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Detection of *Toxoplasma gondii* Tachyzoites and Bradyzoites in Blood, Urine, and Brains of Infected Mice

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Different techniques for identifying *Toxoplasma gondii* were compared. PCR was used to amplify part of the major surface antigen *P30* gene of *T. gondii*. Amplified-DNA detection with the DNA enzyme immunoassay (PCR-DEIA) was more sensitive than ethidium bromide staining after agarose gel electrophoresis and as sensitive as nested PCR. PCR-DEIA, using common enzyme-linked immunosorbent assay (ELISA) methods, avoids agarose gel electrophoresis for the identification of amplified products. *T. gondii* can also be detected with equal sensitivity in infected fibroblasts, but only after at least 8 days of cell culture. PCR-DEIA is thus recommended because of its sensitivity and convenience for detecting early parasitemia in the surveillance of toxoplasmosis among pregnant women and immunocompromised hosts. The courses of infection in mice infected with two strains of *T. gondii* were compared. Tachyzoites of the virulent strain *T. gondii* RH, killing the host in 4 days, were identified in urine specimens and blood samples of mice 24 to 94 h after inoculation but not in brains, but no antibodies were detected. After intraperitoneal inoculation with cysts of the low-level virulence Beverley strain of *T. gondii*, parasites were identified in blood samples 4 days later and up to 17 days (but not in urine specimens) and in the brain from day 6 through day 525. By ELISA, high antibody titers were found from day 11 to day 525, with parasitemia preceding the appearance of antibodies. The usefulness of PCR-DEIA tests in conjunction with the search for circulating antibodies for the early diagnosis of toxoplasmosis in humans is discussed.

In congenital infection, *Toxoplasma gondii* may cause fetal death or severe neurological sequelae, such as hydrocephalus, microcephalus, or blindness (25). Fetal contamination is estimated to occur in 0.1 to 0.5% of infected pregnant women in the United Kingdom (1), 0.2 to 0.3% in southern Finland (19), 1% in France (24), 0.2 to 0.8% in Canada (4), and 0.2 to 0.6% in the United States (11). Prevention of congenital toxoplasmosis in pregnant women has been based mainly on serological tests for anti-*Toxoplasma* antibodies. In acquired diseases, specific antibodies belong to the immunoglobulin M (IgM), IgA, and IgG classes (6, 8, 28, 30). However, infection should be diagnosed at the early acute stage, when treatment is more effective. In this study, PCR, cell culture, and enzyme-linked immunosorbent assay (ELISA) were used to detect *T. gondii* in urine specimens, blood samples, and brains of mice experimentally infected by virulent and weakly virulent strains of *T. gondii* and were compared. Our data suggest that PCR-DNA enzyme immunoassay (PCR-DEIA) is a highly sensitive method for detecting this parasite in biological samples. Since PCR-DEIA is as sensitive as cell culture but much more convenient and rapid, it should be used for the early diagnosis of human congenital toxoplasmosis.

MATERIALS AND METHODS

Mice and *T. gondii* infection. NMRI female mice (6 to 8 weeks old) were used in these experiments. Mice were obtained from the animal facility of Catholic University of Louvain, Brussels, Belgium. Sera were tested by ELISA (5) to confirm the absence of *T. gondii* antibodies before the experiments.

Cysts of the low-level virulence Beverley strain of *T. gondii* were obtained from chronically infected NMRI female mice. The source of the Beverley strain was isolated by J. K. A. Beverley and kindly provided by G. Desmonts from the Institute of Puériculture, Paris, France, in 1977. The animals were sacrificed with diethyl ether [$(C_2H_5)_2O$; Merck Laboratory], and their brains were removed and homogenized in 3 ml of sterile phosphate-buffered saline (PBS) (pH 7.2) by using a mortar and pestle. For each brain suspension, the mean number of cysts from 10 samples (10 μl each) was then determined by light microscopy under a magnification of ×10. After appropriate dilution in PBS, each mouse was inoculated intraperitoneally with 20 cysts. The virulent RH strain of *T. gondii* was maintained by continuous passage in female NMRI mice injected intraperitoneally with about 10^4 tachyzoites in 0.5 ml of PBS.

