Detection of *Toxoplasma gondii* Parasitemia by Gene Amplification, Cell Culture, and Mouse Inoculation

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Diagnosis of *Toxoplasma gondii* infection is difficult, especially in immunosuppressed people. The AIDS epidemic has increased the number of people at risk and increased the need for better diagnostic methods. We have compared three methods for detection of *T. gondii* parasitemia. Rabbits were infected subcutaneously with 10^4 *T. gondii* tachyzoites. Blood samples were obtained, and buffy coat or leukocyte fractions were prepared. We sought the *T. gondii* B1 gene by gene amplification by the polymerase chain reaction, and we sought viable *T. gondii* cells by inoculating fibroblast cell cultures and by mouse inoculation. Thirty-two blood samples were obtained from seven infected rabbits, and 18 were obtained from four control, uninfected rabbits. Parasitemia was detected in 20 of 32 samples (62%) from infected samples by mouse inoculation, 12 of 32 samples (37%) by gene amplification and detection, and 8 of 32 samples (25%) by cell culture. Mouse inoculation requires use of live animals and has a long turnaround time. Currently, cell culture is the least sensitive but most practical and widely available method for the detection of *T. gondii* parasitemia. Gene amplification and detection was more sensitive than cell culture and may become available in clinical laboratories as techniques are developed further and automated.

Toxoplasma gondii infects up to half of all the people in the world, and toxoplasmosis is becoming increasingly common with the spread of AIDS. The diagnosis of toxoplasmosis is difficult because most people with severe disease are immunosuppressed and suffer from reactivation of chronic (dormant) infection. In such patients, antibodies are already present in serum and their numbers do not increase in response to reactivation.

Detection of the organism in solid tissue may be misleading unless it is done with a test that differentiates between tachyzoites, the proliferative form of the parasite, and cysts, which persist in chronic infection. In contrast, detection of the organism in blood implies active infection, since parasites are not thought to circulate during chronic infection. Cell culture (9, 20, 21), mouse inoculation (5, 6), and gene amplification (10, 23) have all been used to detect parasitemia, but these techniques have not been extensively compared. We studied *T. gondii* infection in rabbits and compared these three techniques under carefully controlled conditions.

MATERIALS AND METHODS

Rabbit infection. *T. gondii* C56 tachyzoites were prepared as previously described (12). Briefly, *T. gondii* was passaged through specific-pathogen-free BALB/c mice (obtained from the contract facilities of the National Cancer Institute, Bethesda, Md.), and tachyzoites were purified from peritoneal exudate. New Zealand White rabbits (3 to 4 kg; Birchwood Valley Farm, Red Wing, Minn.) were injected subcutaneously with 10^4 tachyzoites in 1 ml of minimum essential medium (GIBCO, Grand Island, N.Y.) containing 2% fetal bovine serum (GIBCO) or with vehicle alone. The rabbits were then monitored daily. We assigned rabbits an illness score with 1 point for each of the following observations:

elevated temperature ($>104^{\circ}\text{F}$ [40°C]), greatly elevated temperature ($\geq 105.5^{\circ}\text{F}$ [40.8°C]), nasal exudate or ruffled fur, and food intake less than half of control levels. Rabbits with 3 or 4 points (4 is the maximum possible number of points) were considered severely ill. Blood was obtained at intervals for detection of parasitemia.

Purification of leukocytes from rabbit blood. For buffy coat leukocytes, 2 ml of heparinized blood was centrifuged at $600 \times g$ for 10 min, and the buffy coat was removed. Buffy coat leukocytes were then washed twice with phosphate-buffered saline (PBS; pH 7.4) and used to inoculate mice. For purified leukocytes, 6 ml of heparinized blood was added to an equal volume of PBS and then layered atop 10 ml of a solution containing 6 g of polysucrose and 16.7 g of sodium diatrizoate per dl (Histopaque 1.119; Sigma Chemical Co., St. Louis, Mo.). Erythrocytes were then separated from leukocytes by centrifugation at $800 \times g$ for 25 min. Leukocytes from the interface were collected, washed twice, and separated into three aliquots, which were used for gene amplification, mouse inoculation, and cell culture, separately. In preliminary experiments, we determined that mononuclear cells infected in vitro with *T. gondii* and then reintroduced into whole blood were efficiently separated from erythrocytes by this technique (unpublished data).

