

# DETECTION OF *TOXOPLASMA GONDII* DNA BY PCR FOLLOWING MICROWAVE TREATMENT OF SERUM AND WHOLE BLOOD

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**Abstract.** Detection of *Toxoplasma gondii* in blood by means of the polymerase chain reaction (PCR) may facilitate early diagnosis of toxoplasmosis in different groups of patients. We evaluated this approach in 42 patients presenting with ocular or psychotic diseases by comparing the sensitivity and specificity of PCR after heat treatment using a microwave oven with a standard genomic DNA extraction method for paired serum and whole blood samples. The presence of serum IgM and IgG antibodies against *T. gondii* was detected using a standard commercial enzyme-linked immunosorbent assay and enzyme immunoassay for IgG avidity test. Of 42 whole blood samples, PCR after microwave treatment was positive in 8 samples with a sensitivity of 73% and specificity of 100% compared to 11 samples positive by the extraction method. Although none of 42 sera samples was PCR positive by the extraction method, 7 specimens were positive after microwave treatment. This is the first study to use a microwave heat treatment, which is simple, rapid and a promising alternative method, in detecting small amounts of *T. gondii* DNA in human blood. Furthermore, irradiation of blood samples with microwaves allows incorporation of PCR into a practical tool for routine clinical assessment of patients with *Toxoplasma* infection.

**Key words:** *Toxoplasma gondii*, PCR, microwave treatment, extraction method, human blood

## INTRODUCTION

*Toxoplasma gondii* an obligate intracellular protozoan, is one of the most common parasites that infects warm blooded animals including man. Approximately half

of the world's population is predisposed to this parasitic infection (Remington and Mcleod, 1992). Toxoplasmosis is generally asymptomatic; however, this parasitic disease can cause symptomatic and/or life-threatening conditions in congenital toxoplasmosis or immunosuppressed patients such as organ transplant recipients, cancer patients and HIV/AIDS patients (Gavinet *et al*, 1997; Nissapatorn *et al*, 2004; Herold *et al*, 2009).

The laboratory diagnosis of toxoplasmosis can be performed in several ways

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(Fleck and Kwantes, 1980) including histological examination, the isolation of the parasite after inoculation, and several serological methods. None of these methods offer a good and reliable result for toxoplasmosis, particularly in the fetus and immunosuppressed patients (Savva *et al*, 1990). The polymerase chain reaction (PCR) has been used as an alternative to serology by amplification of *Toxoplasma* DNA sequences present in various clinical samples, such as amniotic fluid (Jenum *et al*, 1998), cerebrospinal fluid (Cingolani *et al*, 1996), tissues (Held *et al*, 2000), aqueous humor (Mahalakshmi *et al*, 2006), and human blood (Kompalic-Cristo *et al*, 2007). This molecular technique provides important information on the pathobiology of *Toxoplasma* infection. Despite its great diagnostic power, however, PCR not widely used in clinical setting for routine diagnosis or for therapeutic management of patients with *Toxoplasma* infection, as seen in most of developing countries including Malaysia. In general practice, PCR is often applied after a standard genomic DNA extraction method which is time consuming and very expensive kit.

Since 1991, it has been suggested that treatment of serum samples with microwaves increases the ability of PCR to amplify hepatitis B virus (HBV) DNA sequences, either cloned or contained in viral particles, when diluted in human serum (Cheyrou *et al*, 1991). Irradiation of serum samples with microwaves provides a simple, rapid, and less time consuming means to facilitate the detection of small amounts of HBV-DNA (Costa *et al*, 1995). So far, only one study has used PCR after microwave treatment to detect *T. gondii* DNA in whole blood from pregnant women (el-Awady *et al*, 2000).

This study used PCR to amplify *Toxoplasma* DNA from the B1 gene to detect

*T. gondii* in whole blood and serum samples obtained from patients with confirmed clinical toxoplasmosis and positive specific anti-*Toxoplasma* (IgG, IgG and IgM, or IgM) antibodies. The purpose was to compare the sensitivity and specificity of PCR detection of *T. gondii* DNA after heat treatment using microwave oven (MW-PCR) with a standard extraction method (E-PCR) of genomic DNA using a commercial kit (Qiagen, Germany) for both paired serum and whole blood samples.