Preparation of samples and cell cultures. Heart blood samples from mice (under anesthesia with diethyl ether) were collected by cardiac puncture. A volume of between 100 and 1,500 μl of blood was collected in EDTA (15), and erythrocytes were sedimented through Hypaque-methylcellulose (Sigma, St. Louis, Mo.) (2). Briefly, leukocytes were washed three times in sterile PBS (pH 7.4); half of the final pellet was resuspended in distilled water and stored at −20°C until used for PCR, with the other half resuspended in RPMI 1640 for culture on MRC5 fibroblasts (from the NIBSC strain [United Kingdom]; provided by Bioproducts Boehringer). Mice were anesthetized with diethyl ether, and their necks were pinned by dissecting forceps for the collection of urine specimens in sterile petri dishes. Urine samples were washed three times in sterile PBS (pH 7.4). Between 10 and 40 μg of brain tissue was removed and homogenized in sterile PBS with a syringe as previously described (26).

For culture identification of *T. gondii*, cell line MRC5 (7, 16, 22) was maintained in RPMI 1640 (Gibco, Paisley, Scotland) containing 10^5 fibroblasts seeded onto 12-mm-diameter coverslips (Nunc, Naperville, Ill.). After 2 or 3 days, the density of the cell culture was confluent. Each tissue suspension was inoculated into a cell culture well (2 ml per well) in parallel with five parasites inoculated as positive controls. After 8 or more days, *T. gondii* organisms in cell cultures were detected by indirect immunofluorescence assay (7).

PCR. The targets of PCR were sequences of the major surface antigen *P30* gene of *T. gondii*. The following oligonucleotide primers were chosen, with the numbering based on the published sequence (3): 5′-CGACAGCCGCGGTCATTCTC-3′ (GBI3; bases 505 to 525), 5′-GCAACCAGTCAGCGTCGTCC-3′ (GBI4; bases 1006 to 1025), 5′-AGCTGGTGGACGGGGGATTC-3′ (GBI5; bases 689 to 709), and 5′-GTCTGCACCGTAGGAGCACC-3′ (GBI6; bases 875 to 895). Amplification reactions were performed in a total sample volume of 50 μl, 20 μl of test specimen plus 30 μl of a reagent mix containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 200 mM (each) dATP, dGTP, dCTP, and dTTP, 1 μM (each) two primers, and 2.5 U of *Taq* DNA polymerase. The reaction was carried through 30 cycles, each consisting of 60 s at 94°C, 60 s at 55°C, and 60 s at 72°C in a Techne PHC 3 programmable Dri-block (New Brunswick Scientific, Duxford, Cambridge, United Kingdom),

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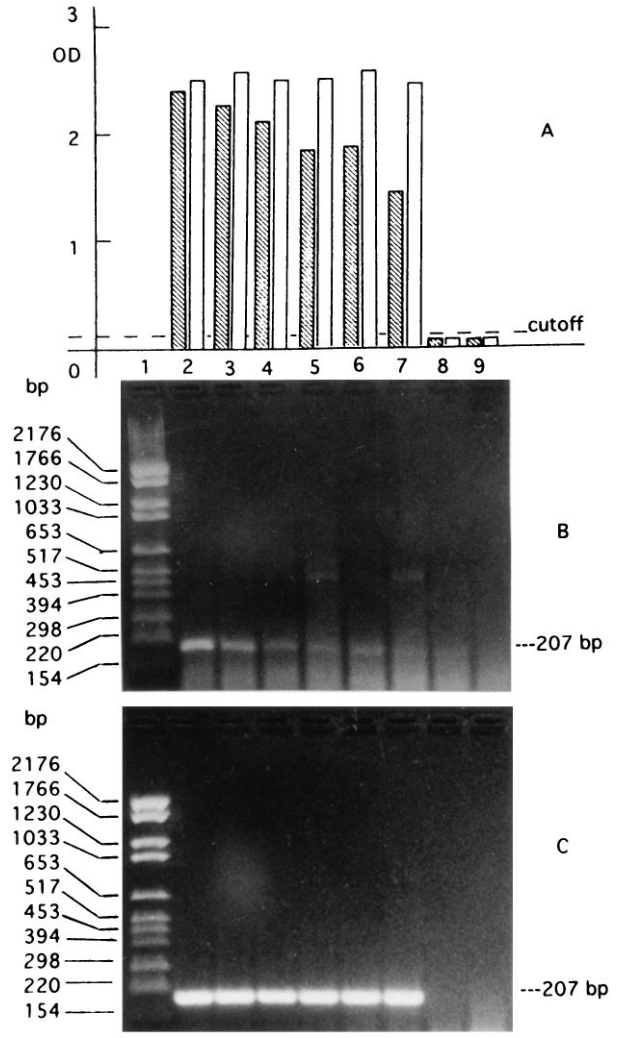

