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Comparison of Cell Culture, Mouse Inoculation, and PCR for Detection of *Toxoplasma gondii*: Effects of Storage Conditions on Sensitivity

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The sensitivity of detection of a wild-type strain of *Toxoplasma gondii* by cell culture, mouse inoculation, and PCR was determined following sample storage under conditions to which clinical specimens may be subjected during transport to the testing laboratory. Sample storage at −20°C significantly decreased the sensitivity of mouse inoculation. The sensitivity of cell culture decreased with sample storage at 4 and −20°C. The sensitivity of PCR was reduced by storage at 4°C for 48 h, freezing, and heating. These findings have implications for the selection of appropriate methods for the direct detection of *T. gondii* organisms in suboptimally transported clinical samples.

Toxoplasma gondii isolates infect 15 to 85% of the human population, and the organism has emerged as a major opportunistic pathogen in immunocompromised patients, in whom it can cause life-threatening disease (14, 19). Toxoplasmosis has become increasingly common with the spread of AIDS (13). Serological diagnosis of active infection is unreliable because reactivation is not always accompanied by changes in antibody levels, and the presence of immunoglobulin M (IgM) does not necessarily indicate recent infection (9).

The "gold standard" for the detection of *T. gondii* organisms in clinical specimens is mouse inoculation and then the detection of *T. gondii*-specific antibodies (9, 14). This method is sensitive and specific but time-consuming, taking up to 6 weeks to obtain a diagnosis (4). Currently, cell culture is the most practical method for the detection of *T. gondii* parasitemia, but this is also relatively slow and may lack sensitivity. PCR has been found to be a sensitive, specific, and rapid method for the detection of *T. gondii* DNA in amniotic fluid (7), blood (5, 10), tissue samples (11), and cerebrospinal fluid (3, 6, 16).

The poor viability of *T. gondii* isolates outside the host is well known (14). Transportation of samples to specialized laboratories is rarely optimal, which is one reason why the laboratory diagnosis of toxoplasmosis remains difficult. In the present study we compared the sensitivity of detection of *T. gondii* organisms by cell culture, mouse inoculation, and PCR under conditions to which clinical specimens may be subjected during transport to a reference laboratory.

For the study we chose a wild-type strain of *T. gondii*, isolated from a wallaby and designated *T. gondii* 002 (provided by David Obendorf), which is not highly virulent in mice and so represents isolates from clinical specimens better than more virulent strains.

T. gondii tachyzoites were maintained in human embryonic lung fibroblast (MRC-5) cells (Commonwealth Serum Laboratories, Melbourne, Victoria, Australia) in Eagle's minimal essential medium (MEM) containing 9% decomplemented fetal calf serum, benzylpenicillin (12 μg/ml), and streptomycin (10 μg/ml). Suspensions of tachyzoites in Eagle's MEM containing 10-fold dilutions from 10^5 to 1 parasite were prepared and divided into five equal portions, which were treated as follows: group A, untreated, intact live tachyzoites; group B, tachyzoites stored at 4°C for 24 h; group C, tachyzoites stored at 4°C for 48 h; group D, tachyzoites stored at −20°C for 48 h; group E, tachyzoites heated at 70°C for 10 min (Fig. 1). Each preparation was divided into 1-ml aliquots for PCR, shell vial cell culture, and mouse inoculation.

One milliliter of each portion of each tachyzoite suspension was inoculated intraperitoneally into three 6-week-old female specific-pathogen-free BALB/c mice (obtained from the SPF Biological Facility, The University of New South Wales, Randwick, New South Wales, Australia). MEM was injected into four control mice. One mouse from each group was sacrificed on days 14, 28, and 42 after inoculation, and their sera were tested by the indirect fluorescent-antibody test (IFAT) for IgG toxoplasma antibodies (ToxoSpot IF; bioMerieux, Marcy l'Etoile, France). Sera demonstrating a titer of less than 16 were considered negative (14).

For the detection of *T. gondii* organisms in culture, 2 ml of a human embryonic lung fibroblast cell (MRC-5) suspension (10^5 cells per ml) was seeded onto coverslips in shell vials, and the vials were incubated at 37°C until the cell sheet was confluent (2). The coverslip cultures were then inoculated with 1 ml of each pretreated portion of each dilution of tachyzoites and the cultures were incubated at 37°C. Coverslip cultures were examined by (i) daily direct examination for the appearance of a cytopathic effect (CPE) with an inverted microscope (magnification, ×400) and (ii) IFAT of inoculated coverslip cultures on days 5 and 10 (15). Coverslips were fixed with cold acetone and were then washed and incubated with antitoxoplasma human serum (IFAT IgG titer, 256) diluted 1/150 in 0.001 M phosphate-buffered saline (PBS). Following washing with PBS, fluorescein isothiocyanate-conjugated goat anti-human IgG (Cappel, Durham, N.C.) diluted 1/150 in PBS was added, and the mixture was incubated at 37°C for 30 min and washed again. The coverslips were mounted onto slides and

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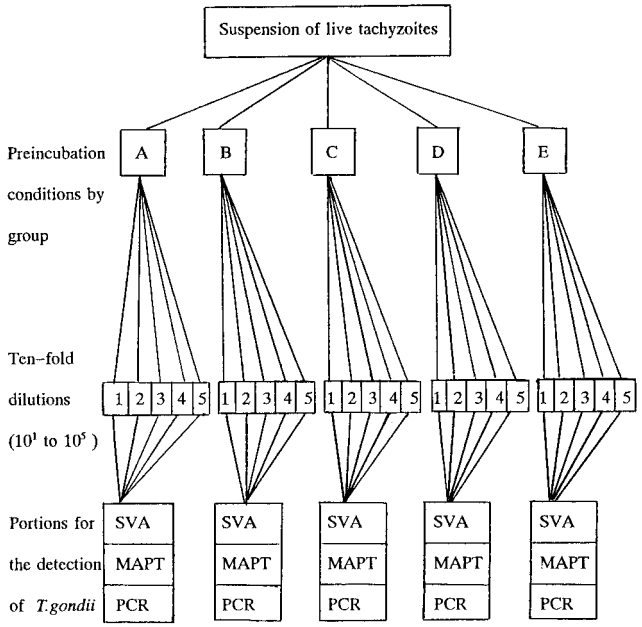

FIG. 1. Structure of the study. The methods used to detect *T. gondii* tachyzoites in different portions of diluted suspensions are indicated. SVA, shell vial assay; MAPT, mouse antibody production test.

were examined for fluorescence at a magnification of ×400. Samples were considered positive if at least two *T. gondii* tachyzoites were stained with the fluorescent antibody and there was no fluorescence of the negative control slides treated in parallel.

For PCR testing, pretreated cell culture tachyzoites and MRC-5 fibroblast cells not infected with *T. gondii* (negative control specimens) were centrifuged at 13,000 × *g* for 15 min at room temperature in a microcentrifuge. The supernatant was removed, and the pellet was resuspended in 100 μl of water. To minimize PCR inhibition, samples were tested undiluted and diluted 1/10 in water. Each sample for PCR had a "shadow" no-DNA control (NDC) consisting of water that was treated in the same manner as the sample during processing. This NDC sample is an indicator of contamination of the samples during processing and must be clear before a result is determined. In addition, each sample had a "run" NDC that was included as an indicator of the occurrence of sporadic contamination at the time that the PCR was set up. Finally, each sample was spiked with 10^2 *T. gondii* tachyzoites as an indicator of PCR inhibitors in the sample. In summary, each of the following samples for PCR was tested: (i) NDC sample, (ii) undiluted sample, (iii) sample diluted 1:10, (iv) sample spiked with tachyzoites, and (v) the run NDC sample.