## MATERIALS AND METHODS

### Patients and samples

Forty-two Malaysian patients (17 males and 25 females, age range 22 to 81 years) with ocular or psychotic diseases presented at the University Malaya Medical Centre (UMMC), Kuala Lumpur were recruited. Thirty-six patients with chronic *Toxoplasma* infection (IgG or IgG/IgM) documented by long lasting positivity for serum anti-*Toxoplasma* antibodies and a high avidity of specific IgG were included. Primary acquired *Toxoplasma* infection evidenced by recent positivity for serum anti-*Toxoplasma* (IgM) antibody was documented in 6 patients. Of this, only 2 cases were clinically confirmed ocular toxoplasmosis. Serum and whole blood samples were collected and stored at -20°C until analyzed.

### IgG and IgM antibodies detection

Specific IgM and IgG antibodies against *T. gondii* were determined using a standard commercial enzyme-linked immunosorbent assay (ELISA, Trinity Biotech, New York, USA) and enzyme immunoassay for IgG avidity test (NovaLisa™, Dietzenbach, Germany), in accordance with the manufacturer's instructions.

### Isolation of DNA

Genomic DNA was isolated from whole blood and serum by a simple and rapid heat treatment procedure using microwave oven which enables direct PCR amplification on desiccated samples (Cheyrou *et al*, 1991). Five  $\mu$ l of whole blood and sera were allocated into thin wall PCR tubes and were exposed to the maximum temperature setting in a 800 watt microwave oven [Sharp, R258L (s), Sharp Roxy Sales Singapore] for 2-3 minutes, or until the whole blood and sera were desiccated. The tubes then were centrifuged briefly and the contents were applied for PCR amplification. DNA also was extracted from both samples using QIAmp DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer's instructions.

### Detection of *T. gondii* by PCR

Detection of *T. gondii* infections was determined by nested PCR amplification targeting the B1 gene (GenBank: AF 146527) (Burg *et al*, 1989). The PCR mixture for the nested reaction consisted of 2X PCR master mix solution with a final concentration of 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M of each dNTP and 2.5 U Taq DNA polymerase (Qiagen, Germany); 50 pmol of each primer; and as a template, 2  $\mu$ l of extracted and 5  $\mu$ l of treated sample DNA for the first reaction and 2  $\mu$ l of a 1:10 dilution of the products of the first amplification for the second reaction, in a final volume of 25  $\mu$ l. PCR reactions were performed in a Mycycler thermal cycler (BioRad, USA). First step PCR was 7 minutes at 94°C, followed by 35 cycles, each cycle consisting of 60 seconds at 94°C, 30 seconds at the annealing temperature for each primer pair, and 60 seconds at 72°C. The final cycle was followed by heating for 7 minutes at 72°C. Primary PCR amplification was performed with outer

primers B1F1 (5'-CCGTTGGTTCCGCCT CCTTC-3') and B1R1 (5'-GCAAAACAG CGGCAG CGTCT-3') at an annealing temperature of 54°C. In the second reaction, the internal primers B1F2 (5'-CCGCC TCCTTCGTCC GTCGT-3') and B1R2 (5'-GTGGGGG CGGACCTCTCTTG-3') were used at an annealing temperature of 60°C. All PCR products were analyzed by 1% agarose gel electrophoresis. The PCR amplification is expected to yield a product of 213 bp for the presence of *T. gondii*. To eradicate contamination, all reagents were stored in aliquots in autoclaved tubes. Reaction mixtures were prepared in a separate room, using disposable aerosol-resistant pipette tips. All reactions were conducted at least in duplicate and positive and negative controls were included.