FIG. 1. Sensitivity of *T. gondii* amplification by PCR and nested PCR and detection by DEIA and ethidium bromide staining of agarose gels. Tachyzoites were serially diluted to yield 64, 32, 16, 8, 4, 2, and 1 parasite per sample (lanes 2 to 8, respectively). No parasite was added to the sample in lane 9. (A) Detection by DEIA (in optical density [OD] units at 450 nm), with the cutoff value shown (see Materials and Methods). ▧, PCR with primers GBI5 and GBI6; □, nested PCR (primary amplification with primers GBI3 and GBI4, followed by amplification with internal primers GBI5 and GBI6). (B) PCR with primers GBI5 and GBI6. Lane 1, molecular size (in base pairs) markers (λ DNA restricted with *Eco*RI and *Hin*dIII), with positions indicated on the left. The position of the expected fragment (207 bp) is indicated on the right. (C) Nested PCR as described for panel A, with molecular size markers in lane 1 as described for panel B.

followed by an extension incubation (5 min at 72°C). In the presence of GBI3 and GBI4 primers, PCR resulted in the amplification of a 522-bp fragment, and nested amplification with GBI5 and GBI6 primers gave a 207-bp fragment. The PCR product was analyzed by electrophoresis and DEIA (see below). Electrophoresis was performed on a 1.5% agarose gel (Sigma) at 140 V for 60 min. PCR products were identified under UV by staining with ethidium bromide (1 μg/ml) added in the electrophoresis buffer (89 mM Tris borate, 2.5 mM EDTA, pH 8.3) (15).

DEIA. Streptavidin-coated microtiter plates (ETI-IEMA; Sorin Biomedica) were used as previously described (21). Briefly, plates were preincubated overnight with 100 μl of biotinylated oligonucleotide probe 5′-TCCCTTGATGCA ACCGACCACAAA-3 (TXB1; bases 788 to 802; sequence between the two previously described primer sets [3]) per well. The wells were washed, and heat-denaturated amplified products were added; hybridized DNA was revealed by monoclonal antibody specific for double-stranded DNA. A sheep anti-mouse IgG conjugated to horseradish peroxidase was used as secondary antibody. After the addition of 100 μl of chromogen (tetramethylbenzidine, 27 g/liter; hydrogen peroxide, 0.1 ml/liter [Sorin Biomedica]) per well, the reaction was stopped with 1 N H_2SO_4. The A_{450} of each sample was read. The results were considered positive when the absorbance was greater than the mean absorbance of sera from healthy mice plus 3 standard deviations (SD) (cutoff value). The average absorbances of samples from 25 healthy mice (in pools of 5) were as follows: for urine specimens, 0.099 with an SD of 0.031; for brains, 0.111 with an SD of 0.016; and for blood samples, 0.094 with an SD of 0.026.

TABLE 1. Presence of parasites, as determined by cell culture and PCR-DEIA, in urine, brain, and blood samples of mice after infection with the virulent RH strain of *T. gondii*

Time postinfection (h)	Presence of parasites in samples[a]					
	Urine		Brain		Blood	
	Culture	PCR-DEIA	Culture	PCR-DEIA	Culture	PCR-DEIA
3	−	−	−	−	−	−
7	−	−	−	−	−	−
17	−	−	−	−	−	−
22	−	−	−	(−)	−	−
24	+	+	−	−	+	+
27	+	+	−	−	+	+
41	+	+	−	(−)	+	+
72	+	+	−	−	+	+
94	+	+	−	−	+	+

[a] +, presence; −, absence; (−), absence of PCR amplification of diluted samples, with amplification of externally added *T. gondii* DNA.

ELISA. ELISA microtiter plates (Immunoplate Maxisorp F96; Nunc, Roskilde, Denmark) were coated by overnight incubation at 4°C with 100 μl of a lysate of *T. gondii* (6.5 μg of protein per ml) in PBS (pH 7.2). The plates were washed three times in PBS (pH 7.2). Wells were saturated with 5% fetal calf serum (Gibco) in PBS for 15 min, and 100 μl of serum diluted 1:100 in PBS-Tween 20 (0.5%) was added and incubated at 22°C for 30 min. After three washings in PBS, 100 μl of conjugated rabbit anti-mouse immunoglobulins labeled with peroxidase (DAKO, Glostrup, Denmark), diluted 1:1,000 in PBS-Tween 20 (0.5%), was added and incubated for 30 min at 22°C. The plates were washed again before the addition of 100 μl of chromogen (tetramethylbenzidine, 27 g/liter; hydrogen peroxide, 0.1 ml/liter [Sorin Biomedica]) solution. The reaction was stopped with 1 N H_2SO_4. The A_{450} of each sample was read with a Sorin spectrophotometer. Serum samples from healthy mice (n = 25) yielded an average absorbance of 0.115 with an SD of 0.017.

RESULTS

Sensitivity of PCR. To determine PCR sensitivity, serial dilutions of RH tachyzoites were tested (Fig. 1). With the GBI5 and GBI6 primers for 40 cycles, the amplification reaction on an ethidium bromide-stained agarose gel yielded a 207-bp fragment, which was barely detectable in samples containing eight and four parasites (Fig. 1B), whereas two parasites were revealed when the PCR product was subjected to DEIA detection (Fig. 1A [hatched bars]). Nested PCR was performed with the same primers on the product of a previous amplification (yielding no visible bands on ethidium bromide-stained gels) with primers GBI3 and GBI4. Under these conditions, two parasites could be detected by both DEIA and ethidium bromide staining (Fig. 1A [open bars] and C).

Time course of virulent *T. gondii* RH infection in mice. Forty-five mice were intraperitoneally infected with *T. gondii* RH, which killed the animals after 4 days. Urine, brain, and blood samples from five mice were collected and pooled at nine intervals (3 to 94 h) and checked for the presence of this parasite by PCR and cell culture (Table 1). The RH strain appeared simultaneously after 24 h in urine and blood samples, in which they could be identified up to at least 94 h. No antibodies were detected by ELISA. No *T. gondii* organisms were detected in brain samples. Thus, PCR and cell culture showed similar sensitivities for the detection of the RH strain.

Time course of infection in mice inoculated with the weakly virulent Beverley strain of *T. gondii*. Urine, brain, and blood

TABLE 2. Presence of parasites and circulating antibodies after inoculation of mice with cysts of the less virulent Beverley strain of *T. gondii*

Time postinoculation (days)	Presence of parasites in samples[a]						Presence of antibodies[a,b]
	Urine		Brain		Blood		
	Culture	PCR-DEIA	Culture	PCR-DEIA	Culture	PCR-DEIA	
2	−	−	−	−	−	−	−
3	−	−	−	(−)	−	−	−
4	−	−	−	−	+	+	−
5	−	−	−	−	+	+	−
6	−	−	+	(+)	+	+	−
7	−	−	+	+	+	(+)	−
9	−	−	+	+	+	+	−
11	−	−	+	+	+	+	+
13	−	−	+	+	+	+	+
17	−	−	+	+	+	+	+
32	−	−	+	(+)	−	−	+
90	−	−	+	+	−	(−)	+
114	−	−	+	+	−	−	+
525	−	−	+	+	−	−	+

[a] See Table 1, footnote *a*.
[b] Determined by ELISA.

samples from five mice were collected and pooled at 14 intervals after intraperitoneal infection with cysts of the Beverley strain of *T. gondii* (Table 2). This parasite was first detected 4 days after infection in blood samples by both culture and PCR. This positivity was maintained for 13 days. The Beverley strain appeared in brains 6 days after intraperitoneal infection and persisted up to 525 days. Antibodies were detected by ELISA 11 days after infection. The PCR and cell culture results were identical. No culture- or PCR-positive tests were obtained with urine samples.

Figure 2 shows quantitative estimations of antigens (by DEIA of PCR-amplified products) and antibodies (by ELISA) in the blood samples from the experiment whose results are reported in Table 2. PCR was clearly able to detect parasitemia at a very early stage of infection, while antibodies appearing 11 days after infection remained high up to 525 days after infection.

DISCUSSION

In this study, cell culture, nested PCR, and PCR-DEIA were compared for the detection of *T. gondii*. Two parasites could be detected when the PCR product was revealed by DEIA (Fig. 1A [hatched bars]), whereas ethidium bromide staining of an agarose gel was less sensitive (Fig. 1B) and gave a number of additional background bands, some of which may migrate at or very close to the position expected for the PCR product (data not shown) (20, 23). After nested PCR, DEIA and agarose gel staining were equally sensitive (Fig. 1A [open bars] and C). Identical results were obtained by PCR-DEIA and culture for the detection of *T. gondii* organisms in urine, blood, and brain samples (Tables 1 and 2). However, by providing a sensitive answer in less than 1 day, PCR-DEIA has an advantage over culture, which requires at least 8 days. Thus, DEIA detection of the PCR product is recommended as an early diagnostic tool

