JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1990, p. 2297–2301
0095-1137/90/102297-05$02.00/0

Vol. 28, No. 10

Rapid Prenatal Diagnosis of Congenital *Toxoplasma* Infection by Using Polymerase Chain Reaction and Amniotic Fluid

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Received 5 February 1990/Accepted 25 July 1990

Infection of pregnant women with *Toxoplasma gondii* places the developing fetus at risk for congenital infection. We report a prospective study of 43 documented cases of acute maternal *Toxoplasma* infections acquired during gestation in which the polymerase chain reaction (PCR) was evaluated for diagnosis of fetal infection and compared with the current standard methods. On the basis of direct lysis of pelleted amniotic fluid cells followed by amplification of a gene sequence specific for *T. gondii*, PCR correctly identified the presence of *T. gondii* in five of five samples of amniotic fluid from four proven cases of congenital infection. PCR also detected three of five positive cases from a nonprospective group. The two diagnostic methods of comparable speed, detection of specific immunoglobulin M from fetal blood and inoculation of amniotic fluid into tissue culture, correctly identified only 3 and 4 of the 10 positive samples, respectively. The considerably more time-consuming methods of mouse inoculation of amniotic fluid and fetal blood both detected 7 of 10 positive samples. There were no false-positive diagnoses by any of the methods. Therefore, detection of *T. gondii* by PCR appears to be the most promising method for prenatal diagnosis of congenital *Toxoplasma* infection, since it is both extremely rapid and highly sensitive.

Congenital toxoplasmosis is the result of transplacental transmission of *Toxoplasma gondii* from an acutely infected mother. When infection of the fetus occurs early in pregnancy, death in utero or severe neurological damage may result (12). Infection of the fetus later in gestation is more likely to be asymptomatic at birth yet may ultimately result in untoward sequelae in the child (12).

Prenatal diagnosis of congenital *Toxoplasma* infection permits treatment of the fetus (2, 4, 12) as well as informed decision making for therapeutic abortion. However, at present, attempts to diagnose infection of the fetus in utero are rare in the United States (13). This is in part because in the United States, current methods to detect *Toxoplasma* infection in utero can be too time-consuming to permit definitive diagnosis before a decision regarding therapeutic abortion must be made. Since the majority of women who acquire primary *Toxoplasma* infection during the first or second trimester of gestation will not transmit the infection to their offspring, many uninfected fetuses may be aborted unnecessarily under current practices (12).

Definitive diagnosis of congenital infection often requires culture of amniotic fluid and fetal blood in both mice and fibroblast cell culture (5). Fibroblast cell culture may provide the diagnosis in 4 days but is effective in only about half the cases (3). Isolation by mouse inoculation, while more sensitive, requires an additional 3 to 6 weeks and is available in relatively few laboratories in the United States. No single method has proven reliable in more than 64% of cases (2), and it is not uncommon for only one of the four techniques to be positive.

We have established (1) an assay for the detection of the presence of *T. gondii* on the basis of in vitro DNA amplification of a sequence within the 35-fold repetitive B1 gene by using the recently developed polymerase chain reaction (PCR; 14). The amplified gene product is identified by hybridization with a labeled probe. We have previously reported detection of single parasites under ideal laboratory conditions (1). In this report, we describe a prospective study to determine the feasibility of diagnosing *Toxoplasma* infection in utero by using PCR. Diagnosis using PCR on cells pelleted from amniotic fluid is compared with existing techniques for detection of the organism by inoculation of amniotic fluid into mice and tissue culture, by inoculation of fetal blood into mice, and by detection of specific immunoglobulin M (IgM) in fetal blood.

MATERIALS AND METHODS

Patients and controls. A consecutive group of 43 pregnant women in whom acquired *Toxoplasma* infection during pregnancy was detected by systematic serologic screening was referred to the Service de Medecine et de Biologie Foetales (F. Daffos and F. Forestier), Institut de Puericulture, in Paris, France, between 13 December 1988 and 9 February 1989. Diagnosis of fetal infection was established in positive cases by mouse inoculation or cell culture as described below. Confirmation of negative prenatal diagnoses was by placental inoculation and serologic follow-up of the infant (5).