DNA preparation and amplification. DNA was extracted with phenol-chloroform as previously described (17). The two pellets of purified leukocytes prepared from each sample were extracted on separate days. Oligonucleotide primers used to initiate DNA amplification were complementary to segments of the B1 gene of *T. gondii* (5'-GGAAGTGCATC CGTTCATGAG and 5'-TCTTTAAAGCGTTCGTGGTC, as described in reference 3). DNA was amplified by the polymerase chain reaction (PCR) in a solution containing 10 mM Tris (pH 8.3), 2.5 U of *Taq* polymerase (Perkins-Elmer Cetus, Norwalk, Conn.), 50 mM KCl, 1.5 mM MgCl_2 , and 200 mM each deoxynucleoside triphosphate. The reaction was carried through 30 cycles, each consisting of 60 s at

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94°C, 90 s at 55°C, and 60 s at 72°C. Amplified DNA was separated by agarose or polyacrylamide gel electrophoresis and then transferred to nylon membranes. Membranes were exposed to an end-labeled 5'-³²P-labeled oligonucleotide (5'-GGCGACCAATCTGCGAATACACC) complementary to a unique segment of the B1 gene completely within the amplified segment. Membranes were washed, and hybridization was detected by autoradiography.

Strict methods to reduce contamination were employed (11). Positive and negative controls were included in each experiment, and results were considered valid only if control results were as expected. Each extracted DNA sample was tested on at least two separate occasions. Results were accepted only if they were repeatable in accordance with previously published criteria (8).

Cell culture. In preliminary experiments, we tested the suitability of several cell lines and growth media for the detection of *T. gondii*. Human skin fibroblasts (Viomed Laboratories, Minneapolis, Minn.) in Dulbecco's minimum essential medium with 10% fetal bovine serum and 1% HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Sigma) were optimal. Human skin fibroblasts were grown to confluence on coverslips in shell vials. Each purified leukocyte pellet was divided into three portions, which were each added to a shell vial and incubated at 37°C in 5% CO₂ for 7 to 10 days.

Indirect immunofluorescence antibody staining was performed as described previously (14). Briefly, coverslips were fixed and exposed to polyclonal rabbit antiserum to *T. gondii* C56 and then to fluorescein-labeled goat anti-rabbit antiserum (Tago Immunologicals, Burlingame, Calif.). All sections were examined by epifluorescence at magnifications of ×100 and ×400. Preparations were considered positive if areas morphologically consistent with *T. gondii* tachyzoites became stained with the fluorescent antibody and control slides treated in parallel did not.

Mouse inoculation. Mouse inoculation to detect *T. gondii* was performed as previously described (15). Briefly, purified leukocyte or buffy coat leukocyte samples were each suspended in 1.7 ml of minimum essential medium containing 2% fetal bovine serum. Each sample was divided into three portions and injected intraperitoneally into Swiss mice (Harlan Sprague Dawley, Inc., Indianapolis, Ind.). Ill mice were sacrificed, and peritoneal fluids were examined for tachyzoites. Mice that remained well were bled from the retroorbital plexus, and serum was tested for antibody to *T. gondii* by the Sabin-Feldman dye test. Brains from seropositive mice were examined for cysts as previously described (15). Mouse inoculation was considered positive if tachyzoites or cysts were seen or if mice developed antibody to *T. gondii*.

Sabin-Feldman dye test. The Sabin-Feldman dye test was performed to detect *T. gondii* antibody in mouse blood as previously described (16). Fourfold dilutions of sera were examined, beginning at a dilution of 1:4. Human sera that did not contain antibody against *T. gondii* served as a source of complement. Each test included seropositive and seronegative human sera and phosphate-buffered saline alone.

RESULTS

Rabbit illness. Seven rabbits were injected subcutaneously with *T. gondii* in two experiments. Two uninfected rabbits injected with vehicle alone served as controls in each experiment. All rabbits injected with *T. gondii* had ruffled fur and rhinorrhea. Two of four infected rabbits in the first experiment and all three in the second had severe illness, with

illness scores of 3 or 4. The infected rabbits in the second experiment died 8, 11, or 14 days after subcutaneous injection. All four surviving infected rabbits developed antibodies to *T. gondii*. Control rabbits remained well and did not develop antibodies to *T. gondii*.

***T. gondii* DNA detection.** In preliminary experiments, we were able to detect 0.1 to 1.0 pg of purified *T. gondii* DNA mixed with 1 or 2 µg of purified human or rabbit genomic DNA. Since each tachyzoite contains approximately 0.2 pg of DNA (18), this corresponds to 0.5 to 5 organisms.

