Open Access Macedonian Journal of Medical Sciences. 2014 Sep 15; 2(3):415-420.
http://dx.doi.org/10.3889/oamjms.2014.071
Basic Science

Molecular Diagnosis of Toxoplasmosis in Non Immune Pregnant Females

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Citation: Abdel Aal AA, Attia SS, Hanafy NA, Al-Antably AS, Hassan MA, El-Sherbiny W, Nasr AS. Molecular Diagnosis of Toxoplasmosis in Non Immune Pregnant Females. OA Maced J Med Sci. 2014 Sep 15; 2(3):415-420. http://dx.doi.org/10.3889/oamjms.2014.071

Key words: *Toxoplasma gondii*; PCR; Molecular diagnosis; non-immune; pregnancy.

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Received: 09-Jun-2014; **Revised:** 15-Jun-2014; **Accepted:** 03-Jul-2014; **Online first:** 10-Jul-2014

Competing Interests: The authors have declared that no competing interests exist.

Abstract

BACKGROUND AND AIM: Infection with the protozoan parasite *Toxoplasma gondii* has a worldwide distribution. Congenital infection is the most important part of the disease burden due to Toxoplasma infection in humans. Early diagnosis of maternal infection helps to prevent severe complications of toxoplasmosis. In the present study, three PCR assays (conventional, nested & quantitative) were evaluated for diagnosis of recent toxoplasmosis based on detection of Toxoplasma B1 gene.

MATERIAL AND METHODS: The present study was carried out on 150 pregnant females who were serologically negative for anti-Toxoplasma IgG and IgM antibodies.

RESULTS: The results revealed that out of 12 true positive cases (by 2 out of the 3 PCR protocols), 8 cases were positive by cPCR, 11 cases were positive by nPCR and 12 cases were positive by qPCR. Accurate estimation of genomic Toxoplasma DNA in positive samples was achieved by qPCR. In general, PCR assays offer a sensitive alternative of serological methods for diagnosis of recent maternal toxoplasmosis. In addition, qPCR decreases the risk of contamination of PCR products being a closed tube method and helps in estimation of infection load.

CONCLUSIONS: We recommend screening of high-risk pregnant women by qPCR for early diagnosis of toxoplasmosis and proper management.

Introduction

Toxoplasmosis caused by *Toxoplasma gondii* is one of the most prevalent parasitic diseases in human beings [1]. The majority of acquired transmissions to humans are caused either by the ingestion of tissue cysts in infected meat or by the ingestion of soil, water, or food contaminated with sporulated oocysts derived from the environment [2].

Congenital infection is the most important part of the disease burden due to Toxoplasma infection in humans. It occurs when pregnant woman primarily acquires Toxoplasma infection during pregnancy. *Toxoplasma tachyzoites* colonize placental tissues during the dissemination process and from there can gain access to the fetal compartment in about 30% of cases [3].

Toxoplasmic infection during pregnancy may lead to severe or even fatal infection of the fetus [4]. Early diagnosis of infection is extremely important to prevent the severe complications of congenital toxoplasmosis [5].

A practice bulletin from the American College of Obstetricians and Gynecologists on perinatal viral and parasitic infections recommends toxoplasmosis screening only in high-risk persons or those in whom routine ultrasound examination shows findings such as hydrocephalus, intracranial calcifications, microcephaly, fetal growth retardation, ascites, or

hepatosplenomegaly [6].

Many serological methods have been developed aiming at diagnosis of toxoplasmosis including indirect fluorescence antibody tests (IFATs) [7] to hemagglutination; enzyme-linked immunosorbent assays (ELISAs); capture ELISAs allowing the detection of specific isotypes IgM, IgA, or IgE; and immunosorbent agglutination assays (ISAGAs), also suitable for IgM, IgA, or IgE detection [2]. The conventional single serum assay does not make a clear distinction between an acute and a chronic infection [8]. The prevalence of high Toxoplama IgG antibody titers among normal individuals in most populations and the persistence of specific IgM antibodies in some persons for long periods cause another problem in estimating the timing of infection [9, 10].

An alternative method of identifying T. gondii by mouse inoculation or tissue culture of the clinical specimen may confirm the infection by parasites. However, this method usually requires several days to obtain results and is labor-intensive [11]. Thus, a more efficient method is needed to provide rapid and quantitative results for the diagnosis of recent T. gondii infection during pregnancy. PCR-based techniques have been developed for the detection of parasite DNA [12, 13] in blood samples [14] and amniotic fluid [4].