### RESULTS

We examined blood samples from 11 out of 42 patients with ocular and psychotic diseases, as summarized in Table 1. There were 7 females and 4 males with ages ranging from 23 to 77 years. MW-PCR was positive for 8 samples of whole blood when compared to 11 samples of the same specimens using E-PCR with a sensitivity of 73% and specificity of 100%. Although no amplification of *Toxoplasma* DNA fragments using E-PCR from serum samples was observed for all 42 patients, a positive MW-PCR was obtained for 7 patients. We observed a correspondence of positive *Toxoplasma* DNA in whole blood samples between MW-PCR and E-PCR, whereas only positive MW-PCR was detected in serum samples of these 2 patients who had ocular manifestations consistent with toxoplasmosis, were positive for anti-*Toxoplasma* IgG and IgM antibodies, high avidity specific for IgG indicating latent infection, and responded well to anti-*Toxoplasma* therapy when toxoplasmic retino-

Table 1  
Diagnosis of toxoplasmosis by PCR from 11 paired blood samples of patients with ocular or psychotic diseases.

Patient group	Age (years)	Sex	Serology			Clinical symptoms	PCR		
			IgM	IgG	IgG avidity		S-MW	S-E	WB-MW
<b>Psychotic</b>									
P1	33	F	+	+	High	AS	-	+	+
P2	44	M	+	+	High	AS	-	-	+
P3	23	F	+	+	High	AS	-	+	+
P4	40	M	+	+	High	AS	-	+	+
P5	50	M	+	+	High	AS	-	+	+
<b>Ocular</b>									
O1	72	F	+	+	High	AS	-	+	+
O2	77	F	+	+	High	AS	-	-	+
O3	73	M	+	+	High	AT	-	+	+
O4	51	F	+	+	High	AS	-	+	+
O5	79	F	+	+	High	AS	-	-	+
O6	43	F	+	+	High	AT	-	+	+

AS, asymptomatic; AT, active toxoplasmosis; S-MW, DNA isolation from serum by microwave treatment; S-E, DNA extraction from serum using commercial kit; WB-MW, DNA isolation from whole blood by microwave treatment; WB-E, DNA extraction from whole blood using commercial kit; +, positive result; -, negative result

MW-PCR FOR DETECTION OF *T. GONDII* DNA IN BODY FLUIDS

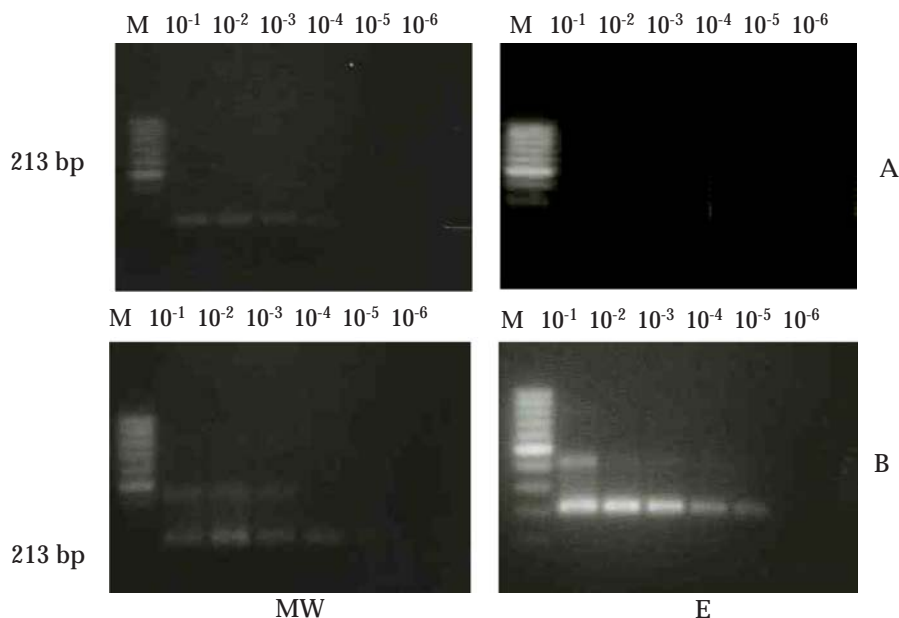


Fig 1—Amplification by nested PCR of *T. gondii* in ten-fold serial dilutions (from 10<sup>-1</sup> to 10<sup>-6</sup>) after microwave treatment with a standard commercial extraction method for serum (A) and whole blood (B). Faint band in lane 10<sup>-5</sup> (panel B-MW) and in lane 10<sup>-4</sup> (panel A-MW) are not reproduced in the photograph. Lane M, molecular weight markers; MW, microwave treatment; E, standard commercial extraction.

