Comparison of PCR Detection Methods for B1, P30, and 18S rDNA Genes of *T. Gondii* in Aqueous Humor

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**PURPOSE.** Comparison of polymerase chain reaction (PCR) amplification of three *Toxoplasma gondii* genes in aqueous humor.

**METHODS.** Nested PCRs carried out using published methods were optimized for maximum sensitivity and specificity. Five pairs of oligonucleotide primers, directed against the B1, P30, and ribosomal genes, were used and compared to determine which sequences were most effective in detecting *T. gondii* DNA. Methods were developed with DNA templates in water and were subsequently applied to both normal and inflamed aqueous.

**RESULTS.** After one round of PCR amplification, P30 and ribosomal primers were able to detect 1 pg genomic *T. gondii* DNA. However, those directed against the B1 gene were able to detect 50 fg (approximately single tachyzoite). This level of sensitivity was also achieved using the P30 primers after a second round of PCR; however, only primers based on the B1 gene maintained this level of sensitivity in both normal and inflamed aqueous. B1-specific primers did not amplify sequences from fungal, bacterial, or human lymphocyte DNA. The sensitivity of *T. gondii* detection using B1 gene-specific primers was not compromised when large amounts of human lymphocyte DNA were present, and application to an ocular sample or retinal section from patients with toxoplasma chorioretinitis was successful in confirming the presence of *T. gondii* DNA.

**CONCLUSIONS.** The B1 PCR protocol appears to be the most sensitive protocol in the detection of *T. gondii* DNA and has been successful in identification of *T. gondii* DNA in ocular fluids and retinal sections. This provides direct evidence of the presence of *T. gondii* within the eye and may therefore help in the management of toxoplasma retinochoroiditis. (*Invest Ophthalmol Vis Sci.* 2000;41:634–644)

Diagnosis of *Toxoplasma gondii* retinochoroiditis can be difficult in atypical cases, as might occur in AIDS patients or in those cases where adequate funduscopy is precluded by overlying vitreous opacity. Because the clinical picture is often that of a panuveitis, associated with marked inflammatory activity in the anterior chamber, and because anterior chamber taps are associated with fewer complications than sampling from the vitreous cavity, an investigation was undertaken to determine whether *T. gondii* DNA could be detected in the aqueous humor. The development of a highly sensitive polymerase chain reaction (PCR) protocol to identify *T. gondii* DNA will help in the early diagnosis of unusual retinochoroiditis and facilitate the institution of appropriate treatment.

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PCR amplification of sequences within the B1, P30, and ribosomal genes of *T. gondii* have been assessed in a variety of tissues, in both immunocompetent and immunocompromised patients. Blood, bronchoalveolar lavage, cerebrospinal fluid, vitreous, liver, urine, amniotic fluid, cardiac tissue, cerebral tissue, and retina have all been investigated using these methods.

Several groups have reported the use of PCR analysis of aqueous humor in the diagnosis of ocular toxoplasmosis using oligonucleotide primers for DNA sequences within the B1 gene, the P30 gene, and the ribosomal gene with highly variable results.

In this study we have directly compared the sensitivity of previously described primer sequences in the amplification of specific target sequences from these *T. gondii* genes both in water and aqueous humor and have developed a PCR-based protocol that is capable of detecting a single *T. gondii* tachyzoite.

**MATERIALS AND METHODS**

Unless otherwise stated, all chemicals used were purchased from Sigma Chemical (Poole, UK) and were of the highest grade available.
Preparation of *T. gondii* Tachyzoites, Inoculation of Mice, and Isolation of DNA

Infection-free mice (MF1 strain) were inoculated intraperitoneally with 0.5 ml of RH strain tachyzoites at a concentration of 500,000/μl from a cell line that was originally isolated from a human encephalopathy sample.26 Tachyzoites isolated from peritoneal fluid were counted using a modified Fuchs Rosenthal CSF counting chamber. DNA was extracted after suspension in 2 ml lysis buffer (50 mM Tris–HCl [pH 8.0], 0.1 M NaCl, 10 mM EDTA, 2% sodium dodecyl sulfate, and 0.2 μg/ml proteinase K) and incubation at 50°C for 3 hours. Samples were extracted with phenol:chloroform (1:1), chloroform, precipitated with ethanol in the presence of 300 mM sodium acetate (pH 5.2), and air-dried after washing with 70% ethanol. DNA pellets were resuspended in TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA) and concentration assessed by both UV absorbance at 260 nm and by comparison with *Hind*III-digested lambda DNA standard. The DNA was diluted to 10 ng/μl with water and stored at −20°C.

**Collection of Aqueous Fluid**

The extracocular environment was sterilized with 5% povidone iodine solution before surgery. Approximately 100 to 200 μl of aqueous fluid was withdrawn using a 27-gauge (0.33-mm) needle via a limbal paracentesis before routine cataract surgery in patients with no evidence on funduscopy of *T. gondii* scars.

To test the effect, if any, of PCR amplification in the presence of inflamed aqueous, 150 μl of aqueous was also obtained from a patient with postoperative fibrinous uveitis. Before sampling, informed consent was obtained from all patients. The protocol for collection of aqueous samples was approved by the institutional review board at Moorfields Eye Hospital. This research followed the tenets of the Declaration of Helsinki at all times.

**Retina**

The sample of retinal tissue from an enucleation sample was obtained from the Department of Pathology at the Institute of Ophthalmology at Moorfields Eye Hospital (London, UK). Microscopy had confirmed the presence of *T. gondii* cysts within the tissue sections.

**Nested PCR Protocols**

One microliter of first-round product was used as a template for subsequent nested amplification. The negative control sample from the first-round amplification and a second-round negative control of sterile water only was included in the nested amplification.

