in abortion, prematurity, stillbirth and early postnatal mortality [*4*]. *T. gondii* can also cause serious damage to different tissue organs of its infected host depending on the site where it forms its cyst [*2*].

Early diagnosis of toxoplasmosis in pregnant women allows early intervention and prevention of congenital disorders that usually lead to fetal death. The Sabin–Feldman dye test [5], direct haemagglutination test [*6*], enzyme-linked immunosorbent assay (ELISA) [*7*] and indirect fluorescent antibody test [*2*] are the most common serological and histological assays for detection of toxoplasma infection. Histological diagnosis of toxoplasmosis is carried out by staining the infective stage in tissue biopsy using antibodies raised in mice or rabbits against toxoplasma surface antigen [*8*]. Apart from the difficulties in obtaining suitable biopsy material, histological investigations fail to give informative data on the stage of infection and the effect of the treatment administered.

Different assays have been developed to detect anti-toxoplasma antibodies (IgM, IgG, IgA and IgE) in the sera of pregnant women with a history of repeated abortion, and women suspected of being infected with *T. gondii* [*9*]. Serological tests based on the detection of IgM antibodies cannot differentiate between chronic and acute infection [*10*]. Serodiagnosis of congenital infection depending on the presence of IgG antibodies in the sera of suspected fetuses is not helpful as it is difficult to differentiate between fetal IgG antibodies and maternal IgG antibodies that have migrated across the placenta [*11*]. Toxoplasma-specific IgA and IgE antibodies in sera of pregnant women have also been assayed with ELISA to detect congenital infection. This has proved to be more sensitive than IgM detection during acute infection, but it has not been used by enough laboratories to determine its usefulness in the diagnosis of acute and/or chronic congenital toxoplasmosis [*12*]. Another approach to obtain an accurate serological diagnosis of *T. gondii* infection has been to employ combined serological tests, where IgM, IgG and/or IgA anti-toxoplasma antibodies are used for screening of *T. gondii* acute infection. Paul reported that avidity of anti-toxoplasma IgG antibodies was a useful marker to differentiate between chronic infection and recently acquired infection [*13*].

Serological tests to follow up treatment of toxoplasmosis are not sufficient to evaluate the efficacy of the treatment administered. It is desirable to detect the pathogen directly [*14*]. Recently, accurate detection of *T. gondii* in the peripheral blood of infected individuals has been shown to be possible with amplification of a sequence within the 35-fold repetitive B1 gene of *T. gondii* using polymerase chain reaction (PCR) [*15*]. It has also been shown that PCR for the detection of toxoplasma DNA in the amniotic fluid is a useful tool for identification or exclusion of fetal toxoplasmosis [*16*].

In our study, we used PCR to amplify DNA from the B1 gene to detect circulating *T. gondii* in blood samples obtained from pregnant women with a history of toxoplasmosis, positive specific anti-toxoplasma antibodies IgM and/or IgG and/or with a history of repeated abortion. The purpose was to evaluate PCR detection of *T. gondii* DNA before and after treatment and to compare this method with serological follow-up of treatment.

Patients and methods

Patients and samples

We examined 29 females (age range 20–35 years) with a history of toxoplasmosis, as detected by the presence of anti-toxoplasma specific IgM and/or IgG antibodies, and a history of repeated abortion. Other factors considered to be involved in repeated abortion were excluded. Serum and whole blood samples were collected before and after treatment with spiramycin, a specific anti-toxoplasma agent. The medication was given at a dose of 3 MIU 3 times daily for 21 days [*17*].

IgM and IgG detection

Specific IgM and IgG anti-toxoplasma antibodies were determined using capture ELISA technique (ETI-TIOXOK-M reverse, Sorin Biomedica, France) according to the manufacturer's instructions. Briefly, patient samples, blank, negative and positive controls were diluted with sample diluent and dispensed into their corresponding wells, followed by incubation for 1 hour at 37 °C. The wells were then washed carefully with the wash buffer. The antigen/tracer was dispensed into all wells except the blank well. Wells were incubated for 1 hour at 37 °C, followed by proper washing. Then 100 μL of chromogen substrate were added to each well and incubated at room temperature for 30 minutes. The reaction was stopped by the addition of the blocking reagent. The optical density (absorbance) was determined at 450 nm 2 hours after the addition of the blocking buffer. The presence or absence of the anti-toxoplasma IgM or IgG antibodies was determined by relating the absorbance value of the unknown samples to that of the cut-off control values.