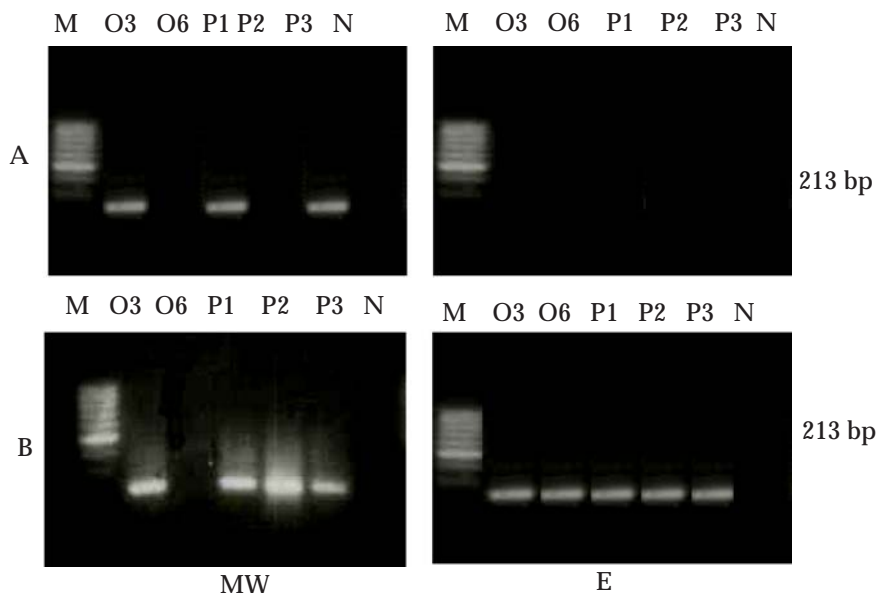


Fig 2—Nested PCR amplification of *T. gondii* B1 gene after microwave treatment and with a standard commercial extraction method for serum (A) and whole blood (B) from 2 patients with active ocular toxoplasmosis (O3 and O6) and 3 patients with asymptomatic condition (P1-P3). Lane M, molecular weight markers; lane N, negative control; MW, microwave treatment; E, standard commercial extraction.

choroiditis was finally confirmed. Among the 9 positive E-PCR patients, positive MW-PCR results were found in 4 blood and 3 serum samples out of 5 patients with psychotic diseases. The remaining 4 patients with ocular diseases, positive MW-PCR results were shown in each 2 whole blood and serum samples.

Amplification of *Toxoplasma* DNA in 5  $\mu$ l of serum and whole blood samples was performed by nested-PCR. PCR amplification was done in ten-fold serial dilutions of serum samples using MW-PCR and E-PCR, and the band of expected size (213 bp) for both MW-PCR and E-PCR was detected in 0.001 pg/ $\mu$ l for whole blood sample, whereas MW-PCR showed visible band in serum diluted to 0.01 pg/ $\mu$ l but no positive serum sample was obtained with E-PCR, as shown in Fig 1.

PCR amplifications of *Toxoplasma* DNA were then done in undiluted serum and whole blood from 2 patients with active ocular toxoplasmosis and 3 patients with asymptomatic condition, as shown in Fig 2.

## DISCUSSION

PCR has been used in an effort to overcome serious deficiencies of other tests for the detection of *Toxoplasma* DNA in infected tissues, particularly in cases of immunosuppressed and immunocompromised individuals (Savva *et al*, 1990). PCR has consistently been performed in the laboratory to detect *T. gondii* DNA in different biological samples (Burg *et al*, 1989; Ho-Yen *et al*, 1992; Dupon *et al*, 1995; Fuentes *et al*, 1996; Cermáková *et al*, 2005; Tonkal, 2008). Up to now, the diagnostic value of PCR for the detection of *T. gondii* in blood samples has been evaluated from both immune-competent and compromised patients (Ho-Yen *et al*, 1992; Bou

*et al*, 1999; Kompalic-Cristo *et al*, 2007).