**Amplification of the B1 Gene**

Two pairs of oligonucleotide primers directed against the B1 gene of *T. gondii*4 were used to perform a nested PCR using purified *T. gondii* DNA as a template (Table 1).

**B1 Gene: First-Round Amplification.** PCRs contained 10 mM Tris–HCl, pH 8.3 (at 25°C), 50 mM KCl, 2 mM MgCl2, 0.1 μM each primer, 0.1 mM each dNTP, 1.25 U Taq DNA polymerase, and varying quantities of purified *T. gondii* DNA. Reactions were cycled 40 times with denaturation at 95°C for 10 seconds followed by annealing at 57°C for 10 seconds and finally an extension step at 72°C for 50 seconds. PCR negative control sample omitted template DNA, which was substituted with sterile water.

**B1 Gene: Nested Amplification.** Nested reactions contained 1 μl first-round product, 10 mM Tris–HCl, pH 8.3 (at 25°C), 50 mM KCl, 3 mM MgCl2, 0.5 μM each primer, 0.1 mM each dNTP, and 1 U Taq DNA polymerase. Nested PCRs were cycled 40 times using a denaturation step of 95°C for 10 seconds, followed by annealing at 62.5°C for 10 seconds and extension at 72°C for 15 seconds. Negative control samples from first-round amplification and an additional second-round negative control of sterile water were included in the nested reactions.

**P30 Gene**

Nested primer sets identical to those used by Savva et al.24 and based on the published sequence of the *T. gondii* P30 gene27 were used to amplify regions of the P30 gene using a nested PCR approach (Table 2).

**P30 Gene: First-Round Amplification.** Each 25-μl PCR mixture consisted of 10 mM Tris–HCl, pH 8.3 (at 25°C), 50 mM KCl, 2 mM MgCl2, 0.2 μM of each primer, 0.1 mM of each dNTP, and 0.5 U Taq DNA polymerase and varying concentrations of purified *T. gondii* DNA. Reactions were cycled 35 times using a denaturation step of 95°C for 1 minute, annealing at 65°C for 1 minute, and extension at 74°C for 3 minutes. PCR negative control sample omitted template DNA, which was substituted with sterile water.

**P30 Gene: Nested Amplification.** One microliter of product was used as template for the nested amplification.
Each 25-μl reaction contained 10 mM Tris–HCl, pH 8.3 (at 25°C), 50 mM KCl, 1 mM MgCl₂, 0.4 μM of each primer, 0.1 mM of each dNTP, and 0.5 U Taq DNA polymerase. Nested reactions consisted of 35 cycles using a denaturation step at 95°C for 1 minute, followed by annealing at 66°C for 1 minute and extension at 74°C for 3 minutes. Negative control samples from first-round amplifications and an additional second-round negative control of sterile water were included in nested PCRs.

**Ribosomal Gene Amplification**

Oligonucleotide primers used to amplify ribosomal DNA were based on those used by Cazenave et al.25 and on the published sequence of the small subunit ribosomal RNA gene of *T. gondii* (Table 3). PCRs (25 μl) consisted of 10 mM Tris–HCl, pH 8.3 (at 25°C), 50 mM KCl, 2.5 mM MgCl₂, 0.2 μM of each primer, 25 μM of each dNTP, and 1.5 U Taq DNA polymerase and varying concentrations of purified *T. gondii* DNA. Reactions were heated at 95°C for 10 minutes, and cycled 35 times using a denaturation step of 95°C for 10 seconds, followed by annealing at 60°C for 30 seconds, and extension at 74°C for 1 minute. PCR negative control sample omitted template DNA, which was substituted with sterile water.

**PCR Amplification of Bacterial and Fungal Genomic DNA Using T. gondii B1 Gene Primers.** To show primer specificity, outer and nested B1 amplification reactions were carried out on 25-μl reaction mixtures that contained 10 ng genomic DNA from a variety of fungal and bacterial species. DNA from the following species was used as template: *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, *Aspergillus fumigatus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Streptococcus viridans*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*. Positive control consisted of 1 ng genomic *T. gondii* DNA. The outer negative control consisted of the 25-μl PCR without added DNA.

**Amplification of Human Lymphocyte Genomic DNA Using T. gondii B1 Gene–Specific Primers.** To ascertain that no human sequences were amplified with B1 gene–specific primers and that the presence of human lymphocyte DNA did not inhibit detection of *T. gondii* B1 template, 1 ng of *T. gondii* genomic DNA was added to different amounts of human DNA in sterile water and amplified using B1 gene primers.

**Amplification of the B1 Gene in Inflamed Aqueous.** To establish the volume of inflamed aqueous responsible for inhibition of B1 gene sequence, 1 ng of *T. gondii* genomic DNA was added to various volumes of inflamed aqueous and subjected to PCR amplification. To establish that the B1 PCR retained its sensitivity even in the presence of inflamed aqueous, various quantities of *T. gondii* genomic DNA were added to 5-μl volumes of aqueous that had been obtained from a patient with postoperative fibrinous uveitis. Samples were subjected to direct PCR analysis without prior DNA extraction.