Several gene targets have been used over the years, with various performances in relation to the use of repeated or single target genes and the means of revealing amplification products [15, 16]. The recent advent of a real-time quantitative PCR (qPCR) technique is a recently introduced technique with promising value in many applications including pathogen detection in addition to accurate quantization [17, 18].

These techniques make use of the most conserved gene sequences among different strains of T. gondii [19], including the B1 gene repetitive sequence, the P30 (SAG1) gene, and ribosomal DNA. The use of the B1 gene for T. gondii detection originated with Burg et al. in 1989 that combined PCR amplification with Southern blotting to detect a specific B1 gene product [20].

The aim of the present study was to diagnose Toxoplasma infection in non-immune pregnant females and compare the technical performances of three PCR assays (conventional PCR, nested PCR and real time PCR) for the molecular diagnosis of toxoplasmosis using Toxoplasma B1 gene (35 fold repeats) as target DNA.

Materials and Methods

This comparative observational cross-sectional study was conducted at the Department of Obstetrics and Gynecology, Kasr El-Aini Hospital, Faculty of Medicine, Cairo University between November 2011 and October 2013.

The Research Ethics Committee approved the study protocol. The study population was recruited from women who booked within one week of their last menstrual period attending the antenatal care clinic for work-up. The study included 150 pregnant women non-immune to toxoplasmosis (pregnant women with positive tests for *T. gondii* antibody, together with those positive or equivocal for *T. gondii* IgM, were excluded from the present study. Informed consents were obtained from all candidates.

All cases were subjected to detailed history taking and complete clinical examination, obstetric ultrasound and blood sampling for serological and molecular assays.

Obstetrics ultrasound

All participants had an ultrasound scans between 6 and 11 weeks to confirm their gestational ages, also those proved to be toxoplasmosis positive by PCR had detailed anatomical scans every 4 weeks starting from their 20th week of gestation to detect any structural anomalies suggestive of congenital toxoplasmosis i.e. ventriculomegaly, intracranial calcifications, microphthalmia, microcephaly, non-immune hydrops, ascites, pleural effusion, pericardial effusion, hepatosplenomegaly and/or placentomegaly.

All scans were done by one operator using sonographic imaging system Toshiba (console model SSA-340A, Toshiba Medical Systems, Tokyo, Japan) equipped with 3.75 MHz Convex transducer.

Serological assay

Collected blood samples were divided in two portions. The first portion was added to EDTA tube to extract DNA. Sera were separated from the remaining part of blood samples and stored frozen at −20°C. Sera were tested for anti- Toxoplasma IgM & IgG antibodies using a commercial ELISA Kit (Cal biotech Inc., CA). The assay was carried out according to the manufacturer's instructions and positive cases were excluded from the study.

Molecular methods for detection of Toxoplasma gondii DNA

DNA extraction and the three PCR assays (conventional PCR, nested PCR and quantitative real time PCR) were performed in the present study in the Department of Clinical Pathology, Faculty of Medicine, Cairo University

DNA extraction

To minimize contamination DNA extraction from blood samples was performed on MagnaPure Compact Instrument for fully automated nucleic acid extraction (Roche Diagnostics GmbH) applying Magnetic beads technology. Final pellets were suspended in 30 µL of TE buffer and stored at –70°C until used.

The reaction conditions of both conventional and nested PCR assays were independently and thoroughly optimized in the presence of human DNA as described by El Awady et al 2000.

Conventional PCR (cPCR) for detection of T.gondii B1 gene

Conventional PCR method used a forward primer (5´-CCG TTG GTT CCG CCT CCT TC-3´) from bases 171 to 190 and a reverse primer (5´-GCA AAA CAG CGG CAG CGT CT-3´) from bases 602 to 583.

The optimized conditions for cPCR were the following: 0.8ul of each primer (50 pmol/ul), 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^{TM}] for a total reaction volume of 50ul including 5ul of sample DNA. The reaction mixtures were cycled in Q cycler Quanta Biotech thermal cycler by using the following conditions: 94 °C for 2 min, followed by 30 cycles of 94°C for 1 min, 57 °C for 2 min and 72 °C for 3 min.