DNA was detected in preparations made from 12 of 32 blood samples, including at least one from each of the seven infected rabbits (Table 1). No control samples were positive. Of the 10 samples obtained from rabbits when they were severely ill, 7 were positive. Of the 22 samples from rabbits that were not severely ill at the time of sample collection, only 5 were positive.

Cell culture. *T. gondii* was detected in cell cultures inoculated with 1 of 18 samples from infected rabbits in the first experiment and in 7 of 8 samples from the second experiment (Table 1). Seven of eight positive samples were obtained when rabbits were severely ill. The eighth was obtained just 1 day before the onset of severe illness in rabbit 5 (Table 1). None of the samples from control rabbits were positive. Plaques became easily detectable 7 to 10 days following inoculation.

Mouse inoculation. Of 32 samples from infected rabbits, 20 were positive by mouse inoculation (Table 1). *T. gondii* was detected in at least one sample from each infected rabbit. All 10 samples from rabbits with severe illness were positive. For 14 of the 20 samples, both buffy coat leukocytes and purified leukocytes were positive. For three, only buffy coat leukocytes were positive, and for two, only purified leukocytes were positive. For one, purified leukocytes were not tested by mouse inoculation.

Of 20 samples positive by mouse inoculation, 18 resulted in mouse illness and tachyzoites were observed in peritoneal exudates of all 18. The remaining two samples, both buffy coat samples, did not make mice ill, but two of the three mice inoculated with each of these samples developed antibody to *T. gondii*. No cysts were detected in the brains from the six mice injected with the latter two samples. No samples from control rabbits were positive.

Comparison of mouse inoculation, cell culture, and gene amplification. Mouse inoculation was the most sensitive test and was positive for 20 samples. Of these, 8 were positive in cell culture and 12 were positive by gene amplification. All of the samples positive by cell culture or gene amplification were also positive by mouse inoculation. Thirteen samples were positive by gene amplification or cell culture. Of these, five were positive by gene amplification only, one was positive by cell culture only, and seven were positive in both.

DISCUSSION

We detected the *T. gondii* B1 gene in the blood of infected rabbits by gene amplification with PCR. Gene amplification was positive in samples from each infected rabbit and in 60% of the samples positive by mouse inoculation. With the strict controls and reproducibility requirements we used, gene amplification was specific. No samples from control rabbits were positive.

In experiments with *Toxoplasma* DNA spiked into large quantities of purified human DNA, we were able to detect the DNA of between 1 and 10 tachyzoites. This is similar to

TABLE 1. Detection of *Toxoplasma* parasitemia in rabbits

Rabbit no. and preparation ^a	Presence of parasitemia on given day after <i>T. gondii</i> infection, and severity of illness ^b								Survival or death ^c
	3	6	7	8	10	11	14	32	
Rabbit 1, expt 1									
Buffy coat (MI)	—	+		+		+	+	—	
Leukocytes (MI)	—	+		—		+	+	—	
Leukocytes (CC)	—	—		+		—	—	—	
Leukocytes (PCR)	—	+		+		—	—	—	
Severity of illness	0	2		4		3	2	1	S
Rabbit 2, expt 1									
Buffy coat (MI)	—	—		—		—	+	—	
Leukocytes (MI)	—	—		—		+	+	+	
Leukocytes (CC)	—	—		—		—	—	—	
Leukocytes (PCR)	—	—		—		—	+	—	
Severity of illness	0	0		1		4	2	1	S
Rabbit 3, expt 1									
Buffy coat (MI)	—	—		—		+	+	—	
Leukocytes (MI)	—	—		—		—	+	—	
Leukocytes (CC)	—	—		—		—	—	—	
Leukocytes (PCR)	—	—		—		+	—	—	
Severity of illness	0	0		0		0	1	1	S
Rabbit 4, expt 1									
Buffy coat (MI)	—	—		+		+	+	—	
Leukocytes (MI)	—	—		—		+	+	—	
Leukocytes (CC)	—	—		—		—	—	—	
Leukocytes (PCR)	—	—		—		+	+	—	
Severity of illness	0	1		0		0	2	1	S
Rabbit 5, expt 2									
Buffy coat (MI)			+	+	+				
Leukocytes (MI)			+	+	+				
Leukocytes (CC)			+	—	+				
Leukocytes (PCR)			—	—	+				
Severity of illness			0	3	3				11
Rabbit 6, expt 2									
Buffy coat (MI)			+	+					
Leukocytes (MI)			ND ^e	+					
Leukocytes (CC)			+	+					
Leukocytes (PCR)			+	+					
Severity of illness			4	3					8
Rabbit 7, expt 2									
Buffy coat (MI)			+	+	+				
Leukocytes (MI)			+	+	+				
Leukocytes (CC)			+	+	+				
Leukocytes (PCR)			+	+	+				
Severity of illness			3	3	4				14

