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Improved Sensitivity of the Polymerase Chain Reaction for Detection of *Toxoplasma gondii* in Biological and Human Clinical Specimens

U. Groß[1]*, A. Roggenkamp[1], K. Janitschke[2], J. Heesemann[1]

The aim of the present study was to improve the sensitivity of the polymerase chain reaction for detection of *Toxoplasma gondii* in biological and clinical specimens. Using a pair of primers amplifying a 634 bp fragment of the B1 gene of this parasite, it was possible to detect ten parasites in 100 µl of sample suspensions containing a high concentration of concomitant host cells. A comparison of different DNA purification methods indicated that cell-rich clinical specimens intended for use as samples for the polymerase chain reaction should be digested with proteinase K prior to DNA amplification. By using the described sample preparation methods and the polymerase chain reaction, *Toxoplasma gondii* DNA was demonstrated in ten of 52 clinical specimens of patients with clinical or serological indications of toxoplasmosis.

Toxoplasma gondii, an obligate, intracellular parasite, is the etiological agent of toxoplasmosis, a disease which generally is asymptomatic in healthy adults. However, primary infection acquired during pregnancy may result in the transmission of this parasite to the fetus, resulting in blindness, cerebral damage or even the death of the congenitally infected infant (1, 2). Recently, generalised toxoplasmosis has been considered to be the cause of death in many AIDS patients (3–5). It is estimated that 3–40 % of HIV-positive patients suffer from toxoplasmic encephalitis, which mostly is considered to result from reactivation of *Toxoplasma* cysts (6–10).

Thus far, only serological methods, radiological examination of the brain, and attempts to isolate *Toxoplasma gondii* from clinical specimens are available for the diagnosis of cerebral toxoplasmosis (11–20). However, serological tests in affected patients often fail to give unequivocal results, and isolation attempts are either unsuccessful or time-consuming, requiring four to eight weeks (7). Since the development of polymerase chain reaction (PCR), this technique has been applied for diagnosis of several different infections (21–26).

Only a few nucleotide sequences of *Toxoplasma gondii* genes are available for designing primers intended for use in PCR (27–31). More recently, *Toxoplasma gondii* has been detected in amniotic fluid by using DNA amplification of a part of the B1 gene, which has been detected by PCR in all *Toxoplasma gondii* strains tested thus far (28, 32). PCR has also been described for amplification of the P30 gene of *Toxoplasma gondii* in vitro (33). However, in all studies analysing PCR for diagnostical purposes of toxoplasmosis, optimal conditions for preparation of the clinical specimen prior to PCR have been lacking, a factor that might result in insufficient sensitivity. In addition, no PCR results have been published for detection of *Toxoplasma gondii* DNA in biopsy materials such as brain or lymph nodes.

Therefore, we attempted to optimize the conditions for PCR and for handling of clinical material by comparing different methods for the preparation of cell-rich biological specimens and applying these techniques to the clinical diagnosis of toxoplasmosis.

Materials and Methods

Reagents. If not mentioned otherwise, all chemicals were from Merck, Germany.

Isolation of Toxoplasma gondii Tachyzoites. Toxoplasma gondii laboratory strains RH and BK, as well as the

[1] Institute of Hygiene and Microbiology, University of Würzburg, Josef-Schneider-Str. 2, D-8700 Würzburg, Germany.
[2] Robert Koch Institute, Nordufer 20, D-1000 Berlin 65, Germany.

clinical isolates ALT, 177, NTE and 561, were grown in cell culture by using the permanent mouse macrophage cell line P388D1 (34) in RPMI 1640 (Biochrom, Germany) supplemented with 5 % fetal calf serum, 300 mg/l L-glutamine and antibiotics. Alternatively, tachyzoites were grown in mouse ascites after intraperitoneal injection. Tachyzoites were separated from host cells by filtering the obtained cell suspension through a 3.0 µm polycarbonate filter membrane (Nucleopore, Sweden). Tachyzoites were washed and resuspended in PBS (10 mmol sodium phosphate, 130 mmol NaCl, pH 7.3) at a concentration of 1 x 10^6 tachyzoites/µl.

