COMPARISON OF FIVE DNA EXTRACTION METHODS AND OPTIMIZATION OF A B1 GENE NESTED PCR (nPCR) FOR DETECTION OF *TOXOPLASMA GONDII* TISSUE CYST IN MOUSE BRAIN

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Abstract. Toxoplasmosis, caused by *Toxoplasma gondii*, is an important parasitic disease worldwide. Different techniques have been developed for *T. gondii* detection. At present, polymerase chain reaction (PCR) has been widely used. However, PCR for identifying *T. gondii* remains unsatisfactory in many laboratories because of lack of standardization and variations in efficiency. In the present study, we optimized a nested PCR protocol (n-PCR) in order to compare the amplification of *T. gondii* DNA, after being extracted from mouse brain by five different DNA extraction methods including phenol chloroform, QIAamp DNA minikit, Genomic DNA purification kit and Chelex with or without proteinase K. All DNA extraction methods were able to extract DNA from a single tissue cyst from mouse brain. However, among the five DNA extraction methods, the Chelex without proteinase K appeared to be the most rapid and easiest.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite that infects a wide range of warm-blooded animals including man (Dubey and Beattie, 1988). However, its only definitive hosts are domestic and wild cats. Apart from placental transmission, humans mainly become infected with *T. gondii* by two routes: by ingesting foods or water contaminated with oocysts and by ingesting undercooked meat contaminated with bradyzoites in tissue cysts. After ingestion, oocysts give rise to sporozoites. These sporozoites or ingested bradyzoites convert to tachyzoites inside the host tissues within 18 hours (Dubey, 1997; Dubey *et al*, 1997). Tissue cysts begin to form during the first week after infection and are thought to persist for life. Infection by this parasite is normally asymtomatic in immunocompetent adults but in immunocompromised individuals or in congenitally infected cases, it can lead to serious illnesses. For example, reactivation of latent infection in patients with acquired immunodeficiency syndrome (AIDS) can lead to fatal toxoplasmosis. The reactivation is thought to be due to transformation from the bradyzoite to the tachyzoite stage. Identification of tissue cysts, therefore, plays an important role for the prognosis of *T. gondii* reactivation.

There are several techniques that can be used to detect *T. gondii* in tissue samples. These include animal inoculation, histological examination and molecular biological techniques, such as polymerase chain reaction (PCR). Until now, the most reliable method of inspecting *T. gondii* tissue cysts has been an *in vivo* biological assay (Gamble and Murrell, 1998; Bastein, 2002). However, this method is costly and time-consuming, and is not appropriate for detection in routine work. For this reason, PCR identification of *T. gondii* is considered a valuable alternative. Among the DNA target genes that have been developed for *T. gondii* detection, the most widely used is the B1 gene. It consists of a 35 repeats of 2,214 nucleotide repeats and is highly

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conserved among different strains of *T. gondii* (Burg *et al*, 1989; Da Silva *et al*, 2001), thus making it suitable for detection by PCR. The detection of *T. gondii* by PCR has considerably improved the diagnosis, but the method remains unsatisfactory due to a lack of standardization and a variation in efficiency among laboratories. Thus, a PCR protocol that yields good results in some laboratories may not work in others (Guy *et al*, 1996; Pelloux *et al*, 1997).

The major inconvenience of PCR is the presence in the samples of inhibitors which interfere with amplification-based techniques. Thus the sample preparation and DNA extraction methods can greatly influence the outcome and reliability of the test (Lachaud *et al*, 2001). Although many commercial kits have been developed to allow easy and rapid DNA extraction, the preferred method for preparing DNA from any source should also be rapid, high yielding, cost-effective and environmentally safe.

In the present study, we optimized a PCR protocol using the B1 gene to detect *T. gondii* tissue cysts in brains from experimentally infected mice, and compared different DNA extraction methods.

