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Design of a one-tube hemi-nested PCR for detection of *Toxoplasma gondii* and comparison of three DNA purification methods

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The aims of the present study were to design an easy and sensitive DNA amplification method for detection of *Toxoplasma gondii* with low risk of accidental contamination, and to find a rapid method for purification of clinical samples containing potential inhibitors of the amplification reaction. With a pair of primers amplifying a 619-bp fragment of the B1 gene of this parasite it was possible to detect DNA equivalent to 10 parasites. When a third primer was added to the same tube, sensitivity increased to 0.1 parasite. In a comparison of different DNA purification methods, the High Pure PCR Template Preparation Kit (Boehringer Mannheim, Germany) gave the best results. With this purification method and the one-tube hemi-nested PCR, *T. gondii* DNA was detected in 14 (87.5%) of 16 clinical specimens (amniotic fluid, broncho-alveolar lavage, bone marrow, blood, liver biopsy) in which the parasite was demonstrated by cell culture.

Introduction

Toxoplasma gondii is the aetiological agent of toxoplasmosis, a disease generally asymptomatic in healthy adults. However, primary infection acquired during pregnancy may result in fatal or severe infection of the fetus [1]. In immunocompromised patients, and especially in those with AIDS, toxoplasmic encephalitis or extracerebral toxoplasmosis may occur, usually resulting from reactivation of chronic infection, with an incidence of up to 50% in HIV-infected patients not receiving specific anti-toxoplasma prophylaxis [2]. Reactivating toxoplasmosis can also cause severe damage in organ and bone marrow transplant recipients [3] and in those with immunodepressive neoplasms [4]. In organ transplant recipients, there is an additional risk of transmission of *T. gondii* from a seropositive donor to a seronegative recipient [5].

T. gondii infections in immunocompromised patients are often rapidly progressive and associated with

significant morbidity and mortality [6]. Serological tests fail to give unequivocal diagnosis in patients with affected immunity [7]. Presently, the diagnosis of toxoplasmosis in these patients is usually suspected on clinical or radiological data, with definite diagnosis being obtained within 2–3 weeks by the regression of the lesions after specific treatment [8, 9]. Isolation of the parasite from several clinical specimens can be achieved, but most isolation techniques (mouse inoculation, tissue culture) require several days or weeks before results can be obtained.

In an attempt to reduce this delay, PCR techniques have been applied to the diagnosis of toxoplasmosis. PCR analysis of amniotic fluid has proved to be the most useful diagnostic method for congenital toxoplasmosis [10] and it is also useful in adult immunocompromised patients, particularly in those with extracerebral disease [11]. However, the most sensitive methods, nested PCR or hybridisation after amplification, are time-consuming or susceptible to accidental cross-contamination of DNA, and a rapid and optimal method for purification of the DNA of the large numbers of clinical specimens available for PCR is lacking. Therefore, this study attempted to optimise the conditions for a rapid and sensitive PCR analysis of clinical material from different origins.

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Materials and methods

Parasite samples

T. gondii tachyzoites of the RH strain were obtained from Swiss albino mice ascites 2 days after intraperitoneal injection of the parasite.

To test the sensitivity of the amplification methods, serial dilutions of parasite DNA were prepared from purified tachyzoites. Parasites collected from ascites were washed and resuspended in PBS at a concentration of 10^5 /ml. DNA was released from the parasites by proteinase K (Boehringer Mannheim, Germany) digestion (300 µg/ml) for 1 h at 55°C. After inactivating the proteinase K at 95°C for 10 min, five 10-fold serial dilutions in PBS were prepared.

To compare the efficiency of different purification methods, suspensions of tachyzoites at 10^3 or 10^4 parasites/ml were prepared in PBS or in a suspension of human blood leucocytes obtained from 4 ml of EDTA blood from a *T. gondii* seronegative donor. Leucocytes were separated from erythrocytes by a lysis and centrifugation method [12]. Briefly, an EDTA blood sample was diluted 1 in 4 with lysis buffer (10 mM KHCO₃, 155 mM NH₄Cl), incubated for 30 min, centrifuged at 400 g for 10 min, and the cell pellet was lysed and centrifuged again with 10 ml of lysis buffer. For each parasite suspension, DNA was released from parasites by means of proteinase K digestion (300 µg/ml) for 1 h at 55°C. Each sample was then divided into three aliquots and submitted to three different DNA purification methods, as described below.

