Direct and Sensitive Detection of a Pathogenic Protozoan, *Toxoplasma gondii*, by Polymerase Chain Reaction

J. LAWRENCE BURG,† CHRISTOPHER M. GROVER, PHILIPPE POULETAY,‡ AND JOHN C. BOOTHROYD*  
Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305-5402  
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We applied the polymerase chain reaction to detection of the pathogenic protozoan *Toxoplasma gondii* based on our identification of a 35-fold-repetitive gene (the B1 gene) as a target. Using this procedure, we were able to amplify and detect the DNA of a single organism directly from a crude cell lysate. This level of sensitivity also allowed us to detect the B1 gene from purified DNA samples containing as few as 10 parasites in the presence of 100,000 human leukocytes. This is representative of the maximal cellular infiltration (10⁷/ml) in 1 ml of cerebrospinal fluid obtained from patients with toxoplastic encephalitis. The B1 gene is present and conserved in all six *T. gondii* strains tested to date, including two isolates from patients with acquired immunodeficiency syndrome. No signal was detected by using this assay and DNAs from a variety of other organisms, including several which might be found in the central nervous system of an immunocompromised host. This combination of sensitivity and specificity should make detection of the B1 gene based on polymerase chain reaction amplification a very useful method for diagnosis of toxoplasmosis both in immunocompromised hosts and in congenitally infected fetuses.

There are many instances in the diagnosis of infectious diseases in which it is desirable to detect a pathogen directly. A prominent example is the plethora of opportunistic infections of patients with acquired immunodeficiency syndrome (AIDS; 2, 42) which cannot be reliably diagnosed by serology because of severe immune system dysfunction. The polymerase chain reaction (PCR; 33) has already been used in detection of human immunodeficiency virus types 1 (17, 25, 27) and 2 (30), human papillomavirus (24, 35), human T-cell lymphoma virus (3, 11), and cytomegalovirus (9, 34) from a variety of clinical specimens, including blood, urine, and formalin-fixed tissue specimens, without prior extraction of DNA. In several instances, PCR has allowed direct diagnosis of viral diseases when serologic methods have failed (13, 19). We describe below the application of PCR to detection of the pathogen protozoan *Toxoplasma gondii*.

*T. gondii* can cause severe neurological disease or death in developing human fetuses (22) and in patients immunosuppressed as a result of drug therapy or disease (12, 15, 20). Particularly at risk are patients with AIDS (16, 23, 26), among whom the prevalence of toxoplasmosis is about 10% (14, 36) with the number of such afflicted persons dramatically increasing as the number of AIDS cases rises. In about 5% of AIDS cases, toxoplasmosis is the first indication of human immunodeficiency virus infection (14).

Fetal toxoplasmosis remains a significant disease as a result of acute parasitic infection in mothers not previously infected; consequences of infection are most severe if it occurs during the first trimester. Effective prenatal diagnosis can permit a decision to terminate the pregnancy or initiate treatment of late-term fetuses in utero (8, 10).

Current diagnosis in utero relies on a combination of detection of specific immunoglobulin M, culture of amniotic fluid and fetal blood, and other nonspecific measurements of infection (10). The only definitive diagnosis is by culture, which takes up to 3 weeks. A more rapid method of direct detection could offer justification to start antibiotic therapy immediately.

Current diagnosis of *T. gondii* infection in patients with AIDS cannot rely on serology but rather relies on computerized tomography or magnetic resonance imaging scans and brain biopsies to identify parasites encysted within the brain (6, 26). Such technology is time-consuming, expensive, and associated with considerable risk. New procedures, such as PCR, are therefore desirable for the diagnosis of toxoplasmosis both in developing fetuses and in immunocompromised hosts.