Polymerase chain reaction

A simple procedure for the isolation of DNA by microwave oven was used. The procedure is rapid and can be completed in as little as 15 minutes. The method has been used to isolate DNA of hepatitis B virus from serum samples where the DNA was reproducibly used for PCR amplification [*18*]. We used the microwave oven to extract DNA of *T. gondii* from whole blood. Thus, 100 μL of whole blood collected on EDTA in a 1.5 mL tube were exposed to the maximum heating power of the microwave for 5 minutes. Then 50 μL sterile water were added to the treated blood and mixed vigorously for 5 minutes, followed by centrifugation for 10 minutes at 2000 rpm. In the PCR reaction 25 μL of the supernatant were used. DNA extracted from *T. gondii* (P-strain) collected from the peritoneal lavage of infected mice was used as positive control. This positive control DNA was extracted by phenol/chloroform extraction methods (P-strain was kindly provided by the Department of Parasitology, Research Institute of Ophthalmology, Cairo). The number of *T. gondii* tachyzoites in suspension was counted on a haemocytometer prior to DNA extraction to evaluate the sensitivity of the test according to the number of tachyzoites.

Nested primer sets were used for amplifying fragments of the B1 gene. The outer primers are from bases 171 to 190 (5′-

CCG TTG GTT CCG CCT CCT TC-3′) and from bases 602 to 583 (5′-GCA AAA CAG CGG CAG CGT CT-3′) producing an amplified product of 432 bp. Inner primers are from bases 180 to 196 (5′-CCG CCT CCT TCG TCC GTC GT-3′) and from bases 392 to 372 (5′-GTG GGG GCG GAC CTC TCT TG-3′) producing an amplified product of 213 bp [*19*]. The first 50 μL PCR reaction mixture contained outer primers at a final concentration of 50 pmol each, 20 mmol/L dNTPs and 1.25 U recombinant taq DNA polymerase in 1 × PCR reaction buffer (50 mmol/L KCl and 10 mmol/L tris-HCl, 1.5 mmol/L Mg_2Cl, 0.1% triton × 100) (DynAzyme™). PCR amplification was performed for 2 minutes at 94 °C for one cycle, followed by 30 cycles using denaturation at 94 °C for 1 minute, annealing for 2 minutes at 57 °C and extension for 3 minutes at 72 °C. The nested PCR reaction was performed using 5 μL of the first PCR reaction in a mixture containing the inner primers at final concentration of 50 pmol each, 20 mmol/L dNTPs, 1.25 U recombinant taq DNA polymerase in 1 × PCR reaction buffer. Amplification was carried out at 94 °C for 2 minutes (one cycle), then followed by 35 cycles each for denaturation at 94 °C for 1 minute, annealing at 58 °C for 1 minute and extension at 72 °C for 1 minute. The run was terminated with a final extension at 72 °C for 10 minutes. The amplification products were detected by gel electrophoresis using 3% agarose gel in 1 × tris-borate-EDTA buffer. DNA bands were visualized using 0.5% ethidium bromide in the presence of ultraviolet light.

Results

Out of 29 patients, 27 had a history of repeated abortion and detectable specific anti-*T. gondii* IgM and/or IgG antibodies. The remaining two patients did not have a history of abortion; one had toxoplasma DNA as detected by PCR, and the other had specific anti-*T. gondii* IgG antibodies only. In all, 22 patients became pregnant during the treatment. The efficacy 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; 14 patients received up to four courses of treatment. Detection of the specific anti-toxoplasma IgM and IgG antibodies was used during the follow-up of patient treatment. All the 22 pregnant patients completed their pregnancies to full term.

Before treatment, 28 (96.6%) out of 29 patients had detectable *T. gondii* DNA in their peripheral blood. After treatment with a single course of spiramycin, 22 (75.0%) patients retained *T. gondii* DNA in their peripheral blood and in 6 (20.7%) patients, *T. gondii* DNA was absent by PCR (Table 1).

Regarding IgG titre (Table 2), 10 out of 27 patients with positive anti-toxoplasma IgG antibodies had increasing titre at the end of the treatment, while the titre decreased in 13 patients and was unchanged in 4 patients.

Comparison between toxoplasma DNA and anti-toxoplasma IgG antibody in monitoring the response to medication showed that 10 of the 22 patients who retained *T. gondii* DNA post-treatment had increasing titres of IgG specific anti-toxoplasma antibodies confirming their unresponsiveness to therapy. The remaining 12 patients showed either decreased or unchanged titres of IgG specific anti-toxoplasma antibodies (false responders). Of the 6 patients in whom *T. gondii* DNA disappeared from peripheral blood, 2 had decreased titres of

anti-toxoplasma IgG antibodies after treatment (true responders). The 4 remaining patients had either increased or unchanged titres of anti-toxoplasma IgG antibodies after treatment indicating the presence of a long-standing humoral response (Table 3).

