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Detection of the Pathogenic Parasite *Toxoplasma gondii* by Specific Amplification of Ribosomal Sequences Using Comultiplex Polymerase Chain Reaction

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Amplification of DNA sequences from ribosomal DNA (rDNA) was tested as a specific and sensitive method for the detection of small numbers of *Toxoplasma gondii* tachyzoite cells. We applied the polymerase chain reaction (PCR) on the basis of detection of the 110-fold repetitive rDNA as a target by using (i) DNA sequences within the small ribosomal subunit known to be universal and conserved in all eukaryotes and (ii) small ribosomal subunit and intergenic spacer rDNA sequences known to be *T. gondii* species specific. The level of sensitivity obtained from a crude cell lysate allowed the detection of as few as one parasite visualized directly as a specific PCR product in agarose gels. By using a combination of universal and *T. gondii* species-specific primers, we propose a comultiplex-based PCR approach as a new diagnostic tool. The combination of sensitivity, specificity, and built-in positive and negative PCR controls should make detection of the rDNA sequences by comultiplex PCR a useful clinical test for the diagnosis of toxoplasmosis and for epidemiological studies. Finally, the idea of a built-in positive control to support or counter the *T. gondii*-specific PCR result is novel and is a notable advance.

Toxoplasma gondii is an obligate intracellular protozoan parasite that has an extremely wide host range and that can survive in all nucleated cells of mammals, including humans (20). The establishment of *T. gondii* within a modified host cell vacuole leads to chronic infection and the production of cysts in skeletal muscle and the central nervous system (22). The parasite is described as an opportunistic pathogen because these cysts, which can exist for the life of its host, occasionally rupture and release highly invasive tachyzoites which may cause a disseminated and potentially fatal disease if the host is in a state of immune deficiency.

In patients with immunosuppression caused by complex therapy or disease, *T. gondii* has become one of the leading causes of central nervous system infections and encephalitis. The reactivation of toxoplasmosis is particularly striking in patients with AIDS, among whom the prevalence is about 10 to 15% (19, 27). An infection acquired during childhood is usually asymptomatic in 80 to 90% of cases. Toxoplasmosis acquired during pregnancy remains an important disease in mothers not previously infected, especially if the disease occurs during the first trimester. Thus, the parasite can cause spontaneous abortion, fetal abnormalities, or perinatal death in several species of mammals, including humans (10).

Current diagnosis of toxoplasmosis is based on isolation and growth of the parasite in mice and fibroblast cell culture and by showing a seroconversion of immunoglobulin G antibodies for primary infection and detection of specific immunoglobulin M (2, 16). All these methods are time-consuming, serodiagnosis of toxoplasma is complicated by the presence of cross-reactive antibodies, and in many patients with immunosuppression such as those with AIDS, serology is not an alternative. One of the rapid alternatives that has been tested on clinical specimens is an assay for the detection of the presence of *T. gondii*. The assay is based on in vitro DNA amplification of a sequence within the repetitive (35-fold) B1 gene by the polymerase chain reaction (PCR) and detection of the amplified product by a radioactive probe (3). Rapid prenatal diagnosis of congenital *Toxoplasma* infection by using PCR and amniotic fluid has been shown to be rapid and highly sensitive by this method (13). In contrast, PCR amplification of the B1 gene identified toxoplasmic genomes in frozen tissue from only one of nine patients with toxoplasmic lymphadenitis (28).

Recently, a PCR-based assay was developed for *T. gondii* by using a repetitive $TGR1_E$ genomic sequence and a sequence within the partly known rRNA (4, 8).

In recent years, rRNA sequences have been shown to be unique to particular organisms or related groups of organisms and, hence, offer targets for hybridization probes or PCR amplification with various specificities (21). Actively growing cells may contain 10^4 ribosomes, each a potential PCR target, making it possible to label and identify a single cell (12). In this report we describe a sensitive and rapid PCR-based method for the detection of *T. gondii*. The approach was based on the detection of the multicopy *T. gondii*-specific ribosomal DNA (rDNA) sequence shown to exist at 110 copies per tachyzoite cell, and the PCR product was visualized directly in agarose gels that included built-in positive and negative controls.