After an analysis of the initial prospective study, five additional positive cases were selected, nonconsecutively, from later cases in the same study group. At the same time, five amniotic fluid samples from pregnant women referred to the Palo Alto Medical Clinic, Palo Alto, Calif., with indications for amniocentesis other than toxoplasmosis screening were obtained to serve as a negative control group.

Prenatal diagnosis. The protocol and techniques for prenatal diagnosis have been described previously (2, 3, 5, 12). Briefly, amniocentesis and fetal cord sampling were performed between 20 and 34 weeks of gestation. After elimination of the possibility of maternal blood contamination, as described previously (7), fetal blood was tested for specific IgM by the so-called immunosorbent agglutination assay (ISAGA) and inoculated into mice (6). Amniotic fluid (10 to

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15 ml) was also inoculated into cell cultures and mice. Cell cultures were examined 4 days after inoculation, as previously described (3). Inoculated mice were examined for *Toxoplasma* infection 3 and 6 weeks after inoculation, as described previously (12).

Processing of amniotic fluid for PCR. Approximately 5 ml of fresh amniotic fluid was centrifuged at 4,000 × *g* for 15 min at room temperature, and the supernatant was discarded. The pellets were frozen and sent to Palo Alto, Calif., for PCR analysis, where they were then thawed, suspended in 250 μl of distilled water, divided into 50-μl aliquots, and refrozen.

Portions of each sample were prepared by direct lysis in distilled water immediately prior to PCR analysis as follows: 50-μl aliquots prepared as described above were thawed, vortexed for 5 s, heated to 94°C for 15 min, vortexed for 5 s, and spun at 14,000 rpm in an Eppendorf microfuge for 10 min to pellet cell debris (preliminary experiments indicated that the cell debris is otherwise inhibitory to PCR). A portion (15 or 25 μl) of the resulting supernatant was removed and amplified as described below. The remaining supernatant was stored frozen.

PCR detection. The target for amplification was the 35-fold repetitive B1 gene (coding function unknown) of *T. gondii* (1). To date, the B1 gene has been detected by PCR amplification in all 21 *Toxoplasma* strains tested (1; C. M. Grover and J. C. Boothroyd, unpublished results), yet it is undetectable in the DNA of a variety of closely related organisms and humans (1). PCR conditions and detection of amplified material were as described previously (1). Briefly, amplification of a 193-base-pair segment was directed by primers (oligonucleotides 1 and 4 of reference 1) in a 100-μl reaction volume with 10 mM Tris chloride (pH 8.3), 2 mM $MgCl_2$, 100 μM deoxynucleoside triphosphates (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 0.01% gelatin, supernatant of whole-cell lysates, and 0.1 μM oligonucleotides. Samples were amplified for 55 cycles in an automated PCR machine (Perkin-Elmer Cetus, Norwalk, Conn.). After an initial 10-min denaturation at 94°C, each cycle consisted of 1 min of denaturation at 94°C, 15 s of annealing at 60°C, and 45 s of extension at 72°C with an additional second of extension added with each successive cycle. The final extension step continued for an additional 7 min.

Reaction products were analyzed exactly as described previously (1). Briefly, one half of each reaction mixture was denatured in formaldehyde, applied to nitrocellulose with a slot blot apparatus (Bio-Rad Laboratories, Richmond, Calif.), and probed with a 5′-^{32}P-labeled oligonucleotide (oligonucleotide 3 from reference 1) specific to the B1 gene and wholly within the region amplified. Autoradiograms were obtained after 1 to 12 h of exposure on X-Omat AR or RP film (Eastman Kodak Co., Rochester, N.Y.) at −70°C.

Positive amplification controls consisted of 3 or 10 tachyzoites sorted directly into reaction tubes by a fluorescence-activated cell sorter. Amplification efficiency was significantly less in positive control tubes (data not shown), because they were originally sorted into a brand of tubes (siliconized tubes; Intermountain Scientific, Bountiful, Utah) different from those subsequently found to be preferable and used for all amniotic fluid samples and negative controls (0.6 ml; Perkin-Elmer). For this reason and because the amniotic fluid pelleted material is partially inhibitory to PCR, direct quantitative comparison of results obtained with different experimental samples and even the control was not possible. Negative amplification controls consisted of deionized water amplified as described above for the amniotic supernatants.

All samples were initially amplified once each at 15- and 25-μl dilutions. Samples which gave readings significantly above background (negative controls) were judged positive, while those at or below background were judged negative. Samples only slightly darker than background were judged indeterminate. A positive signal at both 15- and 25-μl volumes was confirmed by further amplification of a 15-μl sample, and if all three were positive, the results were considered indicative of infection. Samples initially positive at only one dilution and negative or indeterminate at the other dilution were repeated at the positive dilution and judged diagnostic of congenital infection if positive at that dilution a total of three of three times.

Batches of 8 to 15 samples were analyzed together with one positive amplification control and two negative amplification controls. The latter were positioned first (control A) and last (control B) in each batch of tubes so that contamination during dispensing of reagents could be detected. If either negative control showed a positive or indeterminate result, the entire experiment was excluded from the final tabulation. If the positive control failed and no sample showed positive amplification at a level equal to that of a duplicate experiment, the results of the experiment were also not included. All PCR work (subsequent to pelleting the amniotic fluid) and initial interpretation of the results were performed by one individual blinded to the results of the other tests.

Steps taken specifically to avoid contamination (9, 11) included the use of positive displacement pipettes for dispensing of master mixes, minimal positive controls with no plasmid DNA, premixed reagents, disposable gloves, and separate benches and materials for pre- and postamplification work. Negative controls contained only PCR components and water (no target or background DNA) to ensure optimal amplification of any contaminating material.

RESULTS

Preliminary experiments were first performed with amniotic fluid from known cases of congenital infection with *Toxoplasma gondii* (data not shown). In those experiments, PCR analysis using raw amniotic fluid was substantially less sensitive than when the pelleted material from the same fluid was analyzed. Accordingly, we standardized our protocol to include a preliminary pelleting to concentrate any parasites that might be present (see Materials and Methods). Prior to PCR analysis, such pellets were suspended in water, boiled to lyse any organisms, and then cleared of cell debris by centrifugation.

The first group of samples analyzed was from a prospective group of 43 consecutive amniocenteses, using 15 and 25 μl of spun lysate representing approximately 6 and 10% of the pellet from 5 ml of amniotic fluid, respectively. Figure 1 shows an autoradiogram of three representative experiments including the five samples which upon unblinding were from definitively diagnosed congenital infections (samples 5, 25, 27, 30, and 41; see below). All five were positive by PCR. In addition, two samples not judged positive by any of the other procedures (samples 3 and 4) gave positive signals in this experiment; since these were not reproducibly positive, they were ultimately designated PCR negative (see below).

Table 1 shows the results of PCR analysis for the entire prospective group (samples 1 to 43). On the basis of previously established criteria, the group included five samples

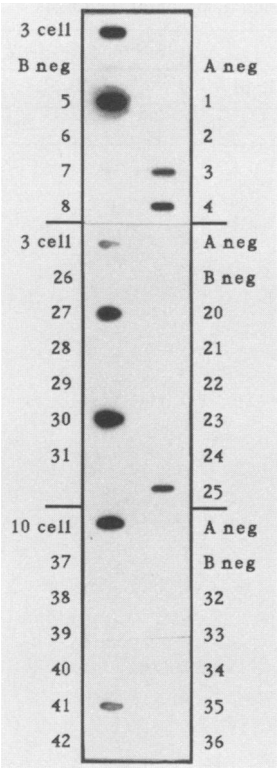

FIG. 1. Sample blots showing PCR detection of *T. gondii* from amniotic fluid of pregnant women with acute toxoplasmosis. Lysates of pelleted amniotic fluid cells were subjected to 55 cycles of amplification, with primers specific for the B1 gene of *T. gondii*, as described in Materials and Methods. PCR products were then applied to nitrocellulose and hybridized with a third radiolabeled oligonucleotide specific to the amplified fragment. The autoradiograph resulting after 1 h of exposure shows results of three experiments (separated by horizontal lines) analyzing samples 1 to 8, 20 to 31, and 32 to 42, with negative controls (A and B) for each experiment. The positive controls consist of amplified product from either 3 or 10 *T. gondii* cells. Samples 3 and 4 are representative of sporadic false-positives as recorded in Table 1.