MATERIALS AND METHODS

Mice and *T. gondii* infection

Six to eight weeks old ICR mice of both sexes were used in this study. Mice were obtained from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand and were infected orally with 20 tissue cysts of *T. gondii* O'Toole strain. After 6 weeks post-infection, all mice were killed by cervical dislocation and their brains were removed and homogenized in 2 ml of sterile phosphate-buffered saline (pH 7.2) by using a mortar and pestle. The concentration of tissue cysts in each brain suspension was calculated by counting under light microscope. The concentration was adjusted to 1 cyst in 200 µl.

Extraction of *T. gondii* DNA

Each 200 µl aliquot of homogenized brain suspension containing 1 tissue cyst was processed in accordance with the DNA extraction methods described below.

Standard method. This method consists of extracting DNA by using the conventional phenol-chloroform method (Sambrook *et al*, 2001). First, the 200 µl of homogenized brain suspension were placed in a 1.5 ml microcentrifuge tube and mixed with 700 µl of lysis buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 20 mM EDTA pH 8.0 and 1% SDS). The next step was to add 20 µl of proteinase K (20 mg/ml; QIAgen) and the mixture was then incubated at 56ºC for an hour during which the tube was inverted 3-5 times every 20 minutes. The solution was added to 600 µl of phenol/chloroform/isoamyl alcohol (25:24:1) solution and was mixed by inverting the tube and then was placed at -20ºC for 10 minutes. The solution was centrifuged for 10 minutes at 12,000 rpm, forming an upper aqueous phase, an inter phase and a lower organic phase. The aqueous phase was transferred to a new microcentrifuge tube and mixed with another 600 µl of phenol/chloroform/isoamyl alcohol (25:24:1) solution, then kept at -20ºC for 10 minutes and centrifuged again at 12,000 rpm for 10 minutes to separate the aqueous phase from the other phases. The aqueous phase was then transfered into a new microcentrifuge tube and 30 µl of 3 M sodium acetate (pH 5.2) were added followed by 600 µl of absolute ethanol. The mixture was placed at -20ºC for 30 minutes. At this stage the DNA was precipitated. The mixture was then centrifuged at 12,000 rpm for 30 minutes. The supernatant was discarded. The precipitated DNA was washed in 500 µl of 70% ethanol and then sedimented at 12,000 rpm for 5 minutes. The ethanol was removed and DNA was dried and dissolved in 100 µl of TE buffer pH 7.6 (10 mM Tris, 1 mM EDTA). The solution was stored at -20ºC until use.

QIAamp DNA mini kit (QIAGEN). Two hundred µl of the brain suspension were added to 20 µl of proteinase K (200 mg/ml; QIAGEN) and ATL lysis buffer and then the mixture was incubated at 56ºC for an hour. After incubation, the QIAamp tissue protocol was performed according to the manufacturer's instructions.

Genomic DNA purification kit (Fermentus, USA). Two hundred µl of the brain suspension

were pulverized in liquid nitrogen using a mortar and pestle. The powder was placed in a 1.5 ml microcentrifuge tube and mixed with 400 µl of the lysis solution (Fermentus, USA) and incubated at 65ºC for 10 minutes. The protocol was then followed according to the manufacturer's protocol.

Chelex method. Two hundred µl of the brain suspension was introduced into a 1.5 ml microcentrifuge tube and mixed with 500 µl of 6% Chelex-100 resin (Bio-Rad) and 20 µl of proteinase K (20 mg/ml; QIAgen). The mixure was incubated at 56ºC for an hour then at 100ºC for 15 minutes. The tube was mixed and then centrifuged at 10,000 rpm for 10 seconds. The supernatant was centrifuged again for 10 minutes to separate the layers. Ten µl of the upper layer were used for each 25 µl of final volume in the PCR reaction mixture.

Chelex method without proteinase K. The same protocol as the Chelex method described above was used but proteinase K was not added.