**Sample Preparation from Paraffin-Embedded Tissue**

Retinal samples containing tissue cysts were deparaffinized according to a technique described by Wright and Manos.29 Briefly, 15-μm sections of paraffin embedded retina were cut, transferred to a 1.5-ml microcentrifuge tube, and deparaffinized by two xylene extractions. One milliliter of xylene was added to each tube, the closed tubes were then mixed at room temperature for approximately 30 minutes. Tissue and residual paraffin were then pelleted by centrifugation at 14,000 rpm for 5 minutes. After a second xylene extraction the xylene was removed by pipette. The samples were then washed twice with 100% ethanol to remove the organic solvent. A 0.5-ml aliquot of 100% ethanol was added to each tube, the contents of which were then mixed by inverting. Samples were then centrifuged for 5 minutes at 14,000 rpm, and the ethanol removed by pipette and the process repeated. The samples were resuspended in 100 μl digestion buffer (50 mM KCl, 10 mM Tris–HCl [pH 8.3], 1 mM EDTA, 2.5 mM MgCl₂, 0.1 mg/ml gelatin, 0.45% octylphenol–ethylene oxide condensate, 0.45% polyoxyethylene sorbitan monolaurate, 200 μg/ml proteinase K) and digested for 3 hours at 55°C. Proteinase K was inactivated by heating for 9 minutes at 95°C. After a 5-minute centrifugation at 14,000 rpm, 10 μl of supernatant was used for PCR amplification. One in 2 and 1 in 10 dilutions of this material also underwent PCR amplification. In addition 10 ng of genomic *T. gondii* DNA in 10 μl sterile water was also subjected to both the deparaffinizing process and the digestion process so as to serve as positive controls for these techniques. Negative extraction controls consisted of sterile water that had undergone both the deparaffinizing and digestion processes. Outer PCR positive controls consisted of 1 ng of genomic *T. gondii* DNA in 10 μl sterile water that underwent PCR without prior extraction or digestion, and a final PCR negative control consisted of sterile water that had not been subjected to either extraction or digestion.

**Visualization and Confirmation of PCR Amplification Products**

Ten-microliter B1 and P30 amplification products were visualized under UV illumination after electrophoresis on 1% to 2% TBE/agarose gels and staining with ethidium bromide. Twenty microliters of ribosomal gene amplification products was visualized on 4% TBE/metaphor agarose gels (Flowgen Instruments, Lichfield, UK) or on 8% to 10% TBE/polyacrylamide gels (Bio-Rad Laboratories, Hemel Hempstead, UK). A molecular weight marker was included in each run (1.0-kb ladder, catalog

**Table 3. Ribosomal Gene Primer Sequences**

<table>
<thead>
<tr>
<th>Oligonucleotide Primer</th>
<th>Sequence Position</th>
</tr>
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<tbody>
<tr>
<td>Sense strand primer</td>
<td>5'-CCTTGCCGCATTAGGTCTAGG-3'</td>
</tr>
<tr>
<td>Nonsense strand primer</td>
<td>5'-TAGGCATTCGGGGTAAAGATTA-3'</td>
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**RESULTS**

**Amplification of B1 Gene from T. gondii DNA**

A single amplicon with a predicted size of 193 bp was amplified using the first-round B1 gene primers. This reaction was capable of detecting 50 fg of *T. gondii* DNA after analysis on ethidium bromide-stained TBE/agarose gels (Fig. 1A). It has been estimated that this is the amount of DNA contained within a single *T. gondii* organism.29 When 1 µl of first-round product was used as template in a nested amplification, a 96-bp target sequence was amplified but showed no increase in the level of detection of *T. gondii* DNA (50 fg). After the nested reaction, however, there was a significant increase in the product yield (Fig. 1B). The nested reaction did not yield product from either the first- or the second-round negative controls. Both first-round and nested PCR products were cycle-sequenced, which confirmed that the amplified products were identical with the published sequence of the *T. gondii* B1 gene.4

**B1 Gene: Amplification of T. gondii DNA in Aqueous**

First-round amplification of *T. gondii* DNA in 20% aqueous (*T. gondii* DNA spiked into 5-µl aqueous in a total reaction volume of 25 µl) was able to detect 1 pg of *T. gondii* DNA (Fig. 1C). After second-round amplification using the nested primers this protocol was effective in detecting 50 fg of *T. gondii* DNA (Fig. 1D).

and visualized after ethidium bromide-staining under UV illumination. (D) Nested amplification of 96-bp product from 193-bp B1 PCR product generated from purified genomic DNA detects 50 fg *T. gondii* DNA in the presence of 20% normal human aqueous. Amplification of 96-bp PCR product from 193-bp product originally generated from varying quantities of *T. gondii* genomic DNA was achieved using nested primers 5'-TGCAATATGTTGCAATCGT-3' and 5'-GGCCAGCAATCTGGAATACC-3'. Lane 1, Molecular size marker (GIBCO-BRL). PCR products used for nested amplification were originally amplified from the following: Lane 2, 1 ng *T. gondii* genomic DNA; lane 3, 100 pg *T. gondii* genomic DNA; lane 4, 10 pg *T. gondii* genomic DNA; lane 5, 1 pg *T. gondii* genomic DNA; lane 6, 100 fg *T. gondii* genomic DNA; lane 7, 50 fg *T. gondii* genomic DNA; and lane 8, nested negative control. PCR products (10 µl) were resolved on 2% agarose/agarose gel and visualized after ethidium bromide-staining under UV illumination.

Before sequencing of PCR products, amplified DNA from PCRs was purified using the Gene clean II kit (BIO 101 Inc., Carlsbad, California). PCR products were excised from agarose/TBE gels, solubilized in sodium iodide and recovered into solution according to the manufacturer's instructions. PCR fragments were directly cycle sequenced in both directions (using the outermost primers for each gene) on an ABI prism automated DNA sequencer (model 377, version 2.1.1). DNA sequences were compared with target sequences and found to be identical in all cases.
Amplification of *T. gondii* P30 Gene

Amplification of the P30 gene using outer primers resulted in a single product of approximately 914 bp. Amplification of the P30 gene using this protocol was able to detect 1 pg of purified *T. gondii* DNA after analysis on ethidium bromide–stained TBE/agarose gels (Fig. 2A). When 1 µl of first-round PCR product was used as template in the nested amplification, a single amplicon of approximately 520 bp was observed. The sensitivity of the reaction was 50 fg of *T. gondii* DNA (Fig. 2B). Both the outer and nested products were subject to cycle sequencing, and confirmed products were identical with published sequences.

Amplification of the P30 Gene from *T. gondii* DNA in Aqueous

First-round amplification of *T. gondii* DNA in 20% aqueous (*T. gondii* DNA spiked into 5 µl aqueous in a total reaction volume of 25 µl) was able to detect 1 ng of *T. gondii* DNA (Fig. 2C). However, second-round amplification using the nested primers was only able to detect 1 pg *T. gondii* DNA in the presence of 20% aqueous humor compared with 50 fg *T. gondii* DNA in the absence of aqueous humor (Fig. 2D).

Amplification of 18S rDNA Gene from *T. gondii* DNA

An 88-bp sequence from the 18S rDNA was used as the target sequence for amplification.²⁵ Using oligonucleotide primers based on those used by Cazenave et al. (1990),²⁵ amplification of the ribosomal gene from *T. gondii* allowed detection of 1 pg *T. gondii* DNA (Fig. 3A). Sequence analysis confirmed the identity of the 88-bp amplicon.

Amplification of 18S rDNA Gene from *T. gondii* DNA in Aqueous

When different concentrations of purified *T. gondii* DNA were supplemented with 5 µl aqueous, ribosomal gene amplification detected only 1 pg of purified *T. gondii* DNA (Fig. 3B).

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**FIGURE 2.** (A) Amplification of 914-bp P30 PCR product from purified genomic DNA detects 1 pg *T. gondii* DNA. Varying quantities of *T. gondii* genomic DNA were subjected to PCR amplification using specific P30-oligonucleotide primers 5'-TTGCGGCGCCCACAGTG-3' and 5'-GGGAGGACAGGCGTAGT-3'. Lane 1, Molecular size marker (Promega UK Ltd.); lane 2, 1 ng *T. gondii* genomic DNA; lane 3, 100 pg *T. gondii* genomic DNA; lane 4, 10 pg *T. gondii* genomic DNA; lane 5, 1 pg *T. gondii* genomic DNA; lane 6, 100 fg *T. gondii* genomic DNA; lane 7, 50 fg *T. gondii* genomic DNA; and lane 8, no *T. gondii* genomic DNA (negative control). PCR products (10 µl) were resolved on 1% agarose/TBE gels and visualized after ethidium bromide-staining under UV illumination. (B) Nested amplification of 522-bp product from 914-bp P30 PCR product generated from purified genomic DNA detects 50 fg *T. gondii* DNA. Amplification of 522-bp PCR product from 914-bp product originally generated from varying quantities of *T. gondii* genomic DNA was achieved using nested primers 5'-CGACAGCCGCGTGACCTGC-3' and 5'-GCAACCAGTGACCTGC-3'. Lane 1, Molecular size marker (Promega UK Ltd.). PCR products used for nested amplification were originally amplified from the following: lane 2, 1 ng *T. gondii* genomic DNA; lane 3, 100 pg *T. gondii* genomic DNA; lane 4, 10 pg *T. gondii* genomic DNA; lane 5, 1 pg *T. gondii* genomic DNA; lane 6, 100 fg *T. gondii* genomic DNA; lane 7, 50 fg *T. gondii* genomic DNA; and lane 8, no *T. gondii* genomic DNA (negative control). PCR products (10 µl) were resolved on 1% agarose/TBE gels and visualized after ethidium bromide-staining under UV illumination. (C) Amplification of 914-bp P30 PCR product from purified genomic DNA detects 1 ng *T. gondii* DNA in the presence of 20% normal human aqueous. Varying quantities of *T. gondii* genomic DNA were subjected to PCR amplification using specific P30-oligonucleotide primers 5'-TTGCGGCGCCCACAGTG-3' and 5'-GGGAGGACAGGCGTAGT-3'. Lane 1, Molecular size marker (Promega UK Ltd.); lane 2, 1 ng *T. gondii* genomic DNA; lane 3, 100 pg *T. gondii* genomic DNA; lane 4, 10 pg *T. gondii* genomic DNA; lane 5, 1 pg *T. gondii* genomic DNA; lane 6, 100 fg *T. gondii* genomic DNA; lane 7, 50 fg *T. gondii* genomic DNA; and lane 8, no *T. gondii* genomic DNA (negative control). PCR products (10 µl) were resolved on 1% agarose/TBE gels and visualized after ethidium bromide-staining under UV illumination.
PCR of T. gondii Genes in Aqueous Humor

fibrous uveitis, product inhibition was seen when the reaction mix contained greater than 20% inflamed aqueous (Fig. 5A). When 1 µl of the outer reaction was added to a final nested reaction mix of 25 µl, a 96-bp amplicon was produced even when the outer reaction contained 60% inflamed aqueous. A decrease in the amount of product however was seen in the nested reaction if the outer reaction contained more than 40% by volume (10 µl) of inflamed aqueous (Fig. 5B). Because previous reports have indicated that 20% of aqueous fluid per PCR can inhibit the reaction,30 we have routinely used 5-µl samples of aqueous per 25 µl reaction for subsequent work. After adding varying amounts of genomic T. gondii DNA to 5 µl of inflamed aqueous, a 193-bp amplicon was produced down to a sensitivity of 1 pg T. gondii DNA (Fig. 5C). When 1 µl of