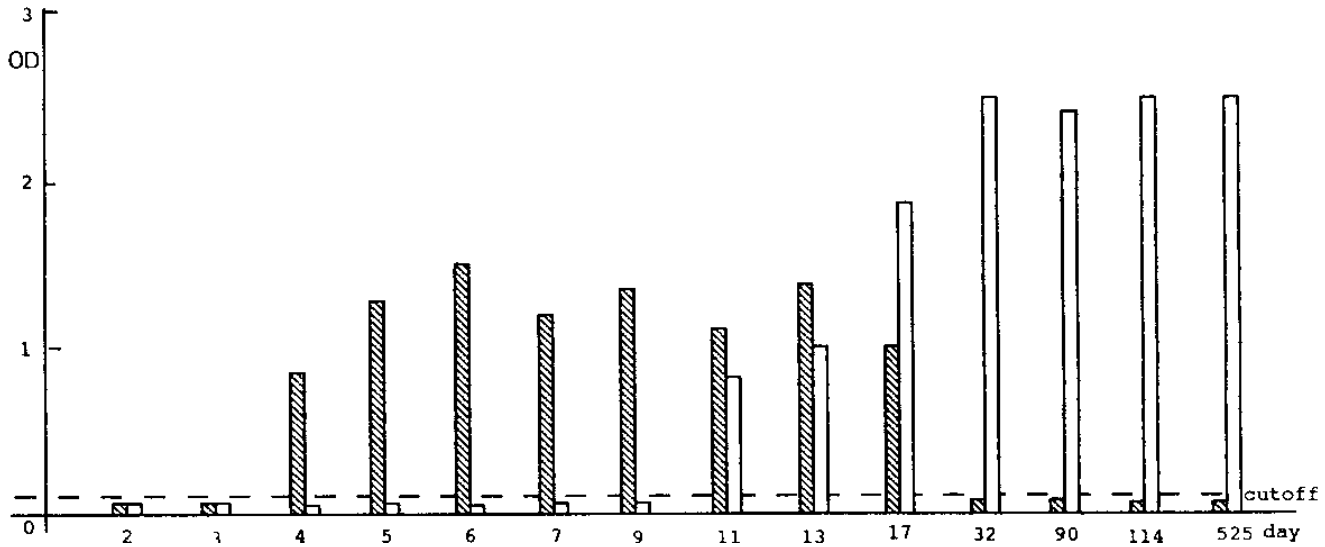

FIG. 2. Quantitative estimations of antigens and antibodies in blood samples of mice infected with the Beverley strain of *T. gondii*. The blood samples of five mice were collected and pooled at the indicated times (in days) after infection. ▨, PCR with antigen detection by DEIA; □, antibody determination by ELISA. The data for both enzymatic reactions, measured at 450 nm, are given in optical density [OD] units, with the cutoff value indicated (see Materials and Methods).

for toxoplasmosis. Nested PCR may be avoided, without loss of sensitivity.

After intraperitoneal injection of the virulent strain *T. gondii* RH, this parasite appeared in blood and urine samples 24 h later and persisted for at least 3 days. Our PCR results confirm the presence of *T. gondii* in mouse blood up to 66 h after infection as described earlier (18). Lysed parasites could account for positive PCR results (17), whereas the reported positive cell culture result indicated the presence of live *T. gondii* organisms in urine specimens. Probably because of the short survival of the infected host (4 days), no circulating antibodies and no parasites in brain samples were detected. After infection by the weakly virulent Beverley strain, which allowed the host to survive, 4 days were needed to observe the presence of this parasite in blood samples and 6 days were needed to detect it in the brain, but the parasite was not detected in urine specimens even after 525 days. Our results are in agreement with the observation of the presence of *T. gondii* DNA in the brains and its absence in the heart blood samples of six mice killed 6 months after inoculation with different Beverley strains (26). ELISA became positive after 11 days, and antibodies persisted for up to 525 days, as did this parasite in the brain.

It is possible that the inhibitors of *Taq* polymerase in some of the brain and blood samples (Tables 1 and 2) were due to the high number of leukocytes ($>10^6$ cells inhibit PCR [18]) or brain cells. We determined the ability of serially diluted brain cell and leukocyte extracts to inhibit PCR amplification of externally added *T. gondii* DNA; the presence of up to 10^6 cells did not interfere with PCR-DEIA, whereas in the presence of 10^5 cells, no amplification product could be detected by agarose gel analysis. We therefore diluted our samples to one-third of the original volume and used PCR-DEIA because of its added sensitivity. We thus obtained control samples with amplification of externally added *T. gondii* DNA (Tables 1 and 2).

Several publications have described the advantages of PCR in the prenatal diagnosis of congenital toxoplasmosis or toxoplasmosis encephalitis of patients with AIDS by using the *P30* (13) or *B1* (10, 12, 29) gene or a segment of 18S ribosomal DNA (9, 27) as a target. The present report adds the convenience of ELISA-type detection, adapted to clinical laboratory diagnostics, thus avoiding the hazardous use and disposal of ethidium bromide in the revelation of amplified fragments on agarose gels. In addition, as noted above, occasional appearances of spurious amplification fragments in agarose gel analysis might require Southern blotting and hybridization with specific probes. This can be avoided by DEIA detection, without loss of specificity.

In preliminary studies of 28 patients during acute and chronic toxoplasmosis infection, as witnessed by positive ELISAs, their blood and urine samples were devoid of antigens, as indicated by negative PCR results. Blood and urine samples of 25 seronegative individuals were also tested for *T. gondii* organisms by PCR-DEIA. One patient, whose blood sample was positive by PCR, had circulating IgM and IgG antibodies 1 month later, suggesting that the time course of the human disease is similar to the one described here for animal infection.

The present study suggests the use of a new PCR technique with improved detection sensitivity, PCR-DEIA, on blood samples of patients suspected of having toxoplasmosis. This simple and rapid test detects the parasite early after infection, before circulating antibodies appear. It has to be used on blood samples because in urine specimens no parasites (Beverley strain) were found. This PCR methodology, currently evaluated in medical diagnostics, seems promising for early detection of toxoplasmosis in pregnant women and immunocompromised hosts.

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