JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2004, p. 1719–1722 Vol. 42, No. 4
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.4.1719–1722.2004


# Comparison of Two Widely Used PCR Primer Systems for Detection of *Toxoplasma* in Amniotic Fluid, Blood, and Tissues

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Received 9 May 2003/Returned for modification 20 July 2003/Accepted 11 January 2004

**The PCR diagnosis of toxoplasmosis suffers from lack of standardization. Interlaboratory comparative studies of PCR methods have been performed, but intralaboratory comparisons are scarce. Here, we optimized and compared the technical performances of two PCR primer systems widely used for *Toxoplasma* detection. The differences between the two methods were visible only at low parasite concentrations (≤1 *Toxoplasma* genome per reaction tube). Nevertheless, when clinical samples were tested, both methods significantly differed in their technical sensitivities and specificities. Only one method appeared adequate for samples containing blood or tissue.**

Toxoplasmosis can cause significant morbidity and mortality in developing fetuses and in immunocompromised patients. For both conditions, a rapid and accurate diagnosis is required in order to start the relatively efficient antiparasitic treatment. The detection of the parasite DNA by PCR has considerably improved diagnosis, particularly the prenatal diagnosis of congenital disease (reviewed in reference 1). Unfortunately, the PCR for identifying *Toxoplasma* remains unsatisfactory for the following reasons. (i) Only in-house PCR assays are available, and they are associated with lack of standardization and variations in efficiency (8, 12, 20). (ii) For most *Toxoplasma* infections, the diagnostic sensitivity of this molecular method remains low, e.g., 50 to 80% for prenatal diagnoses (reviewed in reference 1). (iii) The technical specificity is not high for many assays because of the presence of misleading spurious amplification products, implying the need for an additional step for confirmation of the identity of the PCR product (12, 20; unpublished data). Among the many factors influencing the PCR outcome, the choice of the DNA target and primers is generally considered essential. Few DNA target loci have been described for *Toxoplasma* PCR, but more than 25 different primer pairs have been used in different assays (1), most of them targeting the repetitive 35-copy-number B1 gene (6). In contrast to this diversity of assays, comparative studies are scarce (reviewed in reference 1). Three European collaborative multicenter studies have been implemented in the last few years (8, 12, 20). However, these interlaboratory comparisons cannot distinguish between the many factors influencing the reaction outcome. To establish whether one method is better than another, intralaboratory comparisons, using finely optimized conditions for each method, are necessary, but very few of these have been reported (1, 5, 21). These factors presently render a general consensus, and therefore standardization, impossible. Here, we have compared the two most widely used primer pairs, at least among French groups, in routine hospital diagnosis of toxoplasmosis: primers 1 and 4 of Burg et al. (6), hereafter termed method T, and primers B22 and B23 of Bretagne et al. (4), termed method B, both primer sets targeting the B1 gene. Both methods were validated independently in earlier studies by comparisons with reference methods and clinical criteria and shown to be more sensitive than cell culture and mouse inoculation (1, 2, 10, 11, 13, 15). We have thoroughly optimized the PCR conditions for both methods and then assessed their technical performances with three types of samples: serial dilutions of *Toxoplasma* DNA, mimic (artificially prepared) human samples, and clinical samples.

Human clinical samples were received from gynecology and obstetrics departments throughout the region of Languedoc (Southern France) and from the Infectious Diseases Department of the Centre Hospitalier Universitaire of Montpellier during routine PCR diagnosis for toxoplasmosis. A total of 96 samples were selected to be tested with both PCR methods: 21 amniotic fluid (AF), 38 placenta, 17 cord blood, 15 peripheral blood, and 5 miscellaneous (liver biopsy, aqueous humor, cerebrospinal fluid, and sputum) samples. In addition, negative control samples (10 AF and 6 placenta samples) were obtained from women who were either nonimmune or presented with long-standing immunity against toxoplasmosis and for whom a diagnosis of recent toxoplasmosis was ruled out.

AF samples were prepared as described previously (13). Peripheral blood was collected, and the buffy coat portion was prepared as reported earlier (17). Placentae (300 g) were ground, trypsinized, filtered, and washed three times in 0.9% NaCl. Buffy coat (300-μl) and ground-placenta (1-ml) samples were lysed in 2 and 3 volumes, respectively, of TNN lysis buffer (0.5% Tween 20, 0.5% Nonidet P-40, 10 mM NaOH) with 10 mM Tris and 320 μg of proteinase K/ml for 3 to 16 h. The DNA was extracted by a simplified phenol-chloroform method (17), which was shown by previous comparative experiments to be as efficient as commercial extraction kits (reference 18 and data not shown).

For the preparation of *Toxoplasma* DNA and mimic samples, ascitic fluid containing tachyzoites was drawn from a

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Swiss-Webster female mouse infected with the RH strain of *Toxoplasma gondii*. Parasites were precisely counted and prepared according to a simplified method (13); the DNA was then diluted in distilled water for a *Toxoplasma* DNA serial dilution assay (SDA). Mimic AF and placenta samples were prepared by extracting a pool of known *Toxoplasma*-negative samples under sterile conditions and adding various amounts of *Toxoplasma* DNA. Mimic blood samples were prepared as described previously (17), except that the buffy coat was prepared and the DNA was extracted as described above, and aliquots of this DNA were then mixed with various dilutions of *Toxoplasma* DNA. For the SDA and the three types of mimic samples, the aim was to test the DNA equivalent of 25, 10, 5, 2, 1, 0.5, 0.1, 0.05 and 0.01 parasite genome(s) per PCR tube.

As described above, PCR amplification was carried out in two separate assays using different primer sets (4, 6). The reaction conditions of both assays, termed methods T and B, were independently and thoroughly optimized in the presence of human DNA as described elsewhere (17). The optimized PCR conditions for method T were the following: 5 μl of 10× buffer, 0.6 mg of bovine serum albumin $ml^{-1}$, deoxynucleoside triphosphates at a concentration of 200 μM each, 2 mM $MgCl_2$, 50 pmol of each primer, and 1.5 U of Taq DNA polymerase (Goldstar; Eurogentec), for a total volume of 50 μl including 5 μl of sample DNA. For the B22-B23 primer set, the optimized conditions were identical except that the amounts of primers and Taq polymerase were 10 pmol and 1 U, respectively. The hot-start technique (Dynawax; Eurogentec) was used to increase specificity. The reaction mixtures were cycled in an M.J. Research PTC-100 thermal cycler by using the following conditions: 94°C for 3 min; 40 cycles of 94°C for 30 s, 57°C (for primer set T1-T4) or 59°C (for primer set B22-B23) for 30 s, and 72° for 30 s; and 72°C for 10 min. All samples were tested in quadruplicate and, for some mimic samples, additional replicate experiments were performed to precisely determine the detection limits of the methods. In addition, for each sample, one internal control tube was included for detection of PCR inhibition; it consisted of a minimal amount of purified parasite DNA (equivalent to 0.8 *Toxoplasma* genome) added to the 5 μl of sample DNA. Three negative control tubes that each received 5 μl of $H_2O$ instead of DNA were included in each test to detect carryover contaminations. Extreme physical separation and decontamination procedures (17) were used to avoid contaminations by amplicons.

The reaction products were visualized under UV light after electrophoresis in a 3% agarose gel. The expected fragment sizes were 194 and 114 bp for the T1-T4 and B22-B23 primer methods, repectively. All gels presenting bands of the expected sizes were subjected to Southern blot analysis and hybridized with an $\alpha$-$^{32}$P-labeled B1 gene-specific DNA probe in order to check the specificity of the amplified product. The B1 probe was a generous gift from John Boothroyd and Eduardo Ortega-Barria of the Stanford University School of Medicine (16). It consisted of a 2.2-kb EcoRI genomic DNA fragment comprising a single repeat from the B1 gene. A result was considered positive when one reaction yielded an amplification product hybridizing to the specific DNA probe.

In some instances, PCR products were gel purified by electroelution and cloned into the pGEMT plasmid vector (Promega), and their nucleotide sequences were determined in both directions from double-stranded DNA by use of dye-primer technology with a Vistra (Amersham) automated sequencer. Raw sequences were submitted to BLASTN searches in the GenBank database.