Table 1 **Presence/absence of *Toxoplasma gondii* DNA in the 29 women before and after treatment**

DNA	Before		After	
	No.	%	No.	%
+ve	28	96.6	22	75.9
–ve	1	3.4	7	24.1

Table 2 **Presence/absence of IgG in the 29 women before and after treatment**

IgG	Before		After	
	No.	%	No.	%
+ve	27	93.1	25	86.2
–ve	2	6.9	4	13.8

Regarding toxoplasma DNA versus anti-toxoplasma IgM antibodies, specific anti-toxoplasma IgM antibodies were tested before treatment, and 9 out of 29 patients (31.0%) had detectable levels of IgM specific anti-toxoplasma antibodies in their sera and toxoplasma DNA in their peripheral blood. After treatment, all the 9 patients continued to have toxoplasma DNA. Of these, 5 patients retained detectable levels of IgM specific anti-toxoplasma antibodies in their sera. On the other hand, 5 patients out of the 20 patients with no detectable levels of IgM specific antibodies had detectable levels of IgM specific antibodies after treatment (Tables 4 and 5).

After treatment, 6 patients with no *T. gondii* DNA in peripheral blood had no IgM specific anti-toxoplasma antibodies in their sera. Two of 6 patients with no anti-toxoplasma IgM antibodies before treatment had detectable levels of IgM antibodies after treatment, although *T. gondii* DNA was not detected in their peripheral blood after treatment.

According to the number of patients who received therapy and completed the

Table 3 **Presence/absence of *Toxoplasma gondii* DNA and IgG in the 29 women before and after treatment**

Test	IgG				Total	
	+ve		–ve			
	No.	%	No.	%	No.	%
			Before treatment			
DNA						
+ve	26	92.9	2	7.1	28	100.0
–ve	1	100.0	0	0.0	1	100.0
Total	27	93.1	2	6.9	29	100.0
			After treatment			
DNA						
+ve	19	86.4	3	13.6	22	100.0
–ve	6	85.7	1	14.3	7	100.0
Total	25	86.2	4	13.8	29	100.0

pregnancy period to full term, efficacy of the treatment on the basis of PCR detection of *T. gondii* DNA was statistically significant ($P < 0.011$) (Fisher exact test). It was not statistically significant when anti-toxoplasma specific IgM and IgG antibodies were used to evaluate the efficacy of the treatment.

Discussion

It is crucial in prenatal screening to ascertain whether *T. gondii* infection was acquired before or after conception [12]. Primary congenital toxoplasmosis can lead to parasite transmission to the fetus via the placenta where, the risk of transmission increases during gestation [12]. One way to detect the infection is by testing seroconversion of anti-toxoplasma IgM antibody during pregnancy. Anti-toxoplasma IgM antibodies commonly persist well beyond 6 months, but positive results are very poorly predictive of infections acquired within the previous 2 to 3 months [20].

PCR has been consistently used to detect DNA of *T. gondii* in various biological samples and has shown high sensitivity (single tachyzoite) in the diagnosis [19]. PCR has been shown to be a potentially powerful diagnostic method compared with culture, which is insensitive and time-consuming [19]. The potential of PCR to diagnose active toxoplasmosis is of great help in immunocompromised patients, especially when serological techniques failed [21]. The sensitivity of PCR for purified *T. gondii* DNA has been found to be very high because the B1 gene contains 30–35 copies of repetitive sequences in every *T. gondii* trophozoite [15].

In our study, the sensitivity of PCR detection of *T. gondii* DNA was high enough to detect approximately the DNA of one

Table 4 **Presence/absence of IgM in the 29 women before and after treatment**

IgM	Before		After	
	No.	%	No.	%
+ve	9	31	10	34.5
–ve	20	69	19	65.5

Table 5 **Presence/absence of DNA and IgM in the 29 women before and after treatment**

Test	IgM				Total	
	+ve		–ve			
	No.	%	No.	%	No.	%
			Before treatment			
DNA						
+ve	9	32.1	19	67.9	28	100.0
–ve	0	0.0	1	100.0	1	100.0
Total	9	31.0	20	69.0	29	100.0
			After treatment			
DNA						
+ve	9	33.3	18	66.7	27	100.0
–ve	1	50.0	1	50.0	2	100.0
Total	10	34.5	19	65.5	29	100.0

trophozoite. However, *T. gondii* DNA was detected in only 28 patients (96.6%) out of 29 patients clinically diagnosed as having toxoplasmosis. Also anti-toxoplasma IgG antibody was detected in the sera of 27 patients (93.1%), while anti-toxoplasma IgM antibody was detected in the sera of only 9 patients (31.0%).