**FIGURE 3.** (A) Amplification of an 88-bp PCR product from the 18S rDNA gene from purified genomic DNA detects 1 pg T. gondii DNA. Varying quantities of T. gondii genomic DNA were subjected to PCR amplification using specific 18S rDNA oligonucleotide primers 5'-CCTTGGGCCATAGGTCTAGG-3' and 5'-TAGGCACTCGGGTAAAGATTAGTA-3'. Lane 1, Molecular size marker (GIBCO-BRL); lane 2, 1 ng T. gondii genomic DNA; lane 3, 100 pg T. gondii genomic DNA; lane 4, 10 pg T. gondii genomic DNA; lane 5, 1 pg T. gondii genomic DNA; lane 6, 100 fg T. gondii genomic DNA; lane 7, 50 fg T. gondii genomic DNA; and lane 8, no T. gondii genomic DNA (negative control). PCR products (20 µl) were resolved on 4% TBE/metaphor agarose gels (Flowgen Instruments) and visualized after ethidium bromide–staining under UV illumination. (B) Amplification of an 88-bp PCR product from the 18S ribosomal DNA gene from purified genomic DNA only detected 1 pg T. gondii DNA in the presence of 20% normal human aqueous. Varying quantities of T. gondii genomic DNA were subjected to PCR amplification using specific 18S rDNA oligonucleotide primers 5'-CCTTGGGCCATAGGTCTAGG-3' and 5'-TAGGCACTCGGGTAAAGATTAGTA-3'. Lane 1, Molecular size marker (GIBCO-BRL); lane 2, 1 ng T. gondii genomic DNA; lane 3, 100 pg T. gondii genomic DNA; lane 4, 10 pg T. gondii genomic DNA; lane 5, 1 pg T. gondii genomic DNA; lane 6, 100 fg T. gondii genomic DNA; lane 7, 50 fg T. gondii genomic DNA; and lane 8, no T. gondii genomic DNA (negative control). PCR products (20 µl) were resolved on 4% TBE/metaphor agarose gels (Flowgen Instruments) and visualized after ethidium bromide–staining under UV illumination.

**FIGURE 4.** (A) Amplification of 193-bp B1 PCR product from 1 ng purified T. gondii genomic DNA in 20% normal human aqueous in the presence of human lymphocyte DNA. One nanogram T. gondii genomic DNA in a background of increasing amounts of human genomic DNA was subjected to PCR amplification using specific T1-oligonucleotide primers 5'-GGAACGTGACCTTCTGCTG-3' and 5'-TCTTTAAAAGGGTGTGGTGC-3'. Lane 1, Molecular size marker (GIBCO-BRL); lane 2, 1 ng T. gondii genomic DNA plus 10 ng human DNA; lane 3, 1 ng T. gondii genomic DNA plus 50 ng human DNA; lane 4, 1 ng T. gondii genomic DNA plus 100 ng human DNA; lane 5, 1 ng T. gondii genomic DNA plus 150 ng human DNA; lane 6, no DNA (negative control); lane 7, 150 ng human DNA only; and lane 8, 1 ng T. gondii genomic DNA only (positive control). PCR products (10 µl) were resolved on 2% agarose/TBE gels and visualized after ethidium bromide–staining under UV illumination. (B) Nested amplification of 193-bp B1 PCR product from 1 ng purified T. gondii genomic DNA in 20% normal human aqueous in the presence of human lymphocyte DNA. DNA was added to a first-round reaction mix contained greater than 20% inflamed aqueous (Fig. 5A). When 1 µl of the outer reaction was added to a final nested reaction mix of 25 µl, a 96-bp amplicon was produced even when the outer reaction contained 60% inflamed aqueous. A decrease in the amount of product however was seen in the nested reaction if the outer reaction contained more than 40% by volume (10 µl) of inflamed aqueous (Fig. 5B). Because previous reports have indicated that 20% of aqueous fluid per PCR can inhibit the reaction,30 we have routinely used 5-µl samples of aqueous per 25 µl reaction for subsequent work. After adding varying amounts of genomic T. gondii DNA to 5 µl of inflamed aqueous, a 193-bp amplicon was produced down to a sensitivity of 1 pg T. gondii DNA (Fig. 5C). When 1 µl of

**B1 Gene–Specific Primer Sequences Do not Amplify Sequences from Human, Bacterial, and Fungal Genomic DNAs**

Primer directed against the T. gondii B1 gene did not produce an amplicon when the template genomic DNA was derived from human lymphocytes (Lane 7, Fig. 4B), bacterial or fungal species.

**Amplification of B1 Gene from 1 ng T. gondii DNA in the Presence of Human Lymphocyte DNA**

The presence of increasing amounts of human lymphocyte DNA (10–150 ng) did not inhibit either the amplification of 193-bp B1 PCR product from 1 ng purified T. gondii genomic DNA in 20% normal human aqueous or the nested amplification of 96-bp product generated from 193-bp B1 PCR product (Fig. 4).

**Amplification of the B1 Gene in Inflamed Aqueous**

When 1 ng of genomic T. gondii DNA was added to a first-round PCR containing inflamed aqueous from a patient with...
this outer reaction was added to a nested reaction in a final volume of 25 μl, the reaction was capable of detecting 50 fg of *T. gondii* DNA, which is an identical sensitivity to the detection of *T. gondii* DNA in water (Fig. 5D).