Isolation of Toxoplasma gondii Bradyzoites. The cyst-forming strain NTE was isolated from the brain of experimentally infected NMRI mice using continuous density-gradient centrifugation as described previously (35).

Preparation of Biological Specimens. Tachyzoites of the RH strain grown in P388D1 cell culture were resuspended at a concentration of approximately ten or 100 tachyzoites in 100 µl of a buffer system derived from clinical specimens. Buffer systems consisted of brain buffer or spleen buffer, made by homogenizing the brain or spleen of uninfected mice (also serologically tested by dye test) in 10 ml PBS, resulting in a host cell count of > 2 x 10^6 brain cells/100 µl and > 4 x 10^5 spleen cells/100 µl. Alternatively, a pool of cerebrospinal fluids from patients serologically negative for *Toxoplasma gondii* was used as a clinical buffer system. The respective *Toxoplasma gondii* positive buffer systems, as well as *Toxoplasma gondii* negative buffers, serving as negative controls, were prepared for PCR using three different protocols described below.

Nucleic acids can be purified by using guanidinium thiocyanate and silica (36). Briefly, 50 µl specimens containing either 0, 10 or 100 parasites were resuspended in 900 µl of lysis buffer consisting of 120 g guanidinium thiocyanate (GuSCN) and 2.6 g Triton X-100 in 122 ml of a solution consisting of a final concentration of 82 mM Tris-Cl and 36 mmol EDTA, pH 8.0. Fifty µl of SiO_2 equilibrated in 0.32 % HCl, pH 2.0, was added to bind liberated nucleic acids. After incubation for 10 min at room temperature, the SiO_2-bound DNA was washed in turn with GuSCN wash buffer (120 g GuSCN in 100 ml 0.1 M Tris-Cl, pH 6.4), 80 % ethanol and acetone. Finally, the DNA was eluted by resuspending the SiO_2-DNA mixture in 70 µl of H_2O, centrifuging, and using 50 µl of the supernatant for PCR. In order to analyse whether not only tachyzoites but also bradyzoites can be detected by the PCR technique, a suspension of 12 cysts of the NTE strain was also treated using this method before applying it to PCR.

In an alternative approach, proteinase K was used to prepare the biopsy specimens (37). Specimens containing 0, 20 or 200 parasites were added to a final volume of 100 µl of a buffer consisting of 10 mmol Tris-Cl (pH 8.3), 1.5 mmol $MgCl_2$, 50 mmol KCl, 0.1 mg/ml gelatine, 0.5 % Tween 20 and 20 µg proteinase K and incubated for 1 h at 55 °C. After inactivating proteinase K at 94 °C for 10 min, the suspension was centrifuged at 10,000 x *g* for 10 min, and 50 µl of the supernatant, corresponding to 0, 10 or 100 parasites, was used for PCR.

In a third approach, specimens containing 0, 10 or 100 *Toxoplasma* cells were prepared by just heating for 15 min at 95 °C.

Primers and Conditions for the Polymerase Chain Reaction. Primers derived from the DNA sequence of the well-characterized B1 gene of *Toxoplasma gondii* were chosen because this gene appears to be conserved in all *Toxoplasma gondii* isolates analysed thus far by PCR (32) and it is present at least 35-fold in the genome (28). Although primers derived from this gene have already been published elsewhere (28), we decided to design different primers flanking a 634 bp fragment. The amplification of a larger DNA segment should make it easier to determine the specificity of the PCR product for *Toxoplasma gondii* DNA by restriction enzyme analysis. Primer 1 (5'-ATG-TGC-CAC-CTC-GCC-TCT-TGG-3') and Primer 2 (5'-GAA-CTG-TAA-TGT-GAT-ACT-GTG-3') were synthesized using a Cyclone Plus DNA synthesizer (MilliGen/Biosearch, Germany).