from known cases of congenital infection (representing four separate cases, since samples 5 and 30 were from the same individual at 29 and 35 weeks of gestation, respectively). PCR detected all five of these positive samples by using a 25-μl volume and four of them by using a 15-μl volume. One sample, number 27, which was initially negative at a 15-μl volume and positive at a 25-μl volume, was confirmed to be a true-positive upon retesting twice at 25 μl.

In comparison, the standard methods had a lower efficiency of detection, with three samples positive by IgM, three positive by mouse inoculation of fetal blood, three positive by mouse inoculation of amniotic fluid, and two positive by inoculation of amniotic fluid into tissue culture. In one sample, number 27, PCR detected *T. gondii* in amniotic fluid while the standard methods with amniotic fluid did not (definitive diagnosis was from inoculation of fetal blood).

By using the criterion of three clear positive results necessary for a positive PCR diagnosis, no false-positive diagnoses of 43 samples analyzed by PCR occurred. Although there was a total of 11 instances of sporadic positive signals and 3 indeterminate signals, in no case were these reproducible. Accordingly, these samples were judged negative.

To further test the utility of the assay, five more samples from known positive cases were analyzed. These were from the same prospective study (numbers 115, 118, 129, 130, and 140) but were analyzed in isolation, that is, they were identified as positive by one or more of the standard procedures, coded along with five negative controls, and subjected to PCR analysis. The results of this testing are also shown in Table 1. PCR correctly identified three of five positive samples. In contrast, no positive samples were seen by IgM analysis of fetal blood, and only two positive samples were detected by inoculation of amniotic fluid into tissue culture. However, the more time-consuming standard methods showed greater sensitivity with four positive samples by mouse inoculation of fetal blood and four positive samples by mouse inoculation of amniotic fluid. Results from analysis of the five negative controls are not shown; all were negative by PCR, although there was one instance of a positive signal at a 15-μl volume which was not reproducible; therefore, this sample was judged PCR negative.

The two false-negatives determined by PCR in this second series were numbers 129 and 130. Unlike the other three samples in the series, both were positive by only one of the four standard methods, with number 129 positive only by mouse inoculation of fetal blood (i.e., no parasites were detectable in the amniotic fluid of this patient by any of the methods). Both samples also had exceptionally large amounts of erythrocytes. We had previously noted that in some cases, excessive cell contamination could inhibit the PCR reaction (C. M. Grover and J. C. Boothroyd, unpublished results) such that for a given sample, a smaller volume can give a stronger signal than larger volumes. Therefore, after unblinding, samples 129 and 130 were reanalyzed by using smaller volumes of supernatant (5 μl), but they were again negative (data not shown). This point is further discussed below.

When results of the first prospective series of 43 samples were pooled with those of the second series of 10, a total of 8 of 10 positive samples were correctly diagnosed as positive by PCR with no false-positive diagnoses. This was at least twice as sensitive as the two methods of comparable speed, amniotic fluid culture (40%) and IgM-ISAGA (30%), and marginally more sensitive than the time-consuming methods, amniotic fluid inoculation (70%) and blood inoculation (70%), which take at least 3 weeks to complete.

DISCUSSION

Results of this study indicate that detection of *T. gondii* by in vitro DNA amplification shows considerable promise as a method for prenatal diagnosis of congenital infection from amniotic fluid.

There were two false-negative results by PCR (samples 129 and 130). Several possible explanations exist for these results. First, as already mentioned, samples 129 and 130 were both exceptionally bloody and thus may contain excessive inhibitory material (e.g., heme). Attempts to amplify material from a smaller volume were unsuccessful, but this has the obvious caveat that the smaller volume necessarily contains less target.