MATERIALS AND METHODS

Growth and purification of parasites. The *T. gondii* RH tachyzoites were grown in transformed murine macrophages (P388D1 cell line) (5) in 150-cm^2 flasks in Eagle essential medium (GIBCO-Bethesda Research Laboratories, Mississauga, Ontario, Canada) supplemented with 2 mM glutamine–0.22% $NaHCO_3$–10% fetal calf serum–antibiotics (14, 17). After visual lysis (3 to 7 days), the supernatants

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were collected, concentrated by low-speed centrifugation (1,000 × *g*), and washed twice in 10 mM phosphate-buffered saline (PBS). Tachyzoites were separated from host cell debris and purified by filtration in two steps by using polycarbonate membranes (pore sizes of 5 and 3 μm, respectively; Nuclepore, Toronto, Ontario, Canada) (9). Parasites were concentrated as pellets by centrifugation, resuspended in 1 ml of PBS, and kept frozen at −85°C in 20% glycerol.

Selection of primers and DNA synthesis. The nucleotide sequences of the intergenic spacer (IGS) region and the small subunit (SSU) rRNA gene of *T. gondii* have been described previously (11, 14, 15). Primers were selected on the basis of sequence homology to conserved regions of rRNA from other eukaryotes available in the GenBank and EMBL data banks. The oligonucleotides listed in Table 1 were synthesized by the phosphoramidite method (Pharmacia-LKB Biotechnology, Baie d'Urfé, Québec, Canada) and were visualized by UV shadowing after purification on 7 M urea–20% sequencing gels (1).

Preparation of DNA templates for PCR. DNA templates were prepared from tachyzoites in 100 μl of sterile deionized water by using a freezing-boiling technique. Plasmid DNA was prepared by standard methods and purified by CsCl-ethidium bromide equilibrium density gradient ultracentrifugation (23).

Amplification protocol. The reagents used for PCR amplification (Gene Amp Kit) were purchased from Perkin-Elmer Cetus (Montréal, Québec, Canada), and mixtures were prepared according to the instructions of the manufacturer. Reaction mixture samples of 100 μl were amplified in the DNA thermal cycler (Perkin-Elmer Cetus), which was programmed for 35 cycles of amplification. Parameters for the amplification cycles consisted of 1 min at 94°C (denaturation), 2 min at 50°C (primer annealing), and 3 min at 72°C (primer extension-polymerization). An aliquot of the reaction mixture was analyzed by agarose gel (1%) electrophoresis, and the products were stained with ethidium bromide and visualized under UV light (23).

Determination of rDNA copy number. The copy number of the rDNA unit was determined by comparative hybridization by using a titration of plasmid DNA as described previously (3). A dilution series of 0.75, 3.75, 7.5, 15, and 37.5 ng of pTOX2 plasmid DNA corresponds to 10, 50, 100, 200, and 500 times the number of moles of a single-copy gene found in 1 μg of genomic DNA, respectively. The pTOX2 plasmid DNA samples were mixed with 1 μg of *Eco*RI-digested salmon sperm carrier DNA and three dilutions of genomic DNA (0.25, 0.5, and 1 μg), and the mixture was digested with *Eco*RI and separated by agarose gel electrophoresis. Copy number calculations take into account the fact that pTOX2 is 5,300 bp (pTZ18R plus the known sequence of the 2,397-bp *Bam*HI-*Eco*RI fragment containing the IGS and flanking sequences from pTOX1 shown in Fig. 1) and the fact that the *T. gondii* haploid genome is about 7×10^7 bp (7). The DNA was transferred to nylon membranes (Amersham Corporation, Oakville, Ontario, Canada) and was hybridized with 100 ng of the 800-bp *Bgl*II-*Bgl*II rDNA fragment that was purified by electroelution and radiolabeled with [α-^{32}P]dCTP by random priming (Prime Time C Kit, IBI; Terochem Scientific, Montréal, Québec, Canada). Hybridization was done under high-stringency conditions (42°C, 50% formamide–5× SSC [1× SSC is 0.15 M NaCl plus 0.15 M sodium citrate]–5× Denhardt–50 mM phosphate buffer–1 mg of sonicated and denatured salmon sperm DNA per ml). The filters were washed in 0.1× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7])–0.1% sodium dodecyl sulfate four times at 65°C for 15 min each time and were then autoradiographed (23, 26).

RESULTS

Amplification of *T. gondii* DNA. In a first attempt to demonstrate amplification of the nucleic acids of *T. gondii*, we used PCR to amplify various regions of the rDNA unit from purified pTOX1 DNA. We decided to test a series of oligonucleotides selected within the SSU and IGS sequences, as depicted in Fig. 1, and determine whether a unique amplification product could be detected for each set of primers. In each case, the five sets of primers used (Table 1) could amplify a unique DNA fragment, as shown in Fig. 2, and the sizes obtained for each PCR product are listed in Table 2.

Amplification of DNA from crude tachyzoite lysates. In the next step, we envisaged amplifying segments of the rDNA from whole-cell lysates, but using two distinct pairs of primers; a universal primer set would serve as a PCR positive control and one set was *T. gondii* specific. To determine the specificity of the primers used, we selected a pair of primers (NS 31 and NS 41; B in Table 2) that, when aligned with a collection of ribosomal sequences, spans a conserved region in all eukaryotic sequences (data not shown). The second pair of primers was selected as being *T. gondii* species specific on the basis of (i) the fact that it resides within the IGS, which in all eukaryotic IGS sequences is generally thought of as species specific, and (ii) the fact that the nucleotide sequences from the EH 24 and HE 27 primers selected within the IGS (E in Table 2) showed no significant homology with other DNA sequences available in data banks. In these experiments, we decided to test a serial dilution of tachyzoites starting with approximately 10^2 cells and then diluting them to 10^0 cell. Results obtained after PCR amplification are shown in Fig. 2 and 3. We noted for *T. gondii*-specific primers that the presence of more than 10^6 cells gave no amplified product (data not shown), while amplified DNA fragments from one lysed cell were still easily visible in agarose gels (Fig. 3, lane 4). The results obtained with the comultiplex primers including the universal primer pair also showed amplification of specific *T. gondii* DNA fragments (Fig. 3). Inhibition of PCR was evident when high cell concentrations were used (10^6 cells per ml; data not shown).

Determination of rDNA copy number. Because *T. gondii* primers were an attractive prospect for detecting the parasite by PCR and because we wanted to maximize the sensitivity down to the level of detection of a single tachyzoite by using rDNA primers, we attempted to quantitate the number of rDNA copies in the genome. The autoradiogram presented in Fig. 4 indicates that the *T. gondii* rDNA is 110 copies per genome equivalent.

Sensitivity of *T. gondii* PCR. To better assess the capability of detecting a single tachyzoite cell when using PCR, we estimated the number of tachyzoite cells by microscopic observations and performed a series of PCRs on diluted material. The results of one such experiment are shown in Fig. 3. The signal from one cell was still visible, even with only 35 cycles of amplification.

Since *T. gondii* is an obligate intracellular parasite and since we wanted to optimize the PCR assay with representative clinical samples, we constructed a mock sample by mixing various dilutions of tachyzoites (10^0 to 10^3) with 10^1 to 10^5 human fibroblasts. After 35 cycles of amplification, a signal was still detectable in agarose gels from approximately