In the first method, proteinase K was inactivated at 95°C for 10 min, then nucleic acids were purified by a procedure modified from Loparev *et al.* [13]. Briefly, the resulting crude cell lysate was centrifuged for 10 min at 13 000 rpm with one volume of chloroform: isoamyl alcohol (24:1). The aqueous phase was removed, mixed with one volume of isopropanol and centrifuged for 30 min at 13 000 rpm. The resulting pellet was centrifuged for 10 min at 13 000 rpm in 800 µl of ethanol 70%. The dried pellet was resuspended with 100 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8).

Two other purification methods based on column filtration were used – the silica membrane columns from the High Pure PCR Template Preparation Kit (Boehringer Mannheim) and the Nucleospin C + T kit (Macherey-Nagel, Germany). With both kits, DNA was purified according to the manufacturer's instructions.

Selection of primers

Primers derived from the B1 gene of *T. gondii* were chosen because it appears to be conserved in different parasite strains and is present in 25–50 copies in the

genome [14]. Two different primers flanking a 619-bp fragment (TM1 and TM2) were designed. For the hemi-nested PCR, an internal primer (TM3) giving a 362-bp fragment was designed, taking possible incompatibilities with the latter two into account (Table 1). The theoretical specificity of these primers was determined from the GenBank database.

Processing samples by single-step PCR

Samples (10 µl) of specimen preparations were added to a final volume of 50 µl of PCR buffer consisting of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 200 µM each of dATP, dCTP, dGTP and 400 µM dUTP, primers TM1 and TM2 at 0.5 µM, and 1 U of *Taq*DNA polymerase (Boehringer Mannheim). To minimise the risks of contamination, 0.5 U of uracil-DNA-glycosylase (Boehringer Mannheim) was added. Samples were overlaid with mineral oil and amplified in a thermocycler (MJ Research, MA, USA) for 40 cycles. After an initial denaturation for 3 min at 95°C, the conditions for each cycle were: denaturation at 94°C for 30 s, annealing at 60°C for 30 s and elongation at 72°C for 1 min; the final extension step was continued for a further 10 min. A negative control (de-ionised water) was included. PCR products were visualised on an agarose 2% gel stained with ethidium bromide and compared to a 100-bp DNA ladder (Life Tech, Gibco BRL, MD, USA).

Processing samples by one-tube hemi-nested PCR

Specimen preparation (10 µl) was added to a final volume of 50 µl of PCR buffer consisting of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 200 µM each of dATP, dCTP, dGTP and 400 µM dUTP, primer TM1 at 0.01 µM, primer TM2 at 0.1 µM and primer TM3 at 1 µM, and 2 U of *Taq*DNA polymerase. To minimise the risks of contamination, 0.5 U of uracil-DNA-glycosylase was added. Samples were overlaid with mineral oil and amplified in a thermocycler for two rounds of 30 cycles each. After an initial denaturation for 3 min at 95°C, conditions for each cycle in the first round were: denaturation at 94°C for 30 s, annealing at 65°C for 30 s and elongation at 72°C for 1 min. After an additional extension for 10 min, the second round was performed in the same way as the first, but with an annealing step at 55°C. A negative control (de-ionised water) was included in each experiment. A positive control (RH tachyzoites) was

Table 1. Primers used in the different amplification methods

Primer	Sequence	PCR product
TM1	5'-GAGAGGTCCGCCCCACAAG	...
TM2	5'-CTGCTGGTGCACGGGAGTG	619 bp*
TM3	5'-CAGGAGTTGGATTTGTAGA	362 bp†

*With TM1 and TM2.

†With TM1, TM2 and TM3.

also included when testing patient samples. PCR products were visualised as described above.

Detection of *T. gondii* in human specimens

To assess the specificity of the hemi-nested PCR, 10 EDTA blood samples treated by the lysis-centrifugation method described above from patients serologically negative for *T. gondii* were tested. Also, stored clinical samples (-80°C) from 16 patients in whom *T. gondii* infection had been proved by MRC5 tissue culture [15] were analysed (Table 2). The High Pure PCR Template Preparation Kit was used for DNA purification. A synthetic DNA fragment of 934 bp obtained from the gene encoding the verotoxin-1 of *Escherichia coli* was added to each sample in parallel as a control for the presence of inhibitors.