Second, the probes we used may not be capable of amplifying the B1 gene in the parasites present in samples 129 and 130. Since the B1 gene was detectable with these probes in 21 of 21 previous *Toxoplasma* strains examined (1; C. M. Grover and J. C. Boothroyd, unpublished results), this

TABLE 1. Detection of *T. gondii* in blood and amniotic fluid obtained at prenatal diagnosis[a]

Sample	Time of sampling (wk)	Fetal blood		Amniotic fluid		PCR (amniotic fluid pellet)				Diagnosis (interpretation)
		Mouse inoculation	IgM ISAGA	Tissue culture	Mouse inoculation	15 μl		25 μl		
						1st	2nd	1st	2nd	
1	31	−	−	−	−	−		−		−
2	22	−	−	−	−	−		−		−
3	26	−	−	−	−	−		+	−	−
4	22	−	−	−	−	−		+	−	−
5[b]	29	+	+	−	+	+	+	+		+
6	21	−	−	−	−	−		−		−
7	20	−	−	−	−	+	−	−		−
8	21	−	−	−	−	−		−		−
9	23	−	−	−	−	−		+	−	−
10	20	−	−	−	−	−		−		−
11	23	−	−	−	−	−		−		−
12	20	−	−	−	−	−		−		−
13	26	−	−	−	−	−		−		−
14	21	−	−	−	−	−		−		−
15	21	−	−	−	−	−		+	−	−
16	27	−	−	−	−	+	−	−		−
17	30	−	−	−	−	−		+	−	−
18	22	−	−	−	−	−		−		−
19	31	−	−	−	−	−		−		−
20	23	−	−	−	−	−		−		−
21	20	−	−	−	−	−		−		−
22	21	−	−	−	−	−		−		−
23	21	−	−	−	−	−		−		−
24	22	−	−	−	−	+	−	−		−
25	28	−	+	+	−	+	+	+		+
26	26	−	−	−	−	+	−	−		−
27	21	+	−	−	−	−	−	+	+/+	+
28	24	−	−	−	−	−		−		−
29	21	−	−	−	−	−		−		−
30[b]	35	−	+	+	+	+	+	+		+
31	26	−	−	−	−	−		−		−
32	22	−	−	−	−	−		−		−
33	24	−	−	−	−	−		−		−
34	24	−	−	−	−	−		−		−
35	30	−	−	−	−	+	−	−		−
36	20	−	−	−	−	+	−	−		−
37	30	−	−	−	−	−		−		−
38	22	−	−	−	−	−		−		−
39	27	−	−	−	−	−		−		−
40	22	−	−	−	−	−		−		−
41	25	+	−	−	+	+	+	+		+
42	25	−	−	−	−	−		−		−
43	23	−	−	−	−	−		−		−
115	28	+	−	+	+	+	+	+		+
118	28	+	−	−	+	+	+	+		+
129	29	+	−	−	−	−		−		−
130	34	−	−	−	+	−		−		−
140	26	+	−	+	+	+	+	+		+

[a] Details of the culturing, mouse inoculation, and IgM tests are given in the text. PCR was performed with either 15- or 25-μl samples of cleared lysate from amniotic fluid pellets (see Materials and Methods). When a sample was positive in the first analysis at both volumes, the test was repeated by using a 15-μl volume. If positive at only one volume, the test was repeated at this same volume, and if positive again, the test was repeated at this volume a third time (indicated by +/+ and only necessary for sample number 27). A positive diagnosis by PCR required three PCR analyses to be positive (see Materials and Methods). Time of sampling refers to the estimated gestational age of the fetus when amniocentesis and fetal cord sampling were performed. Samples 1 to 43 are consecutive. The last five samples were from patients identified as positive subsequent to the analysis of the initial cohort of 43 (see Materials and Methods).