B1 Gene PCR Amplification of *T. gondii* DNA from a Vitreous Sample in a Patient with Active Toxoplasmic Retinochoroiditis

Previous work in this laboratory has demonstrated equivalent results for inhibition of PCR by aqueous and vitreous fluids. Therefore, the protocols described herein were applied to a sample of vitreous taken from a patient with active *T. gondii* retinochoroiditis. This sample was boiled at 95°C for 20 minutes and then divided into four 5-μl aliquots. To the first three of these, *T. gondii* genomic DNA was added. No additional DNA was added to the fourth aliquot. The samples then underwent PCR amplification using first-round primers directed against *T. gondii* B1 gene in a 25-μl reaction mix. One microliter of the outer reaction was then added to a 25-μl nested PCR. First-round amplification of 193-bp B1 PCR product from vitreous obtained from a patient with *T. gondii* retinochoroiditis was detected only in the positive control sample that had been spiked with 1 ng *T. gondii* genomic DNA (Fig. 6A). However, nested amplification of 96-bp B1 PCR product generated from vitreous obtained from a patient with *T. gondii* retinochoroiditis was detected in all samples (Fig. 6B).

B1 Gene Amplification of *T. gondii* DNA from Paraffin-Embedded Sections

Four deparaffinized retinal sections from a patient with histopathologic evidence of *T. gondii* cysts, typical of Toxoplasma retinochoroiditis, were subjected to PCR using primers directed against the B1 gene. After first-round amplification a single 193-bp product was visualized from reactions in three of the four sections on 2% agarose gels (Fig. 7A). All negative controls remained negative. Positive and negative controls that had been subjected to the deparaffinizing procedure all remained positive and negative, respectively (Fig. 7B).

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**Figure 5.** (A) First-round amplification of 193-bp B1 PCR product from 1 ng *T. gondii* genomic DNA in inflamed aqueous. One nanogram *T. gondii* genomic DNA spiked into different quantities of inflated aqueous were subjected to PCR amplification using specific B1-oligonucleotide primers 5'-GGAACGTGACATCGGATCATAG-3' and 5'-TCCTTAAGGGTTCGTCGTTAG-3'. Lane 1, Molecular size marker (GIBCO-BRL); lane 2, 1 ng *T. gondii* genomic DNA in 1 μl aqueous; lane 3, 1 ng *T. gondii* genomic DNA in 5 μl aqueous; lane 4, 1 ng *T. gondii* genomic DNA in 10 μl aqueous; lane 5, 1 ng *T. gondii* genomic DNA in 15 μl aqueous; lane 6, 1 ng *T. gondii* genomic DNA in water (positive control); and lane 7, no *T. gondii* genomic DNA (negative control). PCR products (10 μl) were resolved on 2% agarose/TBE gels and visualized after ethidium bromide-staining under UV illumination. (B) Nested amplification of 96-bp B1 PCR product generated from 1 ng purified genomic *T. gondii* DNA in inflamed human aqueous. Amplification of 96-bp PCR product from 193-bp product originally generated from 1 ng *T. gondii* genomic DNA was achieved using nested primers 5'-TGCATAGGCGAATCTGCGAATACACC-3' and 5'-GGCGACCAATCTGCGAATACACC-3'. Lane 1, Molecular size marker (GIBCO-BRL); PCR products used for nested amplification were originally amplified from the following: lane 2, 1 ng *T. gondii* genomic DNA in 1 μl aqueous; lane 3, 1 ng *T. gondii* genomic DNA in 5 μl aqueous; lane 4, 1 ng *T. gondii* genomic DNA in 10 μl aqueous; lane 5, 1 ng *T. gondii* genomic DNA in 15 μl aqueous; lane 6, 1 ng *T. gondii* genomic DNA in water (outer positive control); lane 7, first-round negative control after nested reaction. Additionally, lane 8, 1 ng *T. gondii* genomic DNA second-round amplification only (second-round positive control); lane 9, nested negative control. PCR products (10 μl) were resolved on 2% agarose/TBE gels and visualized after ethidium bromide-staining under UV illumination. (C) Amplification of 193-bp B1 PCR product from purified genomic DNA detects 1 pg *T. gondii* DNA in the presence of 20% inflamed human aqueous. Varying quantities of *T. gondii* genomic DNA were subjected to PCR amplification using specific B1-oligonucleotide primers 5'-GGAACGTGACATCGGATCATAG-3' and 5'-TCCTTAAGGGTTCGTCGTTAG-3'. Lane 1, Molecular size marker (GIBCO-BRL). PCR products used for nested amplification were originally amplified from the following: lane 2, 1 ng *T. gondii* genomic DNA; lane 3, 100 pg *T. gondii* genomic DNA; lane 4, 10 pg *T. gondii* genomic DNA; lane 5, 1 pg *T. gondii* genomic DNA; lane 6, 100 fg *T. gondii* genomic DNA; lane 7, 50 fg *T. gondii* genomic DNA; and lane 8, no *T. gondii* genomic DNA (negative control). PCR products (10 μl) were resolved on 2% agarose/TBE gels and visualized after ethidium bromide-staining under UV illumination. (D) Nested amplification of 96-bp product from 193-bp B1 PCR product generated from purified genomic DNA detects 1 pg *T. gondii* DNA in the presence of 20% inflamed human aqueous. Amplification of 96-bp PCR product from 193-bp product originally generated from varying quantities of *T. gondii* genomic DNA was achieved using nested primers 5'-TGCATAGGCGAATCTGCGAATACACC-3' and 5'-GGCGACCAATCTGCGAATACACC-3'. Lane 1, Molecular size marker (GIBCO-BRL). PCR products used for nested amplification were originally amplified from the following: lane 2, 1 ng *T. gondii* genomic DNA; lane 3, 100 pg *T. gondii* genomic DNA; lane 4, 10 pg *T. gondii* genomic DNA; lane 5, 1 pg *T. gondii* genomic DNA; lane 6, 100 fg *T. gondii* genomic DNA; lane 7, 50 fg *T. gondii* genomic DNA; lane 8, 10 pg *T. gondii* genomic DNA; lane 9, first-round negative control after nested reaction; and lane 10, nested negative control. PCR products (10 μl) were resolved on 2% agarose/TBE gels and visualized after ethidium bromide-staining under UV illumination.
T. gondii infection of the retina is characterized by massive coagulative necrosis of the retina with inflammation of the underlying choroid.\textsuperscript{33,34} Toxoplasma retinochoroiditis is usually a clinical diagnosis based on examination of the retina and recognition of the characteristic lesions; however, this can be difficult in atypical cases or where overlying vitreous opacity precludes adequate fundoscopy.