Results

Sensitivity of the amplification methods

The respective sensitivity of single PCR was estimated from the test with serial dilutions of purified parasitic DNA. With the single-step PCR it was possible to detect DNA extracted from 10 RH tachyzoites, whereas the one-tube hemi-nested PCR method was still positive at a dilution of DNA corresponding to 0.1 parasites (Fig. 1).

Comparison of purification methods

The efficiency of the three DNA purification methods was examined in PBS and leucocyte samples spiked with purified tachyzoites. Each amplification sample contained 10 and 100 parasites in PBS, and 100

Table 2. Hemi-nested PCR of positive samples by cell culture

Sample no.	Type of sample	Date of collection	Clinical condition	Result of PCR
1	Amniotic fluid	Nov. 1991	Pregnancy*	+
2	Amniotic fluid	July 1993	Pregnancy	+
3	Amniotic fluid	Oct. 1993	Pregnancy	+
4	Amniotic fluid	April 1996	Pregnancy	+
5	Amniotic fluid	April 1997	Pregnancy	+
6	Blood	April 1993	HIV infection	+
7	Blood	Aug. 1996	HIV infection	+
8	Liver biopsy	Aug. 1992	HIV infection	+
9	BAL	Feb. 1996	Immunosuppression	+
10	BAL	Aug. 1992	HIV infection	+
11	BAL	Feb. 1993	HIV infection	+
12	BAL	Jan. 1993	HIV infection	+
13	BAL	April 1993	HIV infection	—
14	Bone marrow	Oct. 1991	HIV infection	+
15	Bone marrow	June 1992	BM transplantation	+
16	Bone marrow	Dec. 1993	HIV infection	—

BAL, broncho-alveolar lavage; BM, bone marrow.

*All pregnant patients seroconverted to *T. gondii* during the pregnancy.

Fig. 1. Gel electrophoresis of amplification products after one-tube hemi-nested PCR (primers TM1, TM2 and TM3) for RH strain of *T. gondii*. Lane M, mol.-wt marker; 1, negative control (de-ionised water); 2, DNA from 1000 parasites; 3, DNA from 100 parasites; 4, DNA from 10 parasites; 5, DNA from one parasite; 6, DNA from 0.1 parasite; 7, DNA from 0.01 parasite.

parasites in leucocyte suspension, respectively. After lysis with proteinase K, an aliquot of each sample was submitted to each DNA purification method. A positive signal was obtained in the three methods after one-tube hemi-nested PCR and electrophoresis on an agarose 2% gel stained with ethidium bromide. However, the signals were more intense with the High Pure PCR Template Preparation Kit, particularly in samples containing 10 parasites in PBS (Fig. 2).

In view of these results, it was concluded that the DNA from patient samples should be purified with the High Pure PCR Preparation Kit and amplified with the one-tube hemi-nested PCR method.

Processing of clinical specimens

The PCR analysis of EDTA blood from patients without anti-toxoplasma antibodies was negative. Fourteen (87.5%) of the 16 stored clinical samples in which *T. gondii* had been demonstrated by tissue culture were PCR positive (Fig. 3). Two patients had negative PCR, with no PCR inhibitors identified.

Discussion

PCR has been shown to have good potential as a diagnostic tool for detection of toxoplasmosis in immunodepressed patients. Nested PCR assays [16] or the use of a hybridisation assay after amplification are more sensitive than single-step PCR [11]. However, these methods are time-consuming and, in the case of the two-tube nested PCR, accidental cross-contamination of the sample may occur. In contrast, the one-tube hemi-nested PCR is performed inside a single tube and uracil-DNA-glycosylase can be used to eliminate possible carry-over contamination of PCR products of other positive samples. The one-tube hemi-nested PCR

described above detected DNA equivalent to 0.1 RH tachyzoites (10/ml) and is at least 100-fold more sensitive than the single-step PCR. After proteinase K digestion and with single-step PCR to amplify the B1 gene followed by hybridisation, Khalifa *et al.* [11] were able to detect one parasite genome in 10 μ l of crude DNA sample (100/ml) in the absence of concomitant negative human DNA. Following heat denaturation of parasites, Novati *et al.* [17] detected 100–500 parasite genomes/ml in PBS by using a two-tube nested PCR to amplify the B1 gene.