Nested PCR (nPCR) for detection of T.gondii B1 gene

The nested PCR reaction was performed using 5 µl of the first PCR reaction product under identical conditions in a reaction mixture identical in composition to that of cPCR except that an inner pair of primers were added as follows; a forward primer (5´-CCG CCT CCT TCG TCC GTC GT-3´) from bases 180 to 196 and a reverse primer (5´-GTG GGG GCG GAC CTC TCT TG-3´) from bases 392 to 372. Inner primers were added at final concentration of 50 pmol each to a reaction mixture containing 20 mmol/L dNTPs, 1.25 U recombinant taq DNA polymerase in 1 × PCR reaction buffer. Amplification was done by the following conditions: 94°C for 2 min, followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min and 72°C for 1 min. The run was terminated with a final extension at 72°C for 10 minutes. For each sample, one internal positive control tube was included for detection of PCR inhibition. It consisted of purified parasite DNA equivalent to 0.8 Toxoplasma genome added to 5 ul of sample DNA. Two negative control tubes containing 5 ul of H_2O instead of DNA were included in each test to detect contaminations

The reaction products were visualized using 0.5% ethidium bromide under UV light after electrophoresis in a 3% agarose gel in 1 × tris-borate-EDTA buffer. The expected fragments sizes were 432bp for cPCR and 213bp for nPCR

The semi-quantitative/qualitative assay was used in this study for the estimation of the concentration and quality of DNA in a specimen. The concentration of a sample was determined by comparing the intensity of the fluorescence of the sample to that of the calibration standards (DNA marker) using a gel documentation system which is an U.V safe cabinet connected to computer with the specific soft ware that calculates automatically all DNA parameters according to the standard reference. The image analyzer system permits complete analysis starting from lane creation and band detection with the corresponding molecular weight to the semi quantitative analysis by nanogram

Quantitative real time PCR (qPCR) for detection of Toxoplasma B1 gene:

Quantitative real time PCR was performed with the LightCycler® fastStart DNA Master SYBR Green dye, using the LightCycler® 1.x/ 2.0/ 480 instrument and according to the instruction manual of Roche Diagnostic (Roche Diagnostics, Hoffmann-La Roche Ltd, USA). Primers from bases (5´-CCG TTG GTT CCG CCT CCT TC-3´) and (5´-GCA AAA CAG CGG CAG CGT CT-3´) were used to amplify Toxoplasma B1 gene of 35-fold repeats. The resulting PCR fragment of *T. gondii* was analyzed using the LightCycler® Red 640 (detected in channel 640). The supplied standard row was used to determine the linear range of the reaction and to estimate the quantity of the target sequence in unknown samples. Soft ware data analysis version 3.5.3 was applied as described in the LightCycler® instrument operator's manual. The reaction mixture (20 µl; Master SYBR Green kit; Roche Diagnostic) contained 0.5 µM of each primer, 5 mM $MgCl_2$ and 5 µl templete DNA. All capillaries were sealed, centrifuged at 500 g for 5s, and then amplified in a LightCycler instrument. Amplification was performed for 50 cycles of 95 °C for 5 s, 61 °C for 10 s and 72 °C for 15 s, with an overall ramp rate of 20 °C s. A single fluorescence reading for each sample was taken at the extension step. Quantitative results were expressed by determination of the detection threshold or the crossing point (C_T), which marked the cycle when the fluorescence of the given sample significantly exceeded the baseline signal. They were expressed as a fractional cycle number. Then, the crossing points (C_Ts) were plotted against the known parasite concentration to obtain a standard curve. The parasite count for a given sample was calculated by extrapolation from this standard curve. Positive sample specificity was confirmed by determining the melting curve with different values of melting temperatures (T_m) (95 °C, 4.40 °C/s ramp rate; 40°C, 2.20 °C/s ramp rate; 65°C, 4.40°C/s ramp

rate, 95°C, 0.02°C/s ramp rate continuous measurement).

Results

The present study was carried out on 150 pregnant females. A mid-trimester ultra-sound examination revealed no congenital anomalies suggestive of congenital toxoplasmosis. Sera collected from all cases were negative for anti Toxoplasma IgM & IgG. Serum samples were examined by cPCR & nPCR and qPCR aiming at detection of Toxoplasma B1 gene. A blood sample was considered positive for Toxoplasma if DNA was expressed from the sample by two of the three molecular assays performed. Samples were reported positive for Toxoplasma when the amplified fragments of PCR assays separated by gel electrophoresis were of the expected length (432 bp for cPCR and 213 bp for nPCR) as shown in Figure 1. Out of the 150 cases included in the present study, Toxoplasma B1 gene was expressed in 8 cases by cPCR and in 12 cases in nPCR as shown in Table 1. Regarding specificity of cPCR & nPCR assays, no contamination of the PCR was observed during the course of the study, as inferred from the consistent negativity of negative controls.