Current laboratory methods are limited by their lack of sensitivity and specificity. Serology is of limited value in diagnosing T. gondii as the etiologic agent of disease in the eye. If the patient is antibody negative, T. gondii as the cause of a retinal lesion is improbable,\textsuperscript{35,36} although cases of ocular toxoplasmosis with negative serum titers against T. gondii have been described.\textsuperscript{37,38} Positive serology is a sensitive test but is not specific, because serologic studies suggest that asymptomatic toxoplasmosis occurs with great frequency in the general population.\textsuperscript{35,36,39} Analysis of local antibody production to confirm a suspected clinical diagnosis of toxoplasma retinochoroiditis is a valuable diagnostic tool in the immunocompetent patient; however, false-positive\textsuperscript{33} and false-negative\textsuperscript{36} results can be a problem.

A variety of different patients are at risk from this visually debilitating disease. Immunocompromised hosts, as a result of immunosuppressive therapy, malignancy, or AIDS, are at high risk of disseminated toxoplasmosis. It has been estimated that 1% to 3% of ocular infections in AIDS patients are due to T. gondii.\textsuperscript{40} The disease, which may be bilateral, may be the initial ocular infection in patients, sometimes preceding serologic diagnosis of the HIV disease\textsuperscript{41,42} and is often fulminant and aggressive.\textsuperscript{43} Prominent inflammatory reactions in both the vitreous and anterior chamber are common\textsuperscript{44,45} and in proportion to the patient’s CD4+ count.\textsuperscript{46} Neither the aqueous coefficient nor the serologic tests is helpful in making the diagnosis of ocular toxoplasmosis in AIDS patients.\textsuperscript{41,45,46} The latter may remain negative throughout the clinical course of the disease.\textsuperscript{47}

In the HIV patient population it is essential to establish the diagnosis as rapidly as possible. Early and appropriate treatment of ocular toxoplasmosis in AIDS patients is vital, because not only is the ocular prognosis good\textsuperscript{43} but also because ocular disease often coexists with life-threatening cerebral involvement\textsuperscript{40,41,47} and may well precede neurologic signs.\textsuperscript{40,44} None of which are pathognomonic of T. gondii involvement in the central nervous system.

Molecular biological methods have been investigated to aid in the clinical management of this condition. The use of PCR to amplify and subsequently detect DNA within microorganisms in a range of tissues, and particularly those that are difficult to culture\textsuperscript{48} or for which sample volumes are small, has proven extremely valuable.\textsuperscript{51} The ability to identify T. gondii DNA in ocular samples will provide direct evidence of the presence of the organism within the eye and therefore be helpful in determining the diagnosis of T. gondii infection and subsequent patient management.

**Primer Selection**

The B1, P30, and ribosomal DNA genes are highly conserved in all T. gondii strains tested to date,\textsuperscript{4,8,49} and the B1 and ribosomal genes are multiple copy genes within the T. gondii genome, making them ideal targets for PCR amplification.

The B1 gene is a 35-fold repetitive gene sequence with unknown function. Within eukaryotes, ribosomal DNA is fre-
Selection of B1 Gene Primers

PCR amplification of DNA target sequences within the B1 gene had several advantages. As with the P30 it allowed for greater sensitivity than amplification of the ribosomal gene. However, it has been shown that the P30 primers are less specific than those of the B1 gene, because they have been demonstrated to amplify DNA targets of both the *Nocardia* species and *Mycobacterium tuberculosis* DNA.53 We have shown that the *T. gondii* B1 primers do not amplify DNA from a variety of bacterial and fungal species and that the reaction sensitivity remains unchanged in the presence of increasing amounts of human DNA, and in the presence of the increased levels of protein found in inflamed aqueous. It is, however, possible that other parasites present in the tissue may be amplified by these PCR techniques. To ensure the specificity of the PCR primer, sequences were compared with all other sequenced DNA available from current databases and were found to be noncomplementary to any other known sequence. The possibility does exist, however, that a related organism could be identified using these primers, an organism whose gene sequence remains hitherto unknown. To minimize this possibility the PCR amplification was originally amplified from the following: Lane 1, supernatant from first retinal section that had not been further diluted or had been diluted 1 in 2 (lane 4) or 1 in 10 (lane 6). Lanes 7, 8, and 9, supernatant from third retinal section that had not been further diluted (lane 7) or had been diluted 1 in 2 (lane 8) or 1 in 10 (lane 9). Lanes 10, 11, and 12, supernatant from fourth retinal section that had not been further diluted (lane 10) or had been diluted 1 in 2 (lane 11) or 1 in 10 (lane 12). PCR products (10 μl) were resolved on 2% agarose/TBE gels and visualized after ethidium bromide-staining under UV illumination.

Sensitivity

Nested amplification of the P30 and B1 genes allowed detection of as little as 50 fg of *T. gondii* DNA in water. In the aqueous, only amplification of the B1 gene yielded this level of sensitivity. Nested amplification of the P30 gene in the aqueous allowed detection only to a level of 1 pg *T. gondii* DNA.