**MATERIALS AND METHODS**

**Preparation of *T. gondii* cells and DNA.** *T. gondii* C56 and independent strains A1 and A2 isolated from two patients with AIDS were grown in mouse ascites after intraperitoneal injection (strains provided by J. S. Remington, Palo Alto Medical Foundation, Palo Alto, Calif.). *T. gondii* RH tachyzoites were grown in a monolayer of human foreskin fibroblasts in Eagle minimal essential medium (GIBCO Laboratories) supplemented with 3% fetal bovine serum and antibiotics (28). All strains were purified from host cell material by harvesting lysed cultures, passing the cell suspension through a 27-gauge needle, and filtering through a CF-11 cellulose (Whatman, Inc.) column (37) or a 3-μm nitratecellulose filter (Nucleapore Corp.) (41). Individual *T. gondii* organisms were isolated by using a single-laser fluorescence-activated cell sorter (FACS; Becton Dickinson FACSTAR) after labeling by indirect immunofluorescence with a mouse monoclonal antibody (DG-52) to the p30 antigen of *T. gondii*, followed by fluorescein isothiocyanate-conjugated goat anti-mouse antiseraum. Cells were sorted under a narrow window directly into 0.5-ml microcentrifuge tubes (Sarstedt) and stored frozen.

Whole parasites were prepared for amplification by being heated in microcentrifuge tubes to 94°C for 10 min in 50 μl of deionized water to completely lyse the cells. Positive and negative mock clinical samples (representative of the cellular contents of cerebrospinal fluid [CSF]) were prepared by mixing uninfected human peripheral blood leukocytes (iso-
luted over a 6% glucose gradient column, followed by two 30-s hypotonic lyses in deionized water to rupture residual erythrocytes) with or without whole T. gondii cells. A mixture of human and parasite DNAs was prepared from these samples by incubation for 2 h at 60°C in 200 μg of proteinase K per ml-0.2% sodium dodecyl sulfate-10 mM Tris hydrochloride (pH 7.4)-10 mM NaCl-10 mM EDTA-10 μg of tRNA per ml, followed by phenol-chloroform extraction and ethanol precipitation.

The BI gene. The BI gene was isolated as described elsewhere (4). The complete sequence of the BI gene as cloned in plasmid vector pAT153 (38; to give pTXgB1) was determined by using the chemical modification method of Maxam and Gilbert (21).

The copy number of the BI gene was determined by comparative hybridization using a titration of plasmid DNA. Serially diluted samples of pTXgB1 DNA (5,860 base pairs) were mixed with 1 μg of EcoRI-digested calf thymus carrier DNA and electrophoresed on a 0.7% agarose gel. Two dilutions of genomic DNA (1 and 0.25 μg, containing less than 5% host DNA contamination) were digested with EcoRI and run in parallel (T. gondii is haploid, with a DNA content of about 7 × 10^6 base pairs [7]). The plasmid amounts, 16.8, 8.4, 4.2, 2.1, 1.0, 0.42 ng, correspond to 200, 100, 50, 25, 10, and 5 times, respectively, the number of moles of a single-copy gene found in 1 μg of genomic DNA.

Following electrophoresis, the DNA was transferred to nitrocellulose and hybridized with a nick-translated 2.2-kilobase (kb) BI fragment in 3 × SSC (1 × SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate)-1% Denhardt solution (1 × Denhardt solution contains 0.02% Ficoll 400, 0.02% bovine serum albumin, and 0.02% polyvinylpyrrolidone)-50 μg of carrier DNA (calf thymus) per ml at 65°C. Following autoradiography, the bands were cut out of the nitrocellulose and the radioactivity of each was quantitated in a scintillation counter.

Amplification procedures. Four oligonucleotides (oligos) were synthesized on an Applied Biosystems DNA synthesizer. Two are of the same sense as the sequence presented in Fig. 2: oligo 1 corresponds to BI gene nucleotides 694 to 714 (5'-GGACTGCTTCGCGATTTAGGCT-3'), and oligo 2 (5'-TTATTACGACTCATATAGGTTGCATAGTTGCGCAGTT-3') comprises 40 nucleotides, 20 nucleotides constituting the sense strand promoter sequence for T7 RNA polymerase (underlined), followed by 20 nucleotides of the BI gene sequence (757 to 776). Oligos 3 and 4 are of the opposite sense and correspond to nucleotides 853 to 831 (5'-GGCGACCAATCTGCAGAATACACC-3') and 887 to 868 (5'-CTTTAAAGCGTTCCGTC-3'), respectively, on the antisense strand.