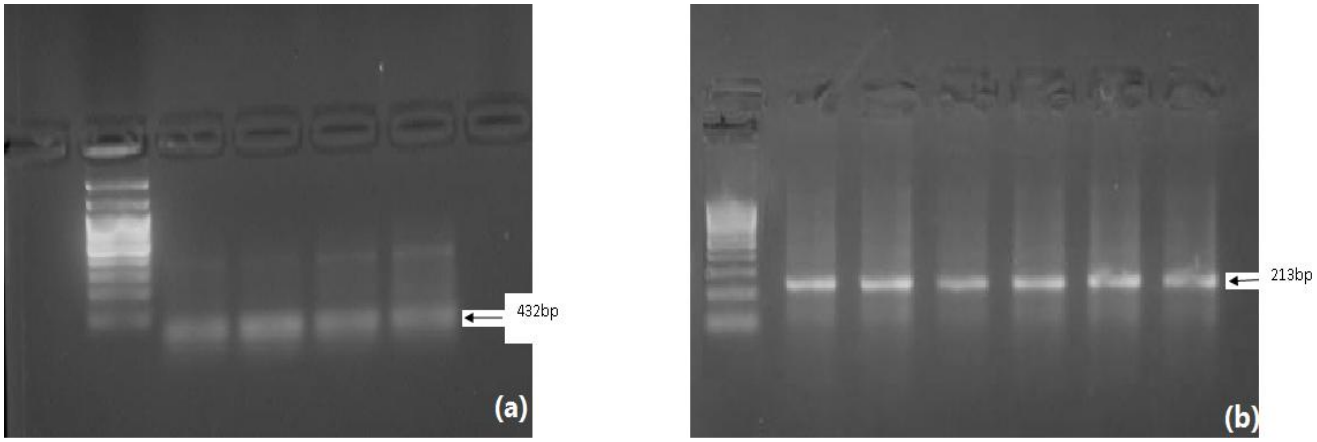

Figure 1: DNA fragments resulting from cPCR (a) and nPCR (b) fractionated on agarose gel followed by visualization with ethidium bromide staining.

Figure 1 DNA fragments resulting from cPCR (a) and nPCR (b) fractionated on agarose gel followed by visualization with ethidium bromide staining.

Table 1: Positive serum samples for *Toxoplasma B1 gene* by cPCR, nPCR & qPCR assays.

Sample number	cPCR	nPCR	qPCR
1	+ve	+ve (1.5X10^1)	+ve
2	-ve	+ve (1.5X10)	+ve
3	+ve	+ve (1.5X10^2)	+ve
4	+ve	-ve	+ve
5	-ve	+ve (1.5X10^2)	+ve
6	+ve	+ve (1.5X10^2)	+ve
7	-ve	+ve (1.5X10)	+ve
8	-ve	+ve (1.5X10^1)	-ve
9	+ve	+ve (1.5X10^2)	+ve
10	+ve	+ve (1.5X10^1)	+ve
11	-ve	+ve (1.5X10)	+ve
12	+ve	+ve (1.5X10^3)	+ve
13	+ve	+ve (1.5X10^2)	+ve
Total	8	12	12

Out of the 150 cases included in the present study, 12 cases were positive for Toxoplasma B1 gene by qPCR that revealed different concentrations and variable C_T (Table 2).

The relative quantity of Toxoplasma DNA in each blood sample was determined using the standard curve provided from Roche Diagnostics.

Quantitative PCR performed in this study showed high specificity as demonstrated by the melting curves applied on the positive samples.

In the present work, a blood sample was considered positive for Toxoplasma if DNA was expressed from the sample by two of the three molecular assays performed. As shown in table (2), a total of 12 true positive cases of toxoplasmosis were detected in the present study being positive by 2 PCR assay. Out of these 12 cases, 8 positive cases were detected by cPCR, 11 positive cases were detected by nPCR while qPCR detected 12 positive cases. Accordingly, the sensitivities of cPCR, nPCR and qPCR in detection of Toxoplasma DNA were 67%, 92% and 100% respectively.

Table 2: The C_Ts and the corresponding *Toxoplasma* quantity in positive samples by qPCR.

Sample number	C_T	qPCR
1	31.91	9.66E^3
2	22.13	3.95E^7
3	32.87	1.69E^3
4	23.48	1.28E^7
5	26.44	1.08E^6
6	24.99	3.63E^6
7	19.06	5.15E^8
8	30.20	1.83E^4
9	32.94	1.62E^3
10	38.68	1.70E^1
11	25.65	2.10E^6
12	29.43	9.30E^4

As shown in Table 1, one sample was positive only by nPCR but was negative by cPCR & qPCR. Similarly, another sample was positive only by qPCR and negative by cPCR & nPCR. Both cases were considered negative for Toxoplasma.