Before amplification, samples must be appropriately prepared to eliminate inhibitors of the *Taq*DNA polymerase. Detection of *T. gondii* DNA is possible by boiling cerebrospinal fluid before the assay [17]. Other human samples can contain inhibitors [18]. Specimens containing potential inhibitors that were prepared with proteinase K digestion before PCR yielded good results [11, 19]. However, the use of proteinase K alone may be insufficient for preparing the clinical samples and some groups prefer to treat them after a purification step with organic diluents or DNA affinity-based methods [16, 20, 21]. These methods allow DNA concentration and, therefore, increase diagnostic sensitivity. Methods based on the use of affinity DNA purification avoid the use of toxic substances. Moreover, these methods are faster than conventional ones, because DNA can be isolated in minutes. With the High Pure PCR Template Preparation Kit, 14 of 16 samples proved to contain *T. gondii* by cell culture were PCR positive. Some of these specimens, such as blood or bone marrow, might contain inhibitors of the amplification reaction even after phenol extraction [18]. No inhibitor was detected in any sample. In one of the two patients with negative PCR, the tissue culture showed very few parasites. The discrepancy between culture and PCR might also be explained by the storage conditions, as the samples

Fig. 2. Comparison of three DNA purification methods. Gel electrophoresis of amplification products after one-tube hemi-nested PCR (primers TM1, TM2 and TM3) for RH strain of *T. gondii*. Lane M, mol.-wt marker; 1–4, organic diluent method; 5–8, Boehringer-Mannheim method; 9–12, Macherey-Nagel method; 1, 5, 9, negative control (deionised water); 2, 6, 10, DNA from 100 parasites; 3, 7, 11, DNA from 10 parasites; 4, 8, 12, DNA from 100 parasites mixed with human leucocytes.

Fig. 3. Gel electrophoresis of amplification products after one-tube hemi-nested PCR for the clinical samples positive by cell culture. (A) Lane M, mol.-wt marker; 1, positive control (RH strain of *T. gondii*); 2, negative control (de-ionised water); 3 and 4, sample 1 with and without inhibition control (IC); 5 and 6, sample 2 with and without IC; 7 and 8, sample 3 with and without IC; 9 and 10, sample 4 with and without IC; 11 and 12, sample 5 with and without IC; 13 and 14, sample 6 with and without IC; 15 and 16, sample 7 with and without IC; 17 and 18, sample 8 with and without IC (bands of IC do not appear with samples 6 and 7, probably due to DNA competition). (B) Lane M, mol.-wt marker; 1, positive control (RH strain of *T. gondii*); 2, negative control (de-ionised water); 3 and 4, sample 9 with and without IC; 5 and 6, sample 10 with and without IC; 7 and 8, sample 11 with and without IC; 9 and 10, sample 12 with and without IC; 11 and 12, sample 13 with and without IC; 13 and 14, sample 14 with and without IC; 15 and 16, sample 15 with and without IC; 17 and 18, sample 16 with and without IC.

tested were stored in dry ice for >24 h before processing for transportation. It is known that storage conditions affect the sensitivity of PCR [22]. Also, the uneven distribution of trophozoites in the samples cannot be ruled out.

In summary, these results suggest that preparation of clinical samples by proteinase K digestion in combination with a silica-based DNA purification method (such as the High Pure PCR Template Preparation Kit), followed by one-tube hemi-nested PCR may provide a rapid, sensitive and reliable method for detection of *T. gondii* DNA.