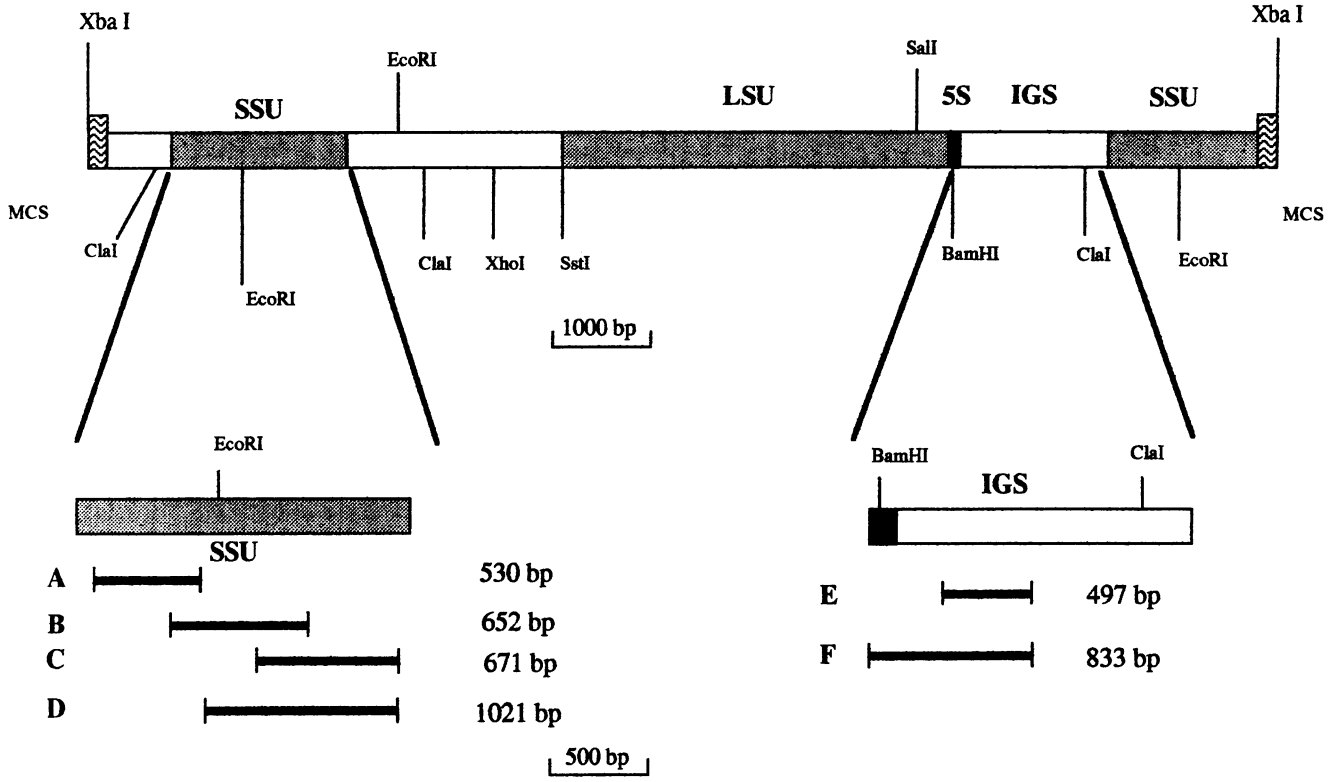

FIG. 1. Physical and genetic map of the 7.5-kb rDNA from *T. gondii*. The rDNA unit is encompassed within a 10-kb *Sau*3A fragment subcloned into the *Xba*I site of pTZ18R and named pTOX1 (14). The rRNA genes are indicated by the shaded boxes, the 5S gene is indicated by the solid box, and the multiple cloning sites are indicated by rectangles with wavy lines. The dark lines labeled A, B, C, D, and E represent the PCR-amplified DNA fragments in the SSU and the IGS. The size of each PCR product is indicated, and the primers used are listed in Tables 1 and 2. Only the *T. gondii* moiety cloned is represented in pTOX1. Abbreviations: SSU, small ribosomal subunit; LSU, large ribosomal subunit; IGS, intergenic spacer; MCS, multiple cloning site; bp, base pair; 5S, 5S ribosomal gene.

10 tachyzoites in the high background of 10^5 human fibroblasts. The signal from 10^0 tachyzoites in the background of 10^4 human fibroblasts was detectable, but the signal in the high background 10^5 human fibroblasts was not detectable (data not shown).

DISCUSSION

Diagnosis of toxoplasmosis is based on a series of serological and time-consuming tests that cannot be performed per se on clinical samples from patients with immunosuppression or AIDS. The results presented here describe an exciting alternative that can be applied directly to clinical material. This alternative is PCR that uses the 110-fold repetitive rDNA unit as a target for amplification. The approach that we developed included a set of universal eukaryotic primers used as PCR-positive controls. It would be highly unlikely that a PCR product would be obtained with the *T. gondii*-specific EH 24-HE 27 primers (E in Table 2) and that a negative result would be obtained with the NS

TABLE 1. Oligonucleotide primers used in the study

Primer	Sequence	Anchor region	Specificity	Reference
SSU 11	5′-TGCCTCTTCCCCTGGAAGGC-3′	18S	*T. gondii*	This study
SSU 5	5′-GGAGAAATCCAGAAGGATGC-3′	18S	*T. gondii*	This study
SSU 51	5′-GCATCCTTCTGGATTTCTCC-3′	18S	*T. gondii*	This study
SSU 6	5′-GAGATAGGAAAACGTCATGC-3′	18S	*T. gondii*	This study
SSU 41	5′-ATTTCGGGCACGAACGCGCC-3′	18S	*T. gondii*	This study
SU5S 1	5′-TTGTAGAGCGATTATCC-3′	5′ of 5S	*T. gondii*	This study
EH 24	5′-GGCACAACGAGCGCCACGC-3′	IGS	*T. gondii*	This study
HE 27	5′-TCATGAAGATCTGTTCATT-3′	IGS	*T. gondii*	This study
B1-1	5′-GGAACTGCATCCGTTCATGAG-3′	B1 gene	*T. gondii*	3
B1-4	5′-TCTTAAAAGCGTTCGTGGTC-3′	B1 gene	*T. gondii*	3
NS 31	5′-TTGGAGGGCAAGTCTGGTGCC-3′	18S	Universal[a]	26a
NS 41	5′-CCCGTGTTGAGTCAAATTA-3′	18S	Universal[a]	26a

[a] Universal primers are sequences found in all eukaryotes.

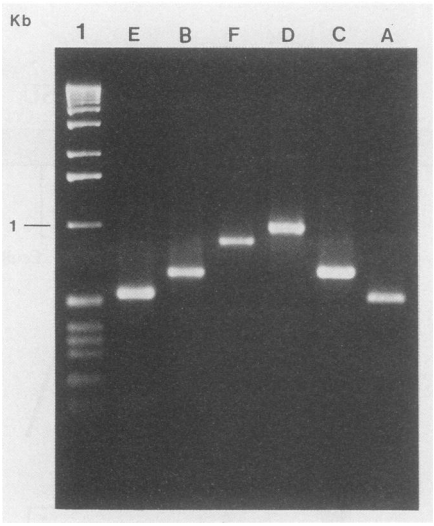

FIG. 2. Agarose gel (1.2%) electrophoresis of nucleic acid amplification products from purified pTOX1 DNA template with a series of universal primers and the IGS-specific *T. gondii* primers listed in Table 1. Lanes: 1, 1-kb DNA ladder (GIBCO-Bethesda Research Laboratories); E, pTOX1 DNA with EH 24 and HE 27 primers; B, pTOX1 DNA with NS 31 and NS 41 primers; F, pTOX1 DNA with EH 24 and SU5S 1 primers; D, pTOX1 DNA with SSU 51 and SSU 41 primers; C, pTOX1 DNA with SSU 6 and SSU 41 primers; A, pTOX1 DNA with SSU 11 and SSU 5 primers. PCR fragments A, B, C, D, E, and F that identify each lane are described in Table 2.