TABLE 1. Comparison of two PCR methods (using artificial samples) in the presence of human DNA

| Primer set | No. of positive reactions/no. of reactions performed with DNA of: | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | AF | | | | | Blood or placenta | | | | |
| | 2[a] | 1 | 0.5 | 0.1 | 0.05 | 2 | 1 | 0.5 | 0.1 | 0.05 |
| B22–B23 | 8/8 | 8/8 | 8/8 | 6/8 | 3/8[b] | 8/8 | 8/8 | 8/8[b] | 6/16[b] | 2/16[b] |
| T1–T4 | 8/8 | 8/8 | 7/8 | 4/8 | 2/8[b] | 8/8 | 6/8[b] | 4/8[b] | 2/16[b] | 0/16 |

[a] Number of PGEs per PCR tube. Theoretically, these values would correspond to 10, 5, 2.5, 0.5, and 0.25 PGE/ml of AF, to 14, 7, 3.5, 0.7, and 0.35 PGE/ml of blood, and to ~60, 30, 15, 3, and 1.5 PGE/g of placenta (from left to right, respectively).
[b] Bands of low staining intensity in ethidium bromide-stained gels.

***Toxoplasma* SDA.** The analytical sensitivities of both assays, determined by a *Toxoplasma* SDA, were very similar and indeed quite high, i.e., at least 0.5 (and as low as 0.05) parasite genome equivalent (PGE) per reaction tube. Below 0.5 PGE per reaction tube, both assays gave inconsistent though reproducible results, i.e., only a portion of the reactions was positive, thus defining the detection limit of these methods. This finding implies that, at these low concentrations, more reactions must be carried out in each experiment to affirm a positive PCR (7, 18, 19). At 0.1 PGE and more so at 0.05 PGE per reaction tube, method B (with six and four positive reactions, respectively, out of eight) performed better than method T (with four and eight positive reactions, respectively, out of eight). None of the methods detected 0.01 PGE.

**Comparison of two PCR methods using artificial human samples.** In order to mimic nonideal conditions of clinical laboratory practice and analyze the effect of host DNA upon the performances of both methods, we then thoroughly optimized the methods with AF DNA as well as placenta and blood DNA. The presence of AF DNA did not alter the performance of either method in comparison to that of the *Toxoplasma* SDA (Table 1): both methods could consistently detect 0.5 PGE/reaction tube (theoretically corresponding to 2 to 3 PGE per ml of AF) and inconsistently down to 0.05 PGE/reaction tube (0.25 PGE/ml). This result compares well with the best sensitivity estimates reported by other authors (8). The difference between the two methods was more visible when tests were performed in the presence of placenta and blood DNA, even though the level of sensitivity was still high for both, i.e., <1 *Toxoplasma* genome/reaction. With placenta and blood DNA, the detection limit of method B was fourfold lower than that of method T with regards to constant detection, i.e., 0.5 and 2 PGEs/PCR tube, respectively. Below these values, as in the SDA, the reactions were inconsistently positive, i.e., at 0.1 to 0.05 PGE and 1 to 0.1 PGE per reaction tube for methods B and T, respectively (Table 1). It should be stressed that the intramethod reproducibility of both assays was high with both the SDA and the seeded samples, including those assays with low parasite concentrations.

**Comparison of PCR methods using clinical samples.** Both methods were then tested using 96 human clinical samples (see

TABLE 2. Comparison of two PCR methods for the detection of *Toxoplasma* in clinical biological samples

| Method B result | No. of samples with indicated result by method T | | | Total no. of samples |
|---|---|---|---|---|
| | 100% positive[a] | Weakly positive[a] | Negative | |
| 100% positive | 20 | 9 | 1 | 30 |
| Weakly positive | 1 | 17 | 15[b] | 33 |
| Negative | 0 | 1 | 31 | 32 |
| Total | 21 | 27 | 47 | 95[c] |

[a] See the text for definitions.
[b] Seven of these samples had to be diluted in order to eliminate PCR inhibitions.
[c] One placenta sample that yielded a reaction inhibition with both methods was not included.

Material and Methods). The relative sensitivities of the methods were assessed based on the number of samples found positive with one or both methods and then validated on clinical grounds and/or by mouse inoculation. For each of the methods, each positive result was grouped into the category of 100% positive or weakly positive. The first category represents PCR amplifications in which all of the tubes yielded a positive signal, whereas the second category represents those in which only a portion of the reactions were positive (e.g., two out of four).

