# SENSITIVITY AND SPECIFICITY OF POLYMERASE CHAIN REACTION FOR THE DETECTION OF *TOXOPLASMA GONDII* DNA ADDED TO LABORATORY SAMPLES

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Abstract. We studied the sensitivity and specificity of PCR to detect *T.gondii* DNA by aliquoting various concentration of tachyzoites into laboratory specimens from 60 positive and 10 negative buffy-coat samples. We were able to detect the specific gene from purified DNA samples containing as few as 0.25 parasites per 100,000 human leukocytes. These results had an impressive initial 100% specificity but later it decreased because of false-negative data.

## INTRODUCTION

The protozoan parasite *Toxoplasma gondii* has emerged as an important opportunistic infectious pathogen effecting organ transplant recipients, AIDS patients, and other immunocompromized patients. The infection occurs through the ingestion of uncooked meat, through the exposure to and/or ingestion of oocysts excreted in the feces of infected cats, or through transplacental transmission from an acutely infected woman to her fetus.

Infection is usually asymtomatic in older children and adults. In newborns and in immunocompromized hosts, there may be persistent or recrudescent clinical manifestations with life-threatening consequences, such as encephalitis, myocarditis or chorioretintis.

The current diagnosis of toxoplasmosis relies mostly on the serological detection of toxoplasma specific immunoglubulin, mouse innoculation or tissue culture of the clinical specimens. Although serological testing has been one of the major diagnostic techniques for toxoplasmosis, it has many limitations. For example, it may fail to detect specific anti-*Toxoplasma* immunoglobulin G (IgG) or IgM during the active phase of *T. gondii* infection because these antibodies may not be produced until after several weeks of parasitemia.

An alternative method of identifying *T. gondii* by mouse inoculation or tissue culture of the clinical specimens may be sensitive and specific (Derouin *et al*, 1987), but this method usually requires a high-skilled technician, is time-consuming and labor-intensive. Thus, a more effective method is needed to provide rapid and quantitative results for the diagnosis of *T.gondii* infection.

Detection of *T. gondii* DNA using polymerase chain reaction (PCR) minimizes the problems faced when using sero-diagnostic or cultured-based assays and facilitates diagnosis in different cases. Detection by the PCR method also saves time and labor.

The PCR method was developed by Burg *et al* in 1989 using a 35-fold repetitive sequence gene by having a B1 gene as a target. Several PCR-based techniques have been developed for the diagnosis of toxoplasmosis using various clinical specimens, including amniotic fluid (Grover *et al*, 1990), blood (Ho-Yen *et al*, 1992; Dupouy-Camet *et al*, 1993), cerebrospinal fluid (Farmly *et al*, 1992), and tissue biopsy (Johnson *et al*, 1993). The major advantage of the PCR technique is that it is able to detect the B1 gene from purified DNA samples containing as few as 10 parasites per 100,000 human leukocytes. PCR is the only method that can detect *T.gondii*

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organisms in low numbers (10 to $10^3$ organisms per ml) and can detect a partly destroyed parasite (James *et al*, 1996).

PCR is a new technique, the sensitivity and specificity of this technique for the detection of *T. gondii* is still in doubt. Guy *et al*, in 1996 investigated the accuracy of PCR for the detection of *Toxoplasma gondii* at five laboratories in Europe. In Thailand, *Toxoplasma* PCR is considerably newer, and there are no reports about sensitivities or specificities of this method yet.

Investigating the sensitivity and specificity of *Toxoplasma gondii* PCR will help us to make decisions regarding the methods suitable for the detection of *T. gondii*, and at what level to use them.

## MATERIALS AND METHODS

### Specimen collection and preparation

*Toxoplasma gondii* trachyzoites of the RH strain maintained in inoculated mice were collected using normal saline solution. A counting chamber was used to estimate number of trachyzoites, then the suspension was diluted to concentrations of approximately 1, 10, $10^2$, $10^3$, $10^4$, and $10^5$ organisms (tachyzoites) per 100 microliters. The solution was then stored until used at -20ºC.

Sixty positive and 10 negative samples were prepared. For the positive samples, 25 ml aliquots of known amounts of *T. gondii*, as described above, were added to each patient's buffy coat specimen acquired from blood samples collected from pregnant woman who attended the ante-natal clinic at Rajvithi Hospital. For negative samples, only nuclease-free water or double distilled water was used. No *T. gondii* was added to the negative samples. Samples were randomized and coded by a technician who was not participating in this experiment, and then were kept at -20ºC until used.

### Isolation of DNA and PCR amplification

All samples were treated in the same way by going through DNA extraction, using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions.

The primer pairs O1 (5′-TCAAGCAGCG TATTGTCGAG-3′), O2 (5′-CCGCAGCGACTT CTATCTCT-3′), N1 (5′-GGAACTGCATCCGTT CATGAG-3′), and C1 (5′-TCTTTAAAGCGTTCG TGGTC-3′) were used to amplify a 197 bp DNA fragment of the B1 gene for *T. gondii*.