The amplification reactions were performed with the thermostable DNA polymerase of Thermus aquaticus (Taq; Cetus Corp.) as previously described (32). Briefly, 2.5 U of Taq polymerase was used in a 100-μl reaction volume with 10 mM Tris (pH 8.3)-2.5 mM MgCl_2-100 μM deoxyribonucleoside triphosphates (Pharmacia)—each oligo at 1.0 μM-0.01% gelatin—whole-cell lysates or purified DNA, as indicated. Samples were overlaid with 100 μl of paraffin oil (J. T. Baker Chemical Co.) in 0.6-ml tubes (Sarstedt) and amplified for 25 to 60 cycles in an automated PCR machine (Perkin-Elmer-Cetus, Eriocmp, or a machine built in our laboratory). Each cycle consisted of 1 min of denaturation at 93°C, 1 to 2 min at the annealing temperature of 55°C, and 1.5 to 3.0 min of extension at 72°C. The final extension step continued for an additional 5 min.

Hybridization assays. Quantitation of amplified products was performed as follows. Various amounts of T. gondii RH genomic DNA (TOXO) or plasmid DNA containing a cloned BI gene (STANDARDS; 4) were digested with EcoRI and analyzed by agarose gel electrophoresis and Southern blot hybridization with a nick-translated 2.2-kb labeled BI probe. The autoradiograph is shown here. The numbers above the standards represent molar equivalents of plasmid DNA relative to the number of moles of a single-copy gene in 1 μg of toxoplasma DNA. The numbers above the TOXO lanes indicate the amounts (micrograms) of genomic DNA loaded.

FIG. 1. Characterization of the BI gene as a target for amplification. Various amounts of T. gondii RH genomic DNA (TOXO) or plasmid DNA containing a cloned BI gene (STANDARDS; 4) were digested with EcoRI and analyzed by agarose gel electrophoresis and Southern blot hybridization with a nick-translated 2.2-kb labeled BI probe. The autoradiograph is shown here. The numbers above the standards represent molar equivalents of plasmid DNA relative to the number of moles of a single-copy gene in 1 μg of toxoplasma DNA. The numbers above the TOXO lanes indicate the amounts (micrograms) of genomic DNA loaded.

RESULTS

Identification and characterization of a repetitive target (BI) in T. gondii. The BI gene was isolated as described elsewhere (4). Briefly, a T. gondii RH genomic library in λgt11 was screened by using mouse polyclonal antiserum to a lysate of T. gondii P tachyzoites. One of the recombinant bacteriophage thus identified contained a 2.2-kb EcoRI genomic fragment which was subsequently found to be tandemly repeated in the genome (4; see below). This gene is arbitrarily referred to as the BI gene. The insert from this phage was subcloned into the EcoRI site of plasmid vector pAT153 (38; to give pTXgB1 and further characterized.

Because of its repetitive nature, the BI gene is an attractive target for detecting T. gondii parasites through amplification of BI-specific DNA. As the first step in exploiting this gene as a target, we sought to precisely quantitate the number of BI repeats in the parasite genome. To do this, quantitative Southern blot analysis was performed by using defined amounts of BI plasmid (mixed with carrier DNA) and total genomic DNAs. The autoradiograph presented in Fig. 1 shows that the BI gene is between 25- and 50-fold repetitive; quantitation (data not shown) of the radioactivity present in each band more precisely indicated that there are about 35 copies of the BI gene in the T. gondii genome.
deletion in the NCBI database, but this was not verified experimentally.}

Figure 2 also indicates the locations of the oligos used for amplification and detection. Oligos 1 and 2 were used as upstream primers for amplification, while oligo 4 corresponds to the opposite strand of the B1 gene and was used as the downstream primer for PCR. Oligo 3 was used as a radiolabeled probe internal to the amplified region.