Discussion

The protozoan *Toxoplasma gondii* is one of the most common infectious pathogenic parasites and can cause severe medical complications in infants [5]. Congenital infection results from primary maternal infection acquired during gestation. The incidence of vertical transmission and the severity of fetal damage depend on the stage of pregnancy when maternal infection occurs [21]. Diagnosis of toxoplasmosis is usually performed by serological methods based on demonstration of a significant increase in specific IgG antibody levels and/or the presence of specific IgM and IgA antibodies [10].

In the present study, all cases were serologically negative for Toxoplasma, yet they were diagnosed positive by the assessed molecular assays. This may be due to the fact that serological tests may fail to detect specific anti-Toxoplasma IgG or IgM during the active phase of T. gondii infection, because these antibodies may not be produced until after several weeks of parasitemia [22].

The detection of Toxoplasma nucleic acids by molecular techniques as polymerase chain reaction (PCR) proved to be a good alternative for serological tests [23]. In blood, the parasite is found during the phase of parasitemia early in the acute phase of infection, and also in reactivated disseminated cases [15]. After infection, Toxoplasma gondii DNA appear earlier than the immunological response (24).

In the present study, three PCR assays were evaluated (cPCR, nPCR & qPCR) for detection of Toxoplasma B1 gene in blood samples. For prenatal diagnosis, Chabbert et al. 2004 validated the choice of B1 gene-based PCR assays as these assays appeared clearly more sensitive than assays targeting the single-copy P30 gene [25]. The sensitivity of PCR for purified Toxoplasma gondii DNA has been found to be very high because B1 gene contains 30-35 copies of repetitive sequences in every trophozoite [20].

In our study, cPCR detected eight cases; nPCR detected 11 cases while qPCR detected 12 cases of toxoplasmosis. Therefore, qPCR was more sensitive than nPCR and cPCR in detection of Toxoplasma B1 gene. Similarly, Lin et al. 2000 demonstrated that real-time PCR of the B1 gene is sensitive and highly reproducible

On the other hand, real time PCR did not appear to be more sensitive than cPCR as stated by other researchers [17, 26]. The difference in results may be explained by lack of standardization of PCR assays and diversity in the protocols used regarding DNA extraction methods, DNA targets for amplification or the primers used [27].

In the present study, we were able to estimate the amount of Toxoplasma DNA in each positive sample by qPCR. Although both nested and real-time PCR are useful in the analysis of clinical specimens and may achieve similar levels of assay sensitivity, the major advantages of real-time PCR are its ability to accurately quantify the infection load of a clinical specimen [5]. Quantification of infection load has been used to assess disease severity and treatment outcome [28].

The negativity of other samples included in our study did not exclude the possibility of acute infection because the exact kinetics of parasitemia in infected people is not well known [29]. The transient and intermittent nature of parasitemia could lead to false-negative results in such cases [30].

In the present study, one sample was positive by nPCR but negative by cPCR and qPCR. This may be due to a possible contamination of this sample. The potential PCR carryover associated with conventional PCR is usually avoided in real-time PCR, since the latter is performed in a closed-tube environment [5].

Another sample was positive only by qPCR but was negative by cPCR and nPCR. This may be explained by higher sensitivity of qPCR over other PCR assays and its ability to detect very low levels of Toxoplasma DNA. Similarly, Li et al. 2004 stated that qPCR could detect Toxoplasma B1 gene at a concentration as low as 0.05 tachyzoite in a 50-ml reaction volume. The use of more than one reaction tubes per patient in research might help to avoid falsely negative results in Toxoplasma PCR [25].

In conclusion, qPCR is a sensitive and efficient method for detection of Toxoplasma B1 gene. The use of commercial PCR kits offers a good alternative to laboratory developed PCR assays being standardized and accredited.

Author contribution

Amany A. Abd El- Aal initiated the idea, made the study design and revised the manuscript. Samar Sayed Attia, Nihal Ahmad Hanafy, Abeer Said Al-Antably, Marwa Adel Hassan shared in study design and interpretation of results, wrote and revised the manuscript.

Walid El Sherbiny shared in study design and interpretation of results, wrote and revised the manuscript.

Aml S Nasr shared in study design and interpretation of results, wrote and revised the manuscript.

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