[b] Samples 5 and 30 are from the same pregnancy at 29 and 35 weeks of gestation, respectively.

result seems least likely, but we tested it by using a different set of primer oligonucleotides for amplification of B1. Although the positive controls worked equally well with this second set of oligonucleotides, samples 129 and 130 were again negative (data not shown). We cannot exclude the possibility that the B1 gene is entirely missing in these two strains, but again, its presence in 21 of 21 strains previously tested argues against this.

Finally, since samples 27 (negative at 15 μl but positive three times at 25 μl), 129, and 130 were each positive by only one of the four standard methods (in contrast to the other seven positive samples), they could represent infections with a smaller number of organisms. In this regard, it is important to note that the tubes for PCR received at most 10% of the material used to infect the mice and thus, because of sampling variation, could contain no parasites. We consider

this the most likely explanation. Attempts at improving the efficiency of PCR by procedures such as DNA extraction and the use of larger volumes of amniotic fluid could overcome this problem, although samples such as number 129, which was negative by all assays from amniotic fluid, suggest that the extraction of DNA from fetal cord blood may also be necessary (9).

The pattern of sporadic, positive signals in this study was most consistent with random contamination events as previously reported (11). These may be explained by the fact that much of the work was performed within a laboratory in which large amounts of *Toxoplasma* DNA and, most notably, the B1 gene in the form of a recombinant plasmid have been handled for several years. This would not be encountered in the environment of a clinical laboratory. Furthermore, as PCR becomes widely used for the detection of infectious diseases, systematic protocols and automated handling should effectively eliminate the risk of contamination in the setting of a diagnostic laboratory. In any case, the sporadic positive amplifications did not interfere with diagnosis using PCR in this study. Of course, we cannot formally exclude the possibility that the sporadic false-positives are from samples containing too few parasites to yield reproducibly positive PCR results (i.e., due to sampling error). However, the fact that these samples were from women whose newborns were not diseased clearly argues that the criterion of PCR reproducibility is valid for purposes of diagnosing disease.

In the United States, the single most important advantage of PCR detection of *T. gondii* may be that it could provide a rapid and definitive diagnosis of congenital infection before week 24 of gestation, currently the latest gestational time a woman can terminate her pregnancy under U.S. law. At present, the pregnant woman who is diagnosed with a *Toxoplasma* infection at 22 to 24 weeks of gestation is in a difficult predicament. She must decide before week 24 whether to terminate the pregnancy or seek treatment for the infection and carry her fetus to term, without waiting the 3 to 6 weeks which may be necessary to establish definitive diagnosis by isolation of the parasite. This delay in definitive diagnosis leads to the termination of many pregnancies, when only about 20% of such fetuses are actually infected (12). These unnecessary abortions, in addition to the difficult predicament of the mother, emphasize the need for methods which will provide more immediate diagnosis of fetal infection.

PCR could meet this need for rapid and definitive diagnosis, since results are available within 1 to 2 days of aminocentesis. Since amniocentesis can easily be performed as early as week 15 of gestation, PCR diagnosis of congenital infections from amniotic fluid between weeks 15 and 19 of gestation may also be possible (8), although it was not tested in this study (sampling at week 20 of gestation was the earliest done in this study). More rapid definitive diagnosis of congenital infections by PCR should also promote earlier treatment of infected fetuses with the most efficacious antibiotics, pyrimethamine in combination with sulfonamides (10, 12). Because of potential hazards for the mother or fetus, use of this antibiotic combination is usually restricted to cases in which fetal infection has been demonstrated.

ACKNOWLEDGMENTS

We thank J. Lawrence Burg for his efforts in the development of PCR detection of the B1 gene and Dyann Wirth for her advice on avoiding contamination. We also thank our many colleagues for the use of uncontaminated bench space and equipment.

This work was supported by Public Health Service grants AI 21423, AI 25732, and AI 04717 from the National Institutes of Health, the John D. and Catherine T. MacArthur Foundation, and the Caisse Nationale de l'Assurance Maladie des Travailleurs Salaries (CM-CYM 135089). J.C.B. is a Burroughs Wellcome Scholar in Molecular Parasitology. C.M.G. was the recipient of a Medical Scholars award from Stanford University School of Medicine.

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