Amplification of cell lysates of different strains. In a first experiment, we used PCR to amplify a segment of the B1 gene from whole-cell lysates of several strains of T. gondii. In one such experiment (Fig. 3), the B1 target was directly amplified from about 100 T. gondii organisms from each of four strains, including two recent isolates from patients with AIDS (A1 and A2) and the RH and C56 strains. After 25 cycles of PCR, the B1 gene was easily detected in all four strains and yielded roughly equivalent signals. The B1 gene has also been amplified from two additional strains, P (39) and C (29) (data not shown). It thus appears to be well conserved among the six strains tested to date, at least over the three regions represented by the oligos used for amplification and detection. Although we did not directly determine the copy number of the B1 gene in each of these strains, these data suggest that it is of similar magnitudes in all of the strains. (Use of 10-fold greater and lesser amounts of cells gave proportionately more or less signal for each strain [data not shown], indicating that amplification did not reach a plateau and that the results were roughly quantitative.) Most importantly, the data show that the B1 gene is sufficiently
repetitive in each isolate to allow PCR detection with roughly similar sensitivity for each.

To determine the specificity of this assay, amplification and probing using the same set of oligos were attempted with DNAs from a variety of closely related organisms, as well as those which might also be found in the central nervous system of an immunocompromised host. These included Sarcocystis, Neospora, Plasmodium, Aspergillus, Candida, Cryptococcus, and Absidia spp. In no case was a signal detectable (data not shown), indicating that this combination of oligos is specific for T. gondii.

**Detection of amplified product from a single organism.** To accurately assess the sensitivity of PCR for detection of the \( B1 \) gene, we used a FACS to place 1, 3, 5, 10, and 100 \( T. gondii \) parasites directly into tubes for amplification as cell lysates. In one such experiment (Fig. 4), 30% of the reaction products were detected by slot blot hybridization after 60 cycles of PCR amplification. The signal from a single cell was easily detected in this autoradiograph in one replicate sample (lane 2) but only barely seen in the other (lane 3) after 17 h of exposure. We attribute the weakness of the signal in lane 3 to the kinetics of amplification (any delay in amplification during the initial cycles has dramatic effects on the final level of the product). In subsequent experiments, we found detection of a single cell to be highly reproducible, both within a single amplification experiment and throughout a series of experiments (data not shown).

To more closely approximate a clinical sample of CSF or blood which would contain not only Toxoplasma cells but also human cells, we isolated human leukocytes from peripheral blood and combined them with our FACS-selected samples of \( T. gondii \). Since we are particularly interested in the possibility of direct detection in CSF, we used levels of human cells that represent the maximum generally observed in CSF from patients with AIDS. In studies with a combined total of 42 patients, Koppel et al. (16) and Snider et al. (36) reported a median of 3 to 4 leukocytes per mm\(^3\) (or 3 \( \times \) 10\(^3\) to 4 \( \times \) 10\(^3\)/ml). We used 100,000 human cells, therefore, to represent an approximate upper limit of the concentration likely to be encountered in such samples.

Initially, attempts were made to directly amplify mixtures of human and \( T. gondii \) cells following heating to 94°C in distilled water. With this procedure, little or no amplification was observed (data not shown). To obtain amplification in the presence of this number of cells, therefore, we tried extracting the DNA from the samples before amplification. Hence, 100,000 leukocytes were added to samples of 1, 10, 100, and 1,000 \( T. gondii \) organisms, and the DNA was extracted as described in Materials and Methods. After 55 cycles of PCR amplification, a signal from as few as 10 toxoplasma organisms was easily detected in the presence of the DNA of 100,000 human leukocytes (Fig. 5; lane 5) after 6 h of exposure, while the signal from a single \( T. gondii \) cell under the same conditions could not be detected in this or in other similar experiments, even after longer exposure of the autoradiograph (lane 4). Lane 3 (0 \( T. gondii \) organisms and 100,000 human leukocytes) indicates that the \( B1 \) target could not be detected by amplification in the human genome; this result was confirmed by repeated experiments.

**DISCUSSION**

Our results show that a single \( T. gondii \) parasite can be directly detected from cell lysate materials by PCR using the 35-fold-repetitive \( B1 \) gene as a target for amplification. With 100,000 human leukocytes and DNA extraction, as few as 10 organisms were detected by using the \( B1 \) gene as a target for amplification. Our results also show that the \( B1 \) gene is highly specific for \( T. gondii \) and is well conserved among all of the strains tested to date, including several isolates from patients with AIDS.