Overall, 64 samples were positive by at least one of the two methods. All but 1 sample (98.4%) were positive with method B, but only 48 samples (75%) were positive with method T (Table 2). Regarding AF samples, both methods performed equally well: nine samples were 100% positive and one was weakly positive by both methods. When blood and placenta clinical samples were used, the differences between the methods were much more obvious. Methods B and T detected 98 and 69%, respectively, of all the positive blood- or tissue-containing samples (Table 2). Among those, method T detected only 53% of the weakly positive samples that were detected by method B.

A second criterion of comparison was the frequency of PCR inhibition. No inhibition was observed with AF DNA, and only one placenta DNA sample yielded PCR inhibition by both methods. Overall, out of 73 blood or tissue samples, only one (1.3%) showed a reaction inhibition with method B, but 15 (20.5%) showed a reaction inhibition with method T. In all these cases, the inhibitions were not absolute (which means that positive controls remain negative and no result can be given, even after dilution of the sample DNA) but only partial, i.e., a result (positive or negative) was determined after a moderate dilution (1/3 to 1/10) of the DNA extract. Thus, the poor performances of method T were due partly to a greater propensity to PCR inhibition when clinical samples were being tested.

With regards to specificity, it is essential to note that no contamination of the PCR was observed during or before the course of the study, as inferred from the consistent negativity of ~1,500 negative controls as well as that of all the negative control samples. The technical specificity was assessed on the presence of spurious amplification products of the expected size. In this respect, method T proved less specific in the absence of specific DNA probe hybridization. Indeed, 31% of the negative or weakly positive samples exhibited spurious amplification products of the expected size with method T compared to 8% with method B. Moreover, in contrast to method T, method B (i) did not yield spurious amplifications with AF samples and (ii) yielded only false weakly positive results, i.e., in only a proportion of the reactions for a given sample. Spurious products obtained with both methods were cloned, sequenced, and compared against the EMBL nucleotide sequence database: their sizes differed by only a few base pairs from the original products, and their sequences matched nonannotated sequences of human chromosomes 3 and 10 for methods T and B, respectively.

**Conclusions.** With regards to the prenatal diagnosis of congenital toxoplasmosis by the use of AF, both methods T and B performed equally well and therefore appear adequate; but, with primer set T, an additional step for confirming the identity of the PCR product (which may also be achieved through an enzyme-linked immunosorbent–PCR assay) is of utter importance. For prenatal diagnosis, the fact that both methods perform equally well would tend to validate the current choice of B1 gene-based PCR assays, which, in any case, appear clearly more sensitive than assays targeting the single-copy P30 gene (reviewed in reference 1). In contrast, for the diagnosis of congenital toxoplasmosis at birth or for the diagnosis of toxoplasmosis in immunocompromised patients, we recommend the use of primers B22 and B23 for their higher sensitivity, specificity, and practicability. The additional step mentioned above may still be necessary with these primers, especially in the presence of weak positives (as defined above). On the other hand, the development of more secure and more efficient methods such as real-time PCR should be encouraged (3, 21), as these methods (i) reduce contamination risks, (ii) ensure specificity, and (iii) suppress the cumbersome processing of gels and Southern blots. However, they do not remove the need for a fine optimization of the PCR itself, as shown by the sensitivities usually reported for B1 gene-based real-time PCR assays, with detection thresholds ranging from 10 to 0.75 PGE per reaction depending on the reports (5, 9, 14, 21), i.e., no better than the results presented here. More intralaboratory comparisons and more DNA targets should continue to be investigated to ensure consistent progress in the molecular diagnosis of toxoplasmosis.

We are grateful to Claudine Rouquairol and Catherine Martinez for technical help with the PCR. We thank all our colleagues at the Centre Hospitalier Universitaire as well as from other clinics in the department for their collaboration. We acknowledge John Boothroyd and Eduardo Ortega-Barria, of the Stanford University School of Medicine, for providing the B1 gene probe. We are also grateful to Jean-Pierre Dedet for continuous encouragement.

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