Where not mentioned otherwise, 50 µl of specimen preparations was added to a final volume of 100 µl PCR buffer consisting of 10 mmol Tris-Cl (pH 8.3), 1.5 mmol $MgCl_2$, 50 mmol KCl, 0.1 mg/ml bovine serum albumin, 100 µmol deoxynucleoside triphosphates (Boehringer Mannheim, Germany), 0.1 µmol of each primer and 2.5 IU of Taq DNA polymerase (Boehringer Mannheim). Samples were overlaid with 100 µl of mineral oil in 1.5 ml Eppendorf tubes and amplified for 40 cycles in a thermic cycler (Biomed, Germany). After an initial 7 min of denaturation, conditions for each cycle were 1.5 min of denaturation at 94 °C, 2 min of annealing at 55 °C and 3 min of extension at 72 °C. The final extension step was continued for an additional 7 min. The PCR products were purified by ethanol precipitation and finally resuspended in 50 µl of H_2O.

Restriction Enzyme Analysis of Amplified DNA. To demonstrate the specificity of the PCR product for *Toxoplasma gondii,* the amplified DNA fragment of the RH strain was digested with AluI, HinfI (Pharmacia, Germany), SstI (BRL, Germany), ClaI and TaqI (Boehringer Mannheim) and analysed on a 3.5 % agarose gel.

Hybridization of the Amplified DNA Fragment. Besides restriction enzyme analysis, the specificity of the amplified DNA fragment for *Toxoplasma gondii* was demonstrated by hybridization. A 98 bp fragment specific to the B1 gene and within the gene sequence amplified was labelled by random primed incorporation of digoxigenin-labelled deoxyuridine triphosphate using the nonradioactive DNA labelling and detection kit as described by the supplier (Boehringer Mannheim). Twenty µl of the PCR product was analysed on a 2.0 % agarose gel (Biozym, Germany) and transferred onto a nylon membrane (Gene Screen Plus, New England Nuclear, USA) by vacuum blotting using a vacuum blot apparatus, LKB 2016 VacuGene (Pharmacia), and the blotting conditions recommended by the supplier. Alternatively, 3 µl of the PCR product was dotted directly onto the nylon membrane. The DNA was fixed to the membrane by baking for 2 h at 80 °C and hybridized with the Dig-dUTP-labelled probe using the nonradioactive DNA detection kit as described by the supplier (Boehringer Mannheim).

Processing of Human Clinical Specimens. Fifty-two clinical specimens were chosen from patients with clinical or serological indications of toxoplasmosis. Twenty-eight of the patients were HIV positive, seven were infants suspected of having congenital toxoplasmosis, and 17 had

either neurological symptoms, lymphadenitis or serological indications of acute toxoplasmosis. A total quantity of 50–500 µl of fluid or homogenized tissue of human origin was centrifuged and the DNA of the pelleted cells isolated by the GuSCN method or by using proteinase K digestion.

Results

Sensitivity and Specificity of the Polymerase Chain Reaction. By heating tachyzoites of the RH strain resuspended in H_2O at 95 °C for 15 min, we were able to detect ten tachyzoites in 100 µl. The negative control, consisting of uninfected P388 macrophages, did not show any amplified DNA (data not shown). To demonstrate the specificity of the amplified DNA fragment for *Toxoplasma gondii*, the PCR product was digested with several restriction enzymes. Analysis of the DNA sequence of the B1 gene showed that the resulting restriction patterns matched the one expected, demonstrating that the amplified DNA fragment indeed was from *Toxoplasma gondii* (data not shown).

The B1 gene seems to be present in all *Toxoplasma gondii* strains, because we were able to amplify the 634 bp segment in the RH strain as well as in five *Toxoplasma gondii* strains isolated in Central Europe (data not shown).

By using PCR, the 634 bp fragment could be amplified from cysts of the NTE strain, demonstrating that bradyzoites can be detected and that the cyst wall has no inhibitory effect for PCR.

Comparison of Methods for Sample Processing. Biological buffer systems composed of either brain or spleen cell homogenates from mice or a pool of human cerebrospinal fluids were used to investigate whether the method of specimen processing is of critical importance for PCR. All experiments were carried out in duplicate. Each specimen contained 0, 10 or 100 tachyzoites of the RH strain (Figure 1). Heating for 15 min at 95 °C did not seem to be sufficient for sample preparations because a positive result was only obtained in one of two reference test specimens harboring 100 tachyzoites. The PCR failed to detect *Toxoplasma gondii* DNA in any of the other biological specimens prepared using this method. An amplified DNA fragment specific for the labelled *Toxoplasma gondii* probe was detected in several of the toxoplasma-containing samples prepared using GuSCN. However, this method was inferior

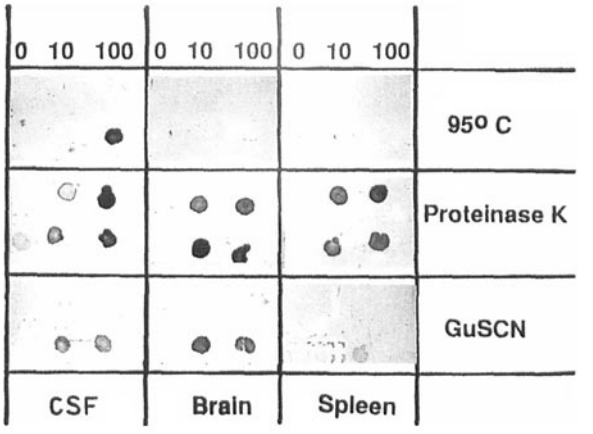

Figure 1: Analysis of different DNA purification methods by hybridization of a *Toxoplasma gondii*-specific probe with amplified DNA from a pool of human CSF or the brain or spleen of mice, each specimen containing 0 (uninfected) 10 or 100 tachyzoites of the RH strain. The samples were treated with GuSCN, proteinase K or just heated for 10 min. Each experiment was carried out in duplicate.

in comparison to proteinase K digestion because not all test results were reproducible and because it was insufficient for detecting ten tachyzoites in spleen cells. Since all specimens artifically inoculated with tachyzoites were detected by using proteinase K digestion and PCR in duplicated experiments, this method of sample preparation seemed to be the method of choice for detection of *Toxoplasma gondii* in clinical specimens by PCR. It even was possible to detect ten tachyzoites in a mixture of spleen cells, although it is known that increased tissue cell counts have an inhibitory effect on PCR (37, 38). However, it must be mentioned that a weak signal was obtained in one of the experiments using uninfected cerebrospinal fluid.

Detection of Toxoplasma gondii in Human Specimens. Fifty-two clinical specimens were analysed using GuSCN or proteinase K and the PCR technique (Table 1). Using proteinase K digestion, an amplified 634 bp DNA fragment was found in six cerebrospinal fluid samples and four tissue biopsies (Table 2). Its specificity for *Toxoplasma gondii* was confirmed by hybridization with the digoxigenin-labelled 98 bp DNA probe (Figure 2). Reliability of the test results was demonstrated by testing all specimens at least twice. Comparable PCR reactions were found in the GuSCN-treated and the proteinase K-treated cerebrospinal fluid samples, whereas the GuSCN method failed to detect *Toxoplasma gondii* DNA in tissue specimens (Table 2).

Table 1: Results of tests for the presence of *Toxoplasma gondii* in clinical specimens and relevant serological characteristics of the patients studied.