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References

- Remington JS, McLeod R, Desmonts G. Toxoplasmosis. In: Remington JS, Klein JO (eds) *Infectious diseases of the fetus and newborn infant*, 4th edn. Philadelphia, WB Saunders. 1995: 140–267.
- Zangerle R, Allerberger F, Pohl P, Fritsch P, Dierich MP. High risk of developing toxoplasmic encephalitis in AIDS patients seropositive to *Toxoplasma gondii*. *Med Microbiol Immunol* 1991; **180**: 59–66.
- Derouin F, Devergie A, Auber P *et al.* Toxoplasmosis in bone marrow-transplant recipients: report of seven cases and review. *Clin Infect Dis* 1992; **15**: 267–270.
- Beaman MH, McCabe RE, Wong S-Y, Remington JS. *Toxoplasma gondii*. In: Mandell GL, Bennett JE, Dolin R (eds) *Mandell, Douglas and Bennett's principles and practice of infectious diseases*, 4th edn. New York, Churchill Livingstone. 1995: 2455–2475.
- Speirs GE, Hakim M, Calne RY, Wreghitt TG. Relative risk of donor-transmitted *Toxoplasma gondii* infection in heart, liver and kidney transplant recipients. *Clin Transplant* 1988; **2**: 257–260.
- Luft BJ, Remington JS. Toxoplasmic encephalitis in AIDS. *Clin Infect Dis* 1992; **15**: 211–222.
- Derouin F, Thulliez P, Garin YJ. [Value and limitations of toxoplasmosis serology in HIV patients.] *Pathol Biol* 1991; **39**: 255–259.
- Dannemann B, McCutchan JA, Israelski D *et al.* Treatment of toxoplasmic encephalitis in patients with AIDS. A randomized trial comparing pyrimetamine plus clindamycin to pyrimetamine plus sulfadiazine. *Ann Intern Med* 1992; **116**: 33–43.
- Raffi F, Aboulker JP, Michelet C *et al.* A prospective study of criteria for the diagnosis of toxoplasmic encephalitis in 186 AIDS patients. *AIDS* 1997; **11**: 177–184.
- Grover CM, Thulliez P, Remington JS, Boothroyd JC. Rapid prenatal diagnosis of congenital *Toxoplasma* infection by using polymerase chain reaction and amniotic fluid. *J Clin Microbiol* 1990; **28**: 2297–2301.

11. Khalifa KES, Roth A, Roth B, Arasteh KN, Janitschke K. Value of PCR for evaluating occurrence of parasitemia in immunocompromised patients with cerebral and extracerebral toxoplasmosis. *J Clin Microbiol* 1994; **32**: 2813–2819.
12. Roth A, Roth B, Hoffken G, Steuber S, Khalifa KI, Janitschke K. Application of the polymerase chain reaction in the diagnosis of pulmonary toxoplasmosis in immunocompromised patients. *Eur J Clin Microbiol Infect Dis* 1992; **11**: 1177–1181.
13. Loparev VN, Cartas MA, Monken CE, Velpandi A, Srinivasan A. An efficient and simple method of DNA extraction from whole blood and cell lines to identify infectious agents. *J Virol Methods* 1991; **34**: 105–112.
14. Burg JL, Grover CM, Pouletty P, Boothroyd JC. Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction. *J Clin Microbiol* 1989; **27**: 1787–1792.
15. Derouin F, Mazon MC, Garin YJF. Comparative study of tissue culture and mouse inoculation methods for demonstration of *Toxoplasma gondii*. *J Clin Microbiol* 1987; **25**: 1597–1600.
16. Verhoshfede C, Reniers S, Colebunders R, van Wanseele F, Plum J. Polymerase chain reaction in the diagnosis of *Toxoplasma* encephalitis. *AIDS* 1993; **7**: 1539–1540.
17. Novati R, Castagna A, Morsica G *et al.* Polymerase chain reaction for *Toxoplasma gondii* DNA in the cerebrospinal fluid of AIDS patients with focal brain lesions. *AIDS* 1994; **8**: 1691–1694.
18. Brisson-Noel A, Aznar C, Chureau C *et al.* Diagnosis of tuberculosis by DNA amplification in clinical practice evaluation. *Lancet* 1991; **338**: 364–366.
19. Gross U, Roggenkamp A, Janitschke K, Heesemann J. Improved sensitivity of the polymerase chain reaction for detection of *Toxoplasma gondii* in biological and human clinical specimens. *Eur J Clin Microbiol Infect Dis* 1992; **11**: 33–39.
20. Lamoril J, Molina J-M, de Gouvello A *et al.* Detection by PCR of *Toxoplasma gondii* in blood in the diagnosis of cerebral toxoplasmosis in patients with AIDS. *J Clin Pathol* 1996; **49**: 89–92.
21. Guy EC, Pelloux H, Lappalainen M *et al.* Interlaboratory comparison of polymerase chain reaction for detection of *Toxoplasma gondii* DNA added to samples of amniotic fluid. *Eur J Clin Microbiol Infect Dis* 1996; **15**: 836–839.
22. James GS, Sintchenko VG, Dickeson DJ, Gilbert GL. Comparison of cell culture, mouse inoculation, and PCR for detection of *Toxoplasma gondii*: effects of storage conditions on sensitivity. *J Clin Microbiol* 1996; **34**: 1572–1575.