The presence of anti-toxoplasma IgM antibody in the chronic stage of infection has been reported, especially in pregnant women [*10*]. Therefore, confirmatory tests are needed either by additional tests or by demonstration of a significant rise in antibody titres in serial serum samples obtained at a minimum of 3-week intervals [*22*]. Moreover, specific IgG antibody titres do not constantly increase during reactivation of the disease, but a change in the titre or the presence of IgG is not helpful [*11*]. In the present study such a change was noticed in some patients, which increased the difficulty in defining the efficacy of the treatment.

One patient showed no evidence of infection by PCR, while anti-toxoplasma IgG antibody was detected. This can be attributed to the presence of a long-standing immunity to toxoplasmosis or a cross-reactive antibody. Since specific IgM antibodies can be detected in both the acute and chronic phases of toxoplasmosis, there is a need for confirmatory evidence in the case of acute infection [*23*], especially after treatment. At the same time anti-toxoplasma IgG antibodies have been reported to persist for a long time, up to years [*24*].

The beneficial effect of prenatal therapy is so convincing that it is considered unethical to withhold it in women who acquire *T. gondii* infection during pregnancy [*17*]. Spiramycin alone may prevent the parasitic colonization of the placenta in 60% of the cases; however, the drug has no effect on a fetus that is already infected. In such cases, the pyrimethamine/sulfonamide combination has been reported to be more effective [*17*]. Administration of only one course of spiramycin helped eradicate *T. gondii* infection in 6 of our patients who had toxoplasma DNA in their peripheral blood before treatment.

Evaluation of treatment efficacy during pregnancy is difficult because it depends on several factors: gestational stage at which the infection occurred, how soon the evaluation was performed after acquiring the infection, whether or not the parasites were transmitted to the fetus, and whether the fetus was affected by the infection. Furthermore, the drugs currently used only slow down the multiplication of parasites and are unable to eliminate toxoplasma cysts in tissues. Other complicating factors are related to the ability of the drugs to cross the placental barrier and to the possibility that they may have teratogenic or adverse effects [*17*].

The extent of damage from toxoplasmosis can be reduced by early treatment and thus rapid diagnosis is essential [*19*]. The diagnosis is routinely based on serological tests. In congenitally infected children and immunocompromised patients, and even in immunocompetent patients, however, serology is inadequate because antibody production either fails or is significantly delayed.

After infection, *T. gondii* DNA appears earlier than the immunological response. This was noticed in one of our patients where specific anti-toxoplasma IgM and IgG antibodies were not detected until after treatment. Also our study included 2 patients with *T. gondii* DNA, one of whom had and positive anti-toxoplasma IgG antibody only and the other who had no IgG antibody. The latter lacked IgG until after treatment which indicates that competence of the immune response may hamper our

ability for correct diagnosis and treatment follow-up.

The appearance of anti-toxoplasma IgM antibodies in the sera of 5 patients after treatment when they had been undetectable before treatment indicates a delayed immune response. This may be due to reinfection or reactivation of a latent cyst. On the other hand, the absence of anti-toxoplasma IgM antibodies after treatment despite persistent positive PCR results may be because although the treatment was effective, circulating remnants of *T. gondii* DNA remain, and there has been a complete isoshift from IgM to IgG. On the other hand, DNA stability represents another possible source of false-positive results since PCR can amplify DNA from dead organisms, e.g. after successful treatment. In the presence of a well developed humoral immune response, circulating parasites are rapidly attenuated or killed, but the genetic component of the cells is not immediately destroyed. In such a situation, a false PCR-positive finding in the absence of viable parasite cannot be excluded. For this reason, PCR-positive findings are inferred to be an indicator of apparent parasitaemia only. In such cases, amplification of more labile RNA is confirmatory [*25*].

When we compared the positive PCR results with rising titre of anti-toxoplasma IgG antibodies during the course of treatment with spiramycin, we found that PCR remained positive in 22 patients out of 28, while the anti-toxoplasma IgG antibody titre was rising only in 10 patients. Based on PCR data, treatment courses were continued in the 22 PCR-positive patients. On the other hand, when relying on rising IgG titre, treatment would be continued in only 10 patients.

In conclusion, nested PCR amplification of the B1 gene of *T. gondii* using whole blood is a rapid, sensitive and specific diagnostic procedure and considered a valuable tool for establishing the diagnosis of *T. gondii* infection in adult females before or during pregnancy. Diagnosis of *T. gondii* infection during pregnancy is very important. Serological immune profile is heterogeneous, and may be delayed or fail to be represented. This makes it an unreliable method for diagnosis and/or treatment follow-up.

Thus, it is advisable to rely on PCR of *T. gondii* DNA for diagnosis and monitoring of infection during treatment. Further studies are recommended to differentiate between live and dead trophozoites either by culture, mice inoculation or detection of *T. gondii* RNA. At the same time, quantitation of *T. gondii* trophozoites, probably by competitive PCR, is of great help to estimate effective strategies of treatment.

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