For PCR amplification, the method of Cazenave et al. (1) was used, with modifications. Briefly, a 100-bp segment of the *T. gondii* P30 gene was amplified with 0.25 μM (each) oligonucleotide primers 5′-GCTCCTTGATTCCTGAAGCA and 5′-GGGAACTTCTCGATTGGAAC in a 12.5-μl volume containing 50 mM KCl, 10 mM Tris HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM $MgCl_2$, and 50 μM (each) dATP, dTTP, dCTP, and dGTP. Following the addition of 5 μl of the appropriate sample, the amplification mixture was heated to 99°C for 10 min and was then quenched on ice. DyNAZyme (0.25 U) thermostable DNA polymerase (Finnzymes Oy, Espoo, Finland) was then introduced into each mixture. Amplification in an automated thermal cycler (FTS-1; Corbett Research, Mortlake, New South Wales, Australia) consisted of 40 cycles: denaturation at 96°C for 5 s and annealing-extension at 60°C for 1 min and 20 s, with the final step continuing for 5 min. Two microliters of the first amplification reaction mixture was reamplified in a 20-μl volume containing the amplification mixture described above along with 3.0 mM $MgCl_2$. The second amplification was carried out in an automated capillary thermal cycler (FTS-1S; Corbett Research) and consisted of 30 cycles: denaturation at 98°C for 2 s and annealing-extension at 60°C for 40 s, with the final step continuing for 5 min. Six microliters of each amplification reaction mixture was analyzed in a 2.5% agarose gel containing 0.5 μg of ethidium bromide ml^{-1}, and the bands were visualized under UV light (17). PCR product sizes were compared with a ϕX174 *Hin*fI molecular mass marker (Promega Corporation, Madison, Wis.). Strict precautions to reduce possible contamination were used (12). Amplification reaction mixtures containing a PCR product that was the exact same size as that in the positive control were deemed positive for *T. gondii.*

None of the BALB/c mice injected with tachyzoites of the 002 strain developed parasite-rich ascites or died from acute infection, although some mice were lethargic. This is consistent with the inoculation of strains of *T. gondii* which were isolated from patients and which usually are not lethal to mice (19). Inoculation of 10^5 live parasites produced a detectable antibody response within 1 week, whereas seroconversion was not detectable until 6 weeks after the inoculation of 10 tachyzoites. For most mice from groups A, B, and C injected with high doses of tachyzoites, antibody was detectable at a low titer in the second week after inoculation and had reached high titers (128 to 512) after 6 weeks (Table 1). The parasites which had been stored at −20°C and those which had been heated (groups D and E, respectively) did not elicit a detectable response except in the mice inoculated with the highest dose of tachyzoites (group D) after 6 weeks of observation. Control mice remained well and did not develop antibodies to *T. gondii.*

Table 2 shows that only live tachyzoites in high concentrations ($\geq 10^3$) were detectable in shell vial culture by observation of a CPE and by IFAT after 5 or 10 days of incubation.

TABLE 1. Mouse antibody responses to inoculation of *T. gondii*

Week after inoculation and group	Titer after inoculation of the following no. of tachyzoites/ml[a]:				
	10^1	10^2	10^3	10^4	10^5
Second week					
A			16	32	64
B		32	64	128	256
C		32	64	64	128
D					
E					
Fourth week					
A		256	256	256	256
B		32	64	128	256
C		64	64	64	128
D					
E					
Sixth week					
A	16	128	512	512	512
B	32	128	256	256	256
C	32	64	64	64	128
D					32
E					

[a] Titers of toxoplasma antibody in mouse sera measured by IFAT.

TABLE 2. Shell vial fibroblast cell culture of *T. gondii*

Day after inoculation and group	CPE/IFAT result after inoculation of the following no. of tachyzoites/ml[a]:				
	10^1	10^2	10^3	10^4	10^5
Fifth day					
A	−/−	−/−	+/+	+/+	+/+
B	−/−	−/−	−/−	−/+	+/+
C	−/−	−/−	−/−	−/−	+/+
D	−/−	−/−	−/−	−/−	−/+
E	−/−	−/−	−/−	−/−	+/−
Tenth day					
A	−/+	−/+	+/+	+/+	+/+
B	−/−	−/+	+/+	+/+	+/+
C	−/−	−/−	+/+	+/+	+/+
D	−/−	−/−	−/−	+/+	+/+
E	−/−	−/−	−/−	−/−	−/−

[a] Symbols indicate the absence or presence of a CPE/negative or positive IFAT result.

The sensitivity of this method for the detection of tachyzoites was significantly reduced by pretreatment of the inocula. After storage at 4°C for 24 h, the culture was positive when the initial inoculum was 10^2 organisms, but after 48 h at 4°C it was positive only when there had been 10^3 organisms in the initial inoculum. Freezing of the parasites at −20°C for 48 h resulted in a further loss of sensitivity: growth occurred only when there were at least 10^4 organisms in the original suspension. Heated parasites did not multiply in culture and produced only a nonspecific CPE because of cell monolayer toxicity.