Amplification of the B1 gene was performed by a two-step PCR (nested PCR). For the primary PCR, samples were subjected to an initial denaturation of 94ºC for 30 seconds, followed by 50 cycles of 94ºC for 15 seconds, 45ºC for 30 seconds, and 72ºC for 45 seconds, with a final extension at 72ºC for 10 minutes. For the secondary PCR, the samples were subjected to an initial denaturation of 94ºC for 30 seconds, followed by 35 cycles of 94ºC for 15 seconds, 45ºC for 30 seconds, and 72ºC for 45 seconds, with a final extension at 72ºC for 10 minutes. A Thermo Hybaid Px2 PCR machine was used throughout the experiment. All the PCR samples were then kept at -20ºC until used.

### PCR product detection

PCR products were separated by horizontal electrophoresis in 3% agarose gels; 1x TAE buffer was used as a running medium, with ethidium bromide staining. The gel was then observed under the ultra-violet light to see the fluorescent bands. The expected *T. gondii* DNA fragment was 197 bp, so the bands were supposed to be seen at around 200 bp. PCR tests were considered positive if amplification of correct DNA fragments occurred.

Procedures for avoiding contamination were strictly followed. DNA extraction, preparation of experimental media and amplification, and positive (DNA extracted from *T. gondii* trachyzoites) and negative (containing only the reaction mixture) controls were tested along with the samples each time.

## RESULTS

Examination of laboratory DNA-added buffy coat demonstrated the limitation of the sensitivity of the PCR method to be as low as 0.25 parasites per 100,000 human leukocytes.

When comparing the set-up and the experimental results, we found no false positives, so

Table 1
Comparative table between set-up and test results.

| Set-up concentration (organisms/ 100 μl) | Number of samples prepared | Number of samples with correct results | Initial specificity (%) |
|---|---|---|---|
| $10^5$ (Full strength) | 10 | 10 | 100 |
| $10^4$ (1:10 dilution) | 10 | 10 | 100 |
| $10^3$ (1:100 dilution) | 10 | 10 | 100 |
| $10^2$ (1:1,000 dilution) | 10 | 10 | 100 |
| 10 (1:10,000 dilution) | 10 | 10 | 100 |
| 1 (1:100,000 dilution) | 10 | 10 | 100 |

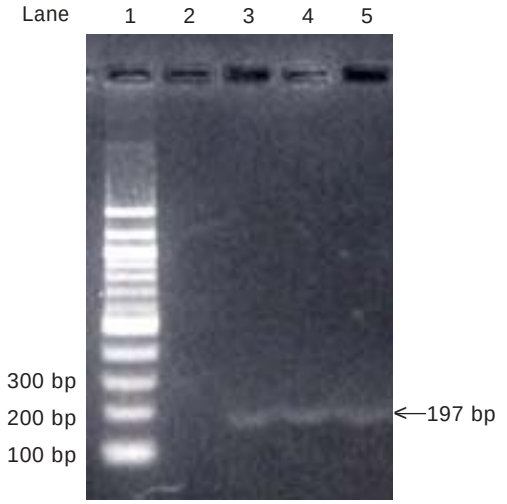


Fig 1–Analysis of PCR product by agarose gel electrophoresis. PCR was based on the amplification of a 197 bp fragment of the B1 gene. Lane 1: molecular weight markers, lane 2: negative control, and lanes 3-5: positive results for the B1 gene (197 bp).

the initial specificity was 100% as showed in Table 1.

## DISCUSSION

Several PCR-based techniques have been developed over the years as an alternative method for the detection of *T. gondii* infection with targets such as the P30 gene, the B1 gene or a segment of 18 S ribosomal DNA. Until now, the most popular DNA target for the diagnosis of toxoplasmosis was the B1 gene, with huge diversity in the PCR protocols and published primers (Reischl *et al*, 2003).

The sensitivity between 10 and 30% were reported by Felice *et al* (1993), Khalifa *et al* (1994), Dupon *et al* (1995), and Guy and Johnson (1995. Dupouy-Camet *et al* (1993) reported a 69% sensitivity.

In our study, all the positive samples had *T.gondii*. We found that our PCR protocol could detect as few as 0.25 parasites per 100,000 human leukocytes, which is very sensitive. Unfortunately, we found that this sensitivity was not stable and caused false-negative data. Sometimes with the same number of parasites we could not detect the expected band on gel electrophoresis. This may be explained by the fact that the level of sensitivity of PCR may be affected by some factors, such as the inhibitory factor porphyrin (Dupon *et al*, 1995), and toxoplasma DNA may be destroyed before or during the process of amplification. We suggest that changing the target to ribosomal DNA (rDNA) which is more repetitive than the target that we used (B1 gene), is a good way to improve the sensitivity of the toxoplasma PCR.

We found that some of our samples, which were positive on the PCR previously were found to be negative upon re-examination the following day or later. The reason for this may be that the RH strain used throughout our experiment has been reported by Nguyen *et al* (1996) to appear in the blood or urine for only about 24 hours, but no longer that 3 days. We also found that if we ran the PCR right after the DNA extraction process, within the same day, there was

a higher yield than if we ran the PCR later.

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