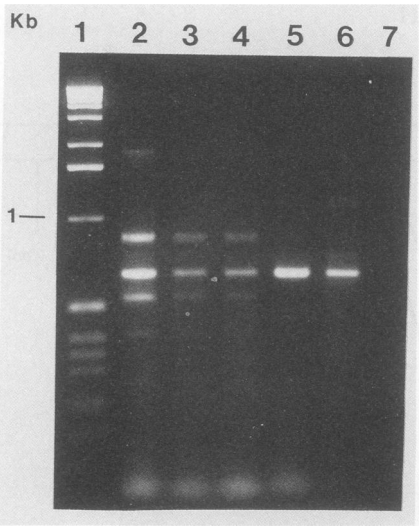

FIG. 3. Agarose gel (1.2%) electrophoresis of nucleic acid amplification products from crude cells lysis DNA templates with the multiplex *T. gondii* primers. These primers are EH 24, HE 27, SU5S 1 (E and F fragments in Table 2; *T. gondii* specific), and NS 31 and NS 41 B fragment (universal). Lanes: 1, 1-kb DNA ladder (GIBCO-Bethesda Research Laboratories); 2, DNA from tachyzoite (100 cells); 3, DNA from tachyzoite cells (10 cells); 4, one tachyzoite DNA; 5, DNA from uninfected murine macrophage (10^4 cells); 6, DNA from human primary fibroblasts (10^4 cells); 7, multiplex primers without template DNA.

31-NS 41 universal primers (B in Table 2). When this is the case, one can assume that the target material used for PCR does not contain DNA or is inhibitory to the reaction (18). There are a number of reports in which PCR inhibition has been associated with high levels of background DNA, blood, glycerol, high GC content in target sequences, and unidentified components that could be present in any clinical specimen (24, 25). In our hands, we did not observe a negative PCR result with universal primers or a positive PCR result with *T. gondii* species-specific primers when tachyzoites or any host cell DNA was present.

To further optimize diagnosis by PCR, we also selected other specific primer sets that should amplify *T. gondii* rDNA (Table 2). Indeed, diagnosis of a parasitic disease on the basis of visualization of a single PCR product is risky, and spurious PCR products have been reported (18). We envisaged that comultiplex PCR (6) with the selected primer pairs listed in Table 2 would give a ladder of amplified fragments of 497, 652, and 833 bp (primer sets E, B, and F, respectively; Table 2), which represents an extremely powerful and viable alternative test for use in a clinical setting. For comparative purposes, we also tested the B1-1 and B1-4 primers reported previously (3). The PCR products could not be visualized directly and easily in a 1% agarose gel because it represents a 174-bp fragment that comigrates with the excess of primers and was undoubtedly detected only by autoradiography. However, it has been accurately assessed

TABLE 2. Oligonucleotide primer pairs used in the study and size of the amplified product

Identification in Fig. 1[a]	Primer pair	Amplification product (bp)
A	SSU 11 and SSU5	530
B	NS 31 and NS 41	652
C	SSU 6 and SSU 41	671
D	SSU 41 and SSU 51	1,021
E	EH 24 and HE 27	497
F	EH 24 and SU5S1	833
	B1-1 and B1-4	130

[a] Pairs B, E, and F were used in the comultiplex assay.

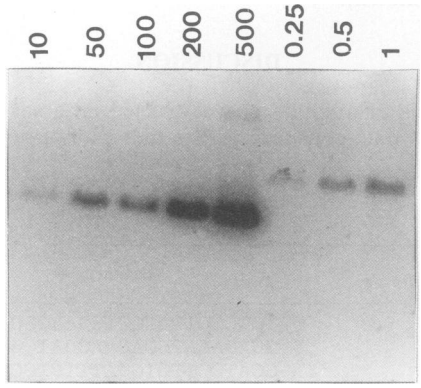

FIG. 4. Determination of the copy number for the *T. gondii* rDNA unit. Various amounts of *T. gondii* RH genomic DNA and the pTOX2 recombinant plasmid DNA were digested with *Eco*RI and were then separated by agarose gel (0.7%) electrophoresis. The autoradiograph obtained after Southern-type gel hybridization with the ^{32}P-labeled IGS rDNA probe is shown. The numbers above the standards (10 to 500) indicate molar equivalents of plasmid DNA (in copies of pTOX2 DNA) relative to the number of moles of a single-copy gene in 1 μg of *T. gondii* DNA, and the number above *T. gondii* lanes (0.25 to 1) indicate the amounts (in micrograms) of DNA used.

that the sensitivity of PCR for detection of the B1 *T. gondii* gene can detect an amplified product from a single organism. The alternative that we proposed here makes the detection of a single tachyzoite even easier, but careful evaluation with clinical specimens is a prerequisite.

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This work is dedicated to the memory of John S. Wolfson, Massachusetts General Hospital and Harvard Medical School, who represented the best of the scientist and the artist in all of us.

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