The ribosomal gene was the least sensitive of these reactions, only allowing detection of the predicted 88-bp target down to a DNA concentration of 1 pg in water. This is perhaps surprising given that it is the most highly repeated of the gene sequences studied, but may be due at least in part to the fact that the ribosomal amplification protocol used only a single reaction.55 Nested reactions increased the yield of product for both the other genes amplified.

Interlaboratory variability is a confounding factor when analyzing results and trying to compare different methodologies used at different centers. This study has used protocols published by others and optimized all three PCRs in the one laboratory. We have concluded that, at least in our hands, the B1 gene primers and PCR protocol are the most sensitive of the three tested.

Figure 7. (A) First-round amplification of 193-bp B1 PCR product from deparaffinized retinal sections obtained from a patient with *T. gondii* retinochoroiditis. Ten microliter quantities of supernatant from deparaffinized retinal sections from a patient with *T. gondii* retinochoroiditis that had undergone protein extraction were subjected to PCR amplification using specific B1-oligonucleotide primers 5'-GGAACTG-CATCCGTTCATGAG-3' and 5'-TCTTTAAGCGTTCGTGGTC-3'. Lanes 1, 2, and 3, supernatant from first retinal section that had not been further diluted (lane 1) or had been diluted 1 in 2 (lane 2) or 1 in 10 (lane 3). Lanes 4, 5, and 6, supernatant from second retinal section that had not been further diluted (lane 4) or had been diluted 1 in 2 (lane 5) or 1 in 10 (lane 6). Lanes 7, 8, and 9, supernatant from third retinal section that had not been further diluted (lane 7) or had been diluted 1 in 2 (lane 8) or 1 in 10 (lane 9). Lanes 10, 11, and 12, supernatant from fourth retinal section that had not been further diluted (lane 10) or had been diluted 1 in 2 (lane 11) or 1 in 10 (lane 12). PCR products (10 μl) were resolved on 2% agarose/TBE gels and visualized after ethidium bromide-staining under UV illumination. (B) Control samples used during first-round amplification of 193-bp B1 PCR product from deparaffinized retinal sections obtained from a patient with *T. gondii* retinochoroiditis. Lanes 1 through 3, *T. gondii*-positive extraction controls; 10 ng genomic *T. gondii* DNA spiked into undiluted supernatant (lane 1) and supernatant that had been diluted 1 in 2 (lane 2) and 1 in 10 (lane 3). Lanes 4 and 5, blank. Lanes 6, 7, and 8, negative extraction controls (supernatant from extraction with no added *T. gondii* DNA) undiluted (lane 6) and diluted 1 in 2 (lane 7) and diluted 1 in 10 (lane 8). Lane 9, blank. Lane 10, 10 ng *T. gondii* DNA spiked into sterile water, PCR positive control. Lane 11, blank. Lane 12, sterile water, no added DNA; PCR-negative control. PCR products (10 μl) were resolved on 2% agarose/TBE gels and visualized after ethidium bromide-staining under UV illumination. (C) Nested amplification of 96-bp B1 PCR product generated from deparaffinized retinal sections obtained from a patient with *T. gondii* retinochoroiditis. Amplification of 96-bp PCR product from deparaffinized retinal sections from a patient with *T. gondii* retinitis was achieved using nested primers 5'-TGATAGGTGCACTGAC-3' and 5'-GGCGAC-CAATCTGCGAATACACC-3'. PCR products used for nested amplification were originally amplified from the following: Lane 1, supernatant from first retinal section that had not been further diluted or had been diluted 1 in 2 (lane 2) or 1 in 10 (lane 3). Lane 4, supernatant from second retinal section that had not been further diluted or had been diluted 1 in 2 (lane 5) or 1 in 10 (lane 6). Lane 7, supernatant from third retinal section that had not been further diluted or had been diluted 1 in 2 (lane 8) or 1 in 10 (lane 9). Lane 10, supernatant from third retinal section that had not been further diluted or had been diluted 1 in 2 (lane 11) or 1 in 10 (lane 12). PCR products (10 μl) were resolved on 2% agarose/TBE gels and visualized after ethidium bromide-staining under UV illumination.
is performed under stringent conditions. Reaction specificity is facilitated by using the highest possible annealing temperatures compatible with maximum reaction sensitivity. Finally, sequencing of all amplified PCR products was performed and confirmed the identity of the genes amplified.

**Application to Clinical Samples**

Although our initial investigations used spiked aqueous samples to assess the efficacy of PCR detection of *T. gondii*, the first clinical sample was vitreous obtained from a patient with suspected *T. gondii* retinochoroiditis. In this case a vitrectomy was performed because the patient had developed a giant retinal tear, but had an active lesion that was clinically suspicious of *T. gondii* retinochoroiditis, and was already receiving prednisolone EC 20 mg bid, and clindamycin 150 mg bid for ocular toxoplasmosis.

Previous work in this laboratory has demonstrated a lack of PCR inhibition if the volume of vitreous does not exceed 20% of the final reaction volume.33 Because the B1 gene primers proved to be both specific and the most sensitive of the primers tested, PCR protocols using this primer set were applied to the detection of *T. gondii* genes in ocular fluids and retinal sections. A positive result was obtained with no “vitreous inhibition” of the PCR, because all the vitreous samples spiked with genomic DNA from *T. gondii* were positive with a sensitivity of 100 fg. The applicability of these protocols to a large number of aqueous samples has yet to be determined.

In conclusion, the B1 PCR protocol appears to be not only highly specific in the amplification of *T. gondii* DNA but also, in our hands, to be the most sensitive protocol in the detection of *T. gondii* and has been successful in the identification of *T. gondii* DNA in both vitreous and retinal sections. The protocols described in this article have potential in aiding the future management of patients with *T. gondii* infection.

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