Nested polymerase chain reaction

Two PCR primer pairs of the B1 gene (Burg *et al*, 1989), F1 (CAAGAGAAGTATTTGAGGTC ATATGC) and R1 (CGGAACTGTAATGTGATACT GTGC) amplifying a 934 bp and F2 (GTGTATT CGAGACAAGAGAGGTCC) and R2 (CATAACA CGCTGTGTCTCCTCTAG) amplifying a 688 bp fragment were used. n-PCR was performed using these two primer pairs as external (F1, R1) and internal (F2, R2) primers for the first and second PCR, respectively. PCR conditions for this study were optimized by changing the concentration of $MgCl_2$ and the annealing temperature. The concentration of $MgCl_2$ was varied from 1.0 to 4.0 mM and the annealing temperature was changed from 52º to 62ºC in each PCR. The PCR reactions were performed in a 25 µl of reaction mixture containing 0.1 µM of each primer, 1X PCR buffer with KCl (Fermentus, USA), 200 µM dNTP and 2.0 U of Taq DNA polymerase (Fermentus, USA). One µl of DNA template was used in the first PCR reaction. The PCR conditions were 2 minutes at 94ºC followed by 35 cycles of 94ºC for 30 seconds, the annealing temperature for 30 seconds, and 72ºC for 2 minutes, and a last extension step at 72ºC for 10 minutes performed in a Px2 thermal cycler (Thermo Hybaid, USA). One µl of the first PCR product was then used as a template in the second PCR using the same conditions as described above. The reaction products were analyzed after electrophoresis in a 1.7 % agarose gel. *T. gondii* RH strain genomic DNA was used as positive control for the PCR. The brain of healthy mice was used as internal negative control and deionized water was used as an external negative control. All of these controls were processed along with all experiments.

RESULTS

All the PCR reactions were found to produce a DNA band of the expected size following optimization of $MgCl_2$ concentration and annealing temperature. However, some differences in background pattern and intensity of the specific signal were observed between each condition; 1.5 mM $MgCl_2$ and 60ºC annealing temperature gave the best result. In this condition the PCR products were found to be specific and there was minimal background smearing (data not shown). Each DNA extraction method described here was successfully applied with the n-PCR protocol without significant differences in the pattern of the PCR products (Fig 1). However, there were differences in the time used for each DNA extraction method (Table 1). The Man-Minute for each method was the time spent for one skilled laboratory assistant to perform each extraction procedure and the completion time

Table 1
Comparison of man-minute and completion time for different methods for DNA extraction.

Methods	Man-Minute[a]	Time for completion[a] (minutes)
Classic	25	185
QIAamp DNA mini kit	20	80
Genomic DNA purification kit	28	38
Chelex	15	85
Chelex without proteinase K	10	27

[a]Average of three samples

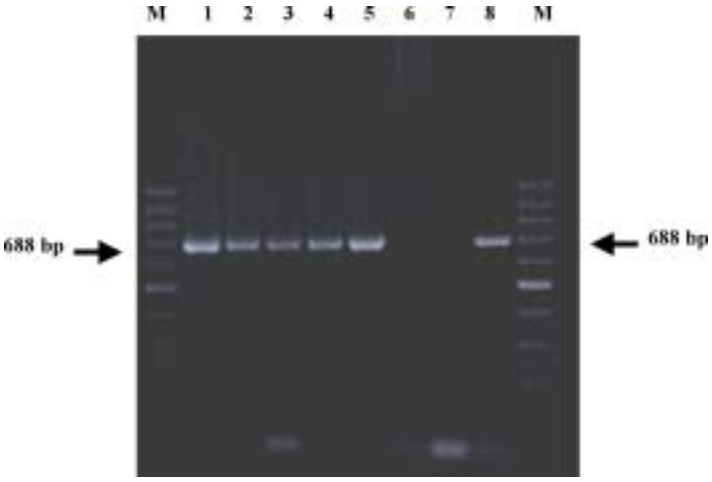

Fig 1–Comparison of n-PCR amplification of *T. gondii* B1 gene by different DNA extraction methods. The expected product is 688 bp amplified. Electrophoresed amplification products are shown in an ethidium bromide-stained agarose gel. M = 100 bp molecular weight marker; lane 1 = standard method; lane 2 = QIAamp DNA mini kit; lane 3 =Fermentus genomic DNA purification kit; lane 4 = Chelex method; lane 5 = Chelex method without proteinase K; lane 6 = negative control (DNA from healthy mouse); lane 7 = amplification reaction in which deionized water was used instead of DNA template; lane 8 = positive control (tachyzoite RH strain).

was the total time required in the laboratory, including incubation periods. No amplification products in both internal and external negative controls were found in the experiment.