No. of samples	Material	PCR	Mouse inoculation	Dye test	IgM-EIA
HIV patients (n = 28)					
11	CSF	–	–	–	–
12	CSF	–	–	< 1:256	–
1	CSF	+	–	1:1000	–
4	CSF	+	+	< 1:256	–
Infants (n = 7)					
3	CSF	–	–	< 1:256	–
3	CSF	–	–	> 1:1000	–
1	CSF	+	–	1:64	–
Adults (n = 17)					
3	CSF	–	–	–	–
1	CSF	–	–	1:64	–
1	CSF	–	–	1:64000	+
2	blood	–	–	> 1:1000	+
2	amniotic fluid	–	–	1:1000	+
1	placenta	–	–	1:4000	+
3	lymph node	–	–	> 1:1000	+
3	lymph node	+	+	> 1:1000	+
1	glandula submandibularis	+	+	1:4000	+

CSF = cerebrospinal fluid.

Table 2: Characteristics of PCR-positive patients.

Case no.	PCR proteinase K	PCR GuSCN	Mouse inoculation	Complement fixation test	Dye test	IgM-EIA
Infant						
1	+	+	–	–	1:64	–
HIV patients						
2	+	+	+	–	1:64	–
3	+	+	+	si	1:25	–
4	+	+	+	1:20	1:16	–
5	+	+	–	si	1:1000	–
6	+	+	+	nt	1:256	–
Adults						
7	+	–	+	1:80	1:4000	+
8	+	–	+	1:80	1:16000	+
9	+	–	+	1:40	1:4000	+
10	+	–	+	1:40	1:4000	+

GuSCN = guanidinium thiocyanate; si = self inhibition; nt = not tested.

The positive cerebrospinal fluid samples were from a congenitally infected infant (case 1) and from five patients suffering from AIDS (cases 2–6). Conventional serological screening of these patients was not definitive for the diagnosis of acute toxoplasmosis because no IgM antibodies could be demonstrated (Table 2). However, the accuracy of the positive PCR results obtained with the cerebrospinal fluid samples was confirmed by the demonstration of tachyzoites in the post mortem examined brain of the infant and by the isolation of viable *Toxoplasma gondii* cells from the cerebrospinal fluid of four AIDS patients using the mouse inoculation method. It was not possible to confirm the positive PCR result by using the mouse inoculation method in

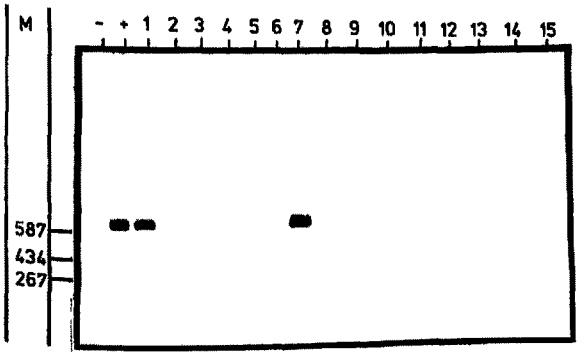

Figure 2: Result of a representative experiment showing the hybridization of a *Toxoplasma gondii*-specific DNA probe with amplified PCR products from human CSF prepared by proteinase K digestion. The CSF samples were from infants suspected of having congenital toxoplasmosis (no. 1–6), adults suffering from AIDS (no. 7–14) and a patient with cerebral cysts of unknown origin (no. 15). - = negative control (PBS); + = positive control (100 tachyzoites of the RH strain in PBS).

one patient (case 5). However, following treatment with pyrimethamine and sulfadiazine the patient's symptoms of toxoplasmic encephalitis declined, indicating the correctness of the PCR result.

The PCR positive tissue specimens were from four adults with lymphadenitis and serological evidence of acute toxoplasmosis. The isolation of *Toxoplasma gondii* by using the mouse inoculation method confirmed the positive PCR results in these patients.

All other patients were negative using either PCR or the mouse inoculation method, although on the basis of serological data the diagnosis of acute toxoplasmosis was likely in nine patients (Table 1).

Discussion

Current diagnosis of *Toxoplasma gondii* infections in infants of recently infected mothers relies mainly on serology and attempts to isolate the parasite from clinical specimens, whereas the diagnosis in patients with AIDS is based on computerized tomography or magnetic resonance imaging scans of the brain and detection of *Toxoplasma gondii* in clinical specimens by mouse or cell culture inoculation (7). However, serological tests and isolation attempts in both patient groups sometimes fail to prove the diagnosis. In addition, diagnosis of toxoplasmic encephalitis cannot rely solely on radiological techniques because lymphomas, metastases or infections with other microbes might mimic brain damage caused by *Toxoplasma gondii*. Therefore, it is necessary to find procedures to improve the diagnosis of toxoplasmosis in such patients.

PCR has been shown to be a new diagnostical tool of high potential for rapid detection of infectious agents. Thus far, the methods of amplification of *Toxoplasma gondii* DNA described were either applied to in vitro models or were from biological material, which contains only a few concomitant host cells (32, 33). Detection of *Toxoplasma gondii* DNA was possible by just boiling the sample prior to PCR. Using mice infected with several clinical *Toxoplasma gondii* isolates, Savva et al. (33) were able to detect *Toxoplasma gondii* DNA in the brain by using sequential PCR. Additional PCR cycles require additional sample handling and could therefore increase the risk of possible cross contamination from carryover of PCR products of different samples (32, 39).

Due to an increasing risk of getting false-positive results, we did not want to use sequential PCR but rather wanted to modify the conditions of sample preparation to detect *Toxoplasma gondii* DNA in cell-rich specimens. As was shown in a study with infected mice, boiling of brain biopsy material is not sufficient for detection of *Toxoplasma gondii* by one-step PCR (33). This observation is in agreement with our study.

To mimic potential biopsy material such as placenta, brain or lymph nodes, we infected buffer systems consisting of brain cells or spleen cells from mice as well as human cerebrospinal fluid with ten or 100 *Toxoplasma gondii* cells. As was shown, only digestion with proteinase K prior to PCR proved to be sufficient for detection of ten or more parasites in all buffer systems tested in this study. This preparation step requires only an additional 90 min and should avoid false-positive results because the test tubes need be opened only once for adding proteinase K buffer. However, a weak signal was obtained in one of two experiments using uninfected cerebrospinal fluid. Since this weak false-positive signal was not reproducible in subsequent experiments, we suggest that proteinase K digestion seemed to be superior to all other preparation methods.

In addition, in order for *Toxoplasma gondii* PCR to become a generally used procedure in all clinical laboratories, a nonradioactive assay for the specific detection of the amplified DNA is important. Our results indicate that a simple dot-

hybridization test on nylon membranes using digoxigenin-labelled probes seems to be an alternative to traditional Southern hybridization with probes labelled with radioisotopes. In addition, this method is specific and can be done within 24 h. As an alternative to time-consuming hybridization, restriction analysis of the PCR product might be of interest for demonstrating the specificity of an amplified DNA fragment.

Using PCR and hybridization, we were able to detect *Toxoplasma gondii* DNA in ten of 52 clinical specimens from patients having clinical or serological indications of toxoplasmosis. Using either the GuSCN or proteinase K method, *Toxoplasma gondii* DNA was detected in six cerebrospinal fluid samples. In contrast, the GuSCN method failed to detect *Toxoplasma gondii* DNA in those four tissue specimens that were positive using proteinase K digestion prior to PCR, indicating the advantage of proteinase K digestion (Table 2). The accuracy of the PCR results was confirmed either by the identification of *Toxoplasma gondii* using the mouse inoculation method, by the demonstration of tachyzoites in histological smears or by clinical improvement following toxoplasma-specific treatment. Isolation attempts failed to demonstrate *Toxoplasma gondii* in the clinical specimens of any of the PCR-negative patients, indicating that the negative PCR results were not false negatives.

These clinical case reports demonstrate that in certain patients PCR might be superior to conventional diagnostical methods. Especially in congenitally infected infants and in AIDS patients, serological methods might be insufficient to confirm the diagnosis of toxoplasmic encephalitis.

In summary, these results suggest that preparation of cell-rich clinical specimens using proteinase K digestion in combination with PCR may potentially be a useful new additional tool for diagnosis of toxoplasmosis.

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