By PCR, *T. gondii* DNA was detected in all concentrations of live tachyzoites (group A) down to 10 organisms per sample. Moreover, we could detect the PCR product after a further 10-fold dilution of the inoculum. *T. gondii* DNA was also detected in all samples from group B (tachyzoites stored at 4°C for 24 h). PCR could detect *T. gondii* DNA from an initial inoculum of 10^2 tachyzoites in groups C (stored at 4°C for 48 h) and D (stored at −20°C for 48 h) and 10^3 tachyzoites in group E (heated). All PCR-positive samples formed a single product that was the exact same size as that in the positive control. The PCR product was not formed in control MRC-5 fibroblast samples or NDC samples.

Our results have shown the relative advantages and disadvantages of mouse inoculation, tissue culture, and PCR for the detection of *T. gondii* organisms. Mouse inoculation is time-consuming but quite sensitive, with the antibody response improving from the second week postinoculation to a maximum sensitivity at 6 weeks. Interestingly, antibody was not detected after inoculation of heat-killed tachyzoites, and antibody was detected only after inoculation of samples stored frozen for 48 h when the initial inoculum was at least 10^5 organisms. Passive immunization with dead tachyzoites did not elicit an antibody response. Therefore, to maintain optimal viability of tachyzoites for the detection of toxoplasmas by mouse inoculation, specimens should be transported at room temperature if the specimen can be delivered in hours or at 4°C if delays are expected.

The sensitivity of cell culture for the detection of *T. gondii* organisms was comparable to that of mouse inoculation. Shell vial assay results were available 10 days postinoculation, which is still clinically relevant. The use of the IFAT improved the sensitivity and discriminatory power compared with the observation of a CPE alone. Cell culture is vulnerable to toxic elements in specimens, which may lead to false-negative results. However, this method is still a good choice for fresh, well-transported samples.

In the present study, PCR was the only method capable of detecting *T. gondii* organisms in low numbers (10 to 10^3 organisms per ml) from samples incubated under all of the conditions evaluated in the present study. PCR can be considered a valuable additional tool for the identification of *T. gondii* infection. The theoretical advantages of PCR for *Toxoplasma* DNA detection over conventional methods have been discussed intensively (8, 9, 18). One of the limitations of PCR is that the procedure analyzes only a small aliquot of DNA. Comparison of animal inoculation and PCR studies suggests that in vivo amplification (isolation from animals) may be more effective in identifying rare toxoplasmic organisms than in vitro amplification (PCR) (19). We used primers for the P30 gene (cDNA) (1) in the PCR, which can detect a single organism (data not shown).

In our study all three methods were approximately equal in sensitivity for fresh live tachyzoites, but PCR demonstrated real advantages in the ability to detect partly destroyed parasites and for samples subjected to conditions which mimic suboptimal conditions of transportation. For the routine laboratory diagnosis of toxoplasma infection, this is important because PCR should yield fewer false-negative results. We have proven that PCR can detect specific gene sequences even after the destruction of viable *T. gondii* organisms and their proteins. Another advantage of the PCR technique is the possibility of detecting not only extracellular tachyzoites but also intracellular tachyzoites. We suggest the use of PCR for the diagnosis of active toxoplasmosis when a result is required urgently (current pregnancy, AIDS, etc.) and/or serological tests are inconclusive. PCR is the method of choice when a very small amount of specimen is available and the condition of the specimen is dubious. Ideally, specimens should be collected for PCR before the initiation of therapy. However, it has been reported that toxoplasma DNA is detectable for some days after the initiation of therapy (5). A sensitive quantitative PCR theoretically could be used to monitor the response to treatment. The detection of *T. gondii* organisms in specimens by PCR permits the more accurate interpretation of serologic data.

Since cost, the time to a result, and sensitivity are important issues, each laboratory must decide the value of these methods on the basis of specimen transportation conditions. From our evaluation, we conclude that although the method is labor-intensive, the turnaround time and exquisite sensitivity of PCR make it the method of choice for the detection of *T. gondii* organisms in immunocompromised or pregnant patients.

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