DISCUSSION

Although precipitation with phenol/chloroform provides DNA fragments of high molecular weight sufficient for any application of molecular biology, this method involves many slow and costly steps. It also includes the use of potentially health hazardous chemicals (*ie* phenol and chloroform) which make this process difficult to carry out on a large scale in laboratory routine work. All extraction methods from commercial kits are easier to perform, safer and more rapid although there are slight differences in the hands-on time from one to another. The Chelex-100 resin without proteinase K method has the shortest time for completion because it does not require the digestion step. This method is as effective as the commercially available kits that were tested in this study.

Since labor costs are the most expensive part of diagnosis, consideration has to be given towards choosing the protocol with the least labor and completion time. The Chelex method has been used to prepare DNA template from many types of protozoa such as *Plasmodium* (Kengne *et al*, 2003), *Giardia* (Mahbubani *et al*, 1998), *Leishmania*, (Cabrera *et al*, 2002) and *Trichomonas* (Chen and Li, 2001) and has performed comparably to or better than the phenol-chloroform method. Joseph *et al* (2002) evaluated the preparation of DNA of *T. gondii* RH strain by the Chelex method in comparison to the Qiagen QIAmp tissue kit and reported that the DNA obtained by both extraction methods gave similar results. However, the DNA purified with Chelex 100 provided reproducible amplification only up to 2 months of storage at -20°C. In contrast, DNA purified with the Qiagen kit provided reproducible amplifications up to 1 year of storage at -20°C. Chelex resin acts as a chelating resin of metallic polyvalent ions in addition to its other roles in DNA purification (Gill *et al*, 1992). Once the ions stabilizing the DNA are removed they lead to the denaturation of double stranded DNA. Thus this method is not as suitable as other commercial kits for other techniques in molecular biology such as RFLP analysis where double stranded DNA is needed.

The B1 gene has theoretical advantages because each *T. gondii* contains approximately 35 copies of this gene (Burg *et al*, 1989). The B1 specific gene probe used for the detection of *T. gondii* does not cross-react with other tissue-resident microorganisms such as *Sarcocystis*, *Neospora*, and *Trichinella* (Da Silva *et al*, 2001). Studies have reported that the B1 gene is able to detect a single tachyzoite in ocular fluids (Burg *et al*, 1989; Bretagne *et al*, 1993). Furthermore the B1 primers do not lose specificity even when large amounts of human lymphocyte

DNA are present. However, not many studies have used the B1 gene for detection *T. gondii* in tissue samples (Steuber *et al*, 1995; Sedlak *et al*, 2004; Sreekumar *et al*, 2004). In these protocols, pepsin digestion of the tissue samples prior to examination has been shown to increase the sensitivity of the PCR protocol (Warnekulasuriya *et al*, 1998). Jauregui *et al* (2001) achieved a sensitivity of 1 bradyzoite in the Toxo Taqman assay by pretreating tissue with pepsin-HCl prior to detection. In our study, the Chelex DNA extraction without proteinase K could detect 1 cyst in infected mouse brain and thus in animals with low cysts number our n-PCR protocol can be used as an alternative to the tedious visual counting. The advantages of the n-PCR assay over mouse inoculation, histology, or other assays are its rapidity, specificity and sensitivity. Procedures can easily be performed in one day.

In summery, the n-PCR assay described here with Chelex extraction of DNA is safer and simpler than the other methods. Moreover, this technique does not require any organic solvent and the entire extraction procedures uses only two microcentrifuge tubes for each sample, decreasing the time for transferring of supernatants between tubes and reducing the possibility of cross contamination. This technique can be used to detect *T. gondii* cysts even when they are only present at low numbers in tissue. However, specimen sample from naturally infected human or animal should be tested for further validation of this technique.

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