We demonstrate here the results of the use of MW-PCR for the identification of *Toxoplasma* DNA in human blood, including whole blood and serum samples from patients with ocular or psychotic diseases. There were 5 psychotic patients who had recently acquired infection without clinical presentation consistent with toxoplasmosis. MW-PCR detected *Toxoplasma* DNA in 4 blood and 3 serum samples when compared to 5 samples of whole blood and none was found in serum using E-PCR. We also have detected *Toxoplasma* DNA using MW-PCR in both whole blood and serum samples of 2 patients with recently acquired *Toxoplasma* infection but without ocular manifestations of toxoplasmosis. These are unexpected results as the patients were in asymptomatic condition but were positive for anti-*Toxoplasma* antibodies with high titers. Nonetheless, there is a possible explanation that a small number of parasites might have been released from tissue into the blood at a subclinical level, and their presence can be detected only by PCR (Bou *et al*, 1999). The positive PCR results indicated that persisting parasitemia is not the reason to produce symptoms, and more extensive studies are required to confirm this conclusion.

An interesting point to be considered is that the presence of positive amplifications with B1 primers of *Toxoplasma* DNA in both patients with clinically confirmed ocular toxoplasmosis was found by MW-PCR in whole blood and sera compared to only whole blood by the extraction method. The clinical diagnosis of ocular toxoplasmosis is based on the presence of a necrotizing lesion on the fundus, response to treatment, and serological determination. Apart from aqueous humor (Brezin *et al*, 1991; Aouizerate *et al*, 1993; Garweg *et al*, 1996; Robert *et al*, 1996;

Verbraak *et al*, 1996; Danise *et al*, 1997; Bou *et al*, 1999; Figueroa *et al*, 2000; Jones *et al*, 2000), which is the most common intraocular fluid used to diagnose ocular toxoplasmosis, blood sample has been performed comparatively to detect *Toxoplasma* DNA with a sensitivity value of between 10 and 35% (Filice *et al*, 1993; Dupon *et al*, 1995; Guy and Joynson, 1995; Franzen *et al*, 1997). This result strongly suggests ocular toxoplasmosis should not be considered a local event, as also shown in AIDS patients with cerebral toxoplasmosis (Dupouy-Camet *et al*, 1993; Bou *et al*, 1999). From these results, it could be explained with the hypothesis that the source of parasitemia is the ocular lesion, or that it is associated with reactivations in other body tissues leading to tachyzoites, or that *T. gondii* DNA released from cysts in these tissues may be the source in blood (Bou *et al*, 1999).

MW-PCR may be used to assist in diagnostic confirmation of ocular toxoplasmosis and also may be helpful in differentiating between ocular toxoplasmosis and other ocular diseases. Moreover, MW-PCR has been proved to be a sensitive technique to detect other pathogens such as hepatitis B virus (HBV-DNA) in serum samples (Cheyrou *et al*, 1991; Costa *et al*, 1995). Nonetheless, cases of active toxoplasmosis by using MW-PCR should be further explored for its diagnostic value in detecting *Toxoplasma* DNA from clinical samples.

Our findings showed high sensitivity and specificity values of MW-PCR in detecting *Toxoplasma* DNA in whole blood samples, but these diagnostic values of MW-PCR in serum samples need to be evaluated in further experiments. Other investigators have reported that sample storage conditions before amplification can have an important influence on PCR sensitivity values (James *et al*, 1996). This

can contribute to the sensitivity values of MW-PCR as our DNA samples were kept at -20°C before the amplification of *Toxoplasma* DNA as immediate processing for these samples was not possible. Since this is the first study of MW-PCR done in a very small number of paired samples from patients, a larger sample size is therefore recommended in order to reduce the possible risk of contamination of serum samples, reactive agents, or laboratory devices leading to false positive results (Costa *et al*, 1995). Moreover, the PCR results must be interpreted with caution because PCR may be too sensitive for some purposes.

In summary the results presented here suggest that it is possible to detect *T. gondii* DNA in body fluids such as venous blood by the MW-PCR with specificity and high sensitivity. This technique is cheaper and much less time-consuming than the existing standard extraction method. The results suggest that MW-PCR may be used routinely to complement serology for clinical studies and diagnostic purposes of toxoplasmosis.

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