Further optimization of extraction, amplification, and detection procedures may enhance the reaction sensitivity to permit detection of a single parasite in the presence of 100,000 human cells. Detection of the \( \beta \)-globin gene after amplification from single human sperm cells (unextracted) in a background of 100 ng of Escherichia coli DNA (18) suggests that the 35-fold-repetitive \( B1 \) target in a single \( T. gondii \) parasite should be detectable within a background of 660 ng of human DNA (the amount of DNA in 100,000 cells). Other investigators, however, have documented decreased amplification of low-titer targets from samples with high levels of background DNA or after extraction. For example, Abbott et al. have shown that 1 \( \mu \)g of negative DNA can have a 50-fold inhibitory effect on amplification of low-titer targets (1), while Shibata et al. found that extraction of cytomegalovirus-positive culture supernatants was variable.

**FIG. 4.** Direct amplification of a single \( T. gondii \) cell lysate as selected by FACS. \( T. gondii \) cells were labeled by indirect immunofluorescence (using fluorescein isothiocyanate-conjugated goat anti-mouse antiserum and a mouse monoclonal antibody [DG-52] to the major surface antigen of \( T. gondii \)) and sorted directly into amplification tubes by a FACS. Samples of 0, 1, 3, 5, and 100 cells were then amplified through 60 cycles of PCR by using oligos 1 and 4 as described in Materials and Methods. For each reaction product, except the 100-cell product which had 5% (i.e., one-sixth of the material in the other slots), 30% was denatured by formaldehyde treatment and detected by slot blot hybridization with oligo 3 as the probe. The filter also contained material in the other slots), (data not shown), indicating that this combination of oligos is specific for \( T. gondii \).

**FIG. 5.** Amplification of DNA prepared from 0, 1, 10, 100, and 1,000 FACS-selected \( T. gondii \) organisms (Toxo) in the presence of 100,000 peripheral blood leukocytes (wbc). DNA was prepared from these samples, suspended in water, and amplified through 55 cycles of PCR with oligos 1 and 4 as described in Materials and Methods. Half of the amplified product is shown in a slot blot hybridization format along with 65 ng of unamplified PAT plasmid plus 1 \( \mu \)g of oligos 1 and 4 (PAT lane). Lane 2 was not extracted; lane 3 was extracted in the presence of 100,000 uninfected peripheral blood leukocytes. Lanes 2 and 3 contained no \( T. gondii \) parasites.
and less sensitive than direct amplification after cell lysis by boiling (34). Our results confirm that some sensitivity is lost in the transition to mock clinical samples, which require extraction and contain high levels of background human DNA. However, most CSF samples contain less than 10% of the human background DNA of our mock clinical samples (16, 26, 36). Thus, we expect that it may be possible to amplify many samples without prior extraction and with minimal preparation.

Our interest in CSF is because of anecdotal reports that tachyzoites of T. gondii are occasionally seen in pelleted material from patients with AIDS who have toxoplasma encephalitis (J. S. Remington, personal communication). However, our results also suggest that the DNA of the parasite may be directly detected in the buffy coat of peripheral blood smears, since T. gondii invades monocytes (40) and can be isolated from blood by inoculation into mice (31). PCR amplification of the B1 gene should also be applicable to the diagnosis of congenitally acquired toxoplasmosis by detection of the parasite in amniotic fluid samples. One study demonstrated that 52% of fetal infections can be identified by xenodiagnosis from amniotic fluid cells (8). An assay based on amplification of the B1 target would be faster than xenodiagnosis and offer the potential for enhanced sensitivity by detection of nonviable as well as viable parasites.

These results indicate that PCR amplification of the B1 gene of T. gondii may be a viable alternative to the more time-consuming and less direct procedures currently used. Assessment of this approach in a carefully controlled analysis of clinical samples is now necessary.

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ADDITIONAL PROOF

Six additional isolates (provided by J. S. Remington) from patients with AIDS were all positive by PCR amplification of the B1 gene. Also positive were strains from Japan (pig), England (sheep), and Australia (human; strains provided by J. K. Frenkel, Kansas University Medical Center, Kansas City).

LITERATURE CITED