^a Abbreviations: leukocytes, cells purified by Histopaque density gradient separation; MI, mouse inoculation; CC, cell culture.

^b Severity of illness is measured from 0 to 4; see text for details.

^c S, survived until euthanasia 90 days after infection. Numbers indicate the day on which death occurred.

^d Mouse produced antibody but did not become ill.

^e ND, not done.

sensitivities reported earlier for similar preparations (3, 19). Others have reported 10-fold less sensitivity with purified DNA, but they were still able to detect the P30 gene in 50% of buffy coat samples from infected mice (23). Other investigators have been unable to detect *T. gondii* DNA in bone marrow from humans (22) or whole blood from mice (19) with toxoplasmosis. Heme (7), heparin (2), and other poorly characterized substances (22) have been reported to de-

crease the efficiency of PCR and may prevent detection of parasitemia if whole-blood samples are tested.

Whether *T. gondii* circulates in the blood of acutely infected animals extracellularly or intracellularly is not known. In our separation procedure, most of the granulocytes and mononuclear cells were separated from erythrocytes. *T. gondii* DNA within these leukocytes should have been detectable. In preliminary experiments, we found that extracellular tachyzoites added to heparinized blood were not concentrated in the leukocyte layer but, rather, were distributed widely, with most remaining in the erythrocyte layer. Therefore, our approach was not likely to result in the detection of most extracellular tachyzoites. However, we are unaware of a practical method of purifying extracellular tachyzoites from whole blood.

In congenitally infected infants and in people with AIDS and toxoplasmosis, circulating antibody and complement will probably kill extracellular tachyzoites. This is the basis for the Sabin-Feldman dye test. DNA from these tachyzoites may be available for gene detection, but it seems more likely that most circulating organisms would be intracellular. As stated above, our method is more likely to detect intracellular than extracellular *Toxoplasma* tachyzoites.

The B1 gene is repeated 35 times in the *T. gondii* genome (3), which probably enhances the sensitivity of this technique. Recently, sequences repeated an estimated 1,000 times have been identified in the *T. gondii* genome (1). If these sequences are unique and can be used as templates for PCR, the sensitivity of gene amplification for the detection of *T. gondii* may be much greater than can be achieved with the B1 gene. Other refinements of the technique may also increase the sensitivity of PCR for detection of pathogens in blood.

Mouse inoculation was positive with 62% of samples, whereas gene amplification was positive for only 37%. Excess DNA inhibits the PCR reaction, and we were able to amplify DNA from only 1/10 the volume of blood that we used for mouse inoculation. Both techniques depend on amplification, but growth within an infected mouse sufficient to stimulate antibody or produce illness may be more efficient than amplification by PCR.

Cell culture was the least sensitive of the techniques we evaluated for the detection of *T. gondii* parasitemia, being positive for 25% of samples and for only 40% of samples positive by mouse inoculation. It has been used successfully to identify *T. gondii* parasitemia in humans (9, 20, 21) and has also been shown to be as sensitive as mouse inoculation when an equal number of purified tachyzoites were added directly to cell culture or inoculated into mice (4). However whole blood or purified leukocytes may be toxic to cell culture monolayers (13). It is possible that leukocytes or other substances present in clinical samples decreased the sensitivity of cell culture in our work.

Cell culture is performed in most clinical virology laboratories for virus isolation. Technicians are not currently trained to recognize *Toxoplasma* tachyzoites, but they could be. The indirect fluorescent-antibody technique could be used when parasitemia is suspected or when cytopathic effects are observed in cell culture. Although it is more sensitive, mouse inoculation requires housing, euthanasia, necropsy, and antibody testing and is not practical for modern clinical laboratories. PCR is much more technically demanding, time-consuming, and expensive. Until PCR is automated, cell culture may be the most practical, readily available current method for detection of *Toxoplasma* parasitemia in clinical laboratories.

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