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Limited Value of PCR for Detection of *Toxoplasma gondii* in Blood from Human Immunodeficiency Virus-Infected Patients

CASPAR FRANZEN,[1]* MARCUS ALTFELD,[1] PETRA HEGENER,[1] PIA HARTMANN,[1] GABRIELE ARENDT,[2] HELMUT JABLONOWSKI,[3] JÜRGEN ROCKSTROH,[4] VOLKER DIEHL,[1] BERND SALZBERGER,[1] AND GERD FÄTKENHEUER[1]

Department of Internal Medicine I, University of Cologne, Cologne,[1] Department of Neurology[2] and Department of Internal Medicine,[3] University of Düsseldorf, Düsseldorf, and Department of Internal Medicine, University of Bonn, Bonn,[4] Germany

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Cerebral toxoplasmosis is a common, opportunistic, and often life-threatening disease in HIV-infected patients. Diagnosis is supported mainly by clinical evidence and computerized tomography or magnetic resonance imaging scans, but brain images may share features with other brain diseases occurring in HIV-infected patients. To determine the diagnostic value of PCR for the detection of *Toxoplasma gondii* in blood from HIV-infected patients, we examined 89 blood samples from 59 HIV-infected patients. PCR and Southern blot hybridization were done with DNA extracted from blood samples from 20 patients with confirmed cerebral toxoplasmosis and from 10 patients with suspected but not confirmed cerebral toxoplasmosis. The samples were taken before and 7 to 10 days after the beginning of antiparasitic therapy. For 9 patients who suffered from cerebral toxoplasmosis more than 6 months prior to the study and for 20 patients without any evidence for toxoplasmosis only one blood sample per patient was examined. PCR gave positive results with 5 of the 20 blood samples from patients who suffered from cerebral toxoplasmosis. After 7 to 10 days of therapy PCR results became negative in all these five cases. No amplification was seen with DNA from blood samples from the other 54 patients as the target. The results presented here show that PCR testing of blood samples from HIV-infected patients is of limited value for the diagnosis of cerebral toxoplasmosis. The sensitivity was only 25%, but the specificity was very high (100%), so this technique may be useful for discriminating between cerebral toxoplasmosis and other brain diseases which may be mistaken for toxoplasmosis.

Cerebral toxoplasmosis is a common, opportunistic, and often life-threatening disease in HIV-infected patients. Diagnosis is supported mainly by clinical evidence and computerized tomography or magnetic resonance imaging scans, but brain images may share features with other brain diseases occurring in HIV-infected patients (e.g., brain lymphoma). A definitive diagnosis of the disease requires the detection of *Toxoplasma gondii* tachyzoites in histological sections of the brain, but brain biopsy is not a suitable procedure for routine use. Serology is not a reliable technique for immunodeficient persons. And techniques for the direct detection of *T. gondii* in blood such as fibroblast cell cultures or mouse inoculation are time consuming, and their sensitivities may be low (6, 15, 16, 20).

PCR has been developed to detect *T. gondii* in different biological samples (1, 3, 5, 6, 9, 13–16, 18, 20). Most studies used cerebrospinal fluid; the sensitivities were between 40 and 50% for this target (6, 8, 21–23, 25). In other studies brain or liver tissue, bronchoalveolar lavage, lymph nodes, and blood samples were used but, especially for the latter, only from limited number of patients (1, 6, 7, 12, 14, 17, 20, 24, 26, 28). Most of these samples can be obtained only by invasive procedures, and only blood can be easily obtained from the patients. So we examined the diagnostic value of PCR for the detection of *T. gondii* in blood samples from a large group of HIV-infected patients with and without cerebral toxoplasmosis.

* Corresponding author. Mailing address: Department of Internal Medicine I, University of Cologne, Joseph Stelzmann Str. 9, 50924 Cologne, Germany. Phone: 49-(0)221-478-4431. Fax: 49-(0)221-478-6456. E-mail: Caspar.Franzen@Uni-Koeln.DE.

MATERIALS AND METHODS

Patients. Eighty-nine blood samples from 59 HIV-infected patients were examined. Anti-*T. gondii* immunoglobulin G, detected by enzyme-linked immunosorbent assay, was present in all patients. The patients were divided into four groups. Group A consisted of 20 patients with confirmed cerebral toxoplasmosis. All these patients showed neurological symptoms, and computerized tomography or magnetic resonance imaging scans showed the typical appearance of cerebral toxoplasmosis. After therapy, symptoms and radiological brain lesions improved for all these patients. One blood sample was taken from these patients before specific antiparasitic therapy was started, and another was taken between days 7 and 10 of treatment. Group B consisted of 10 patients with suspected cerebral toxoplasmosis which was not confirmed. These patients showed no improvement or worsened after 3 weeks of therapy, and later on other diseases such as brain lymphoma, cerebral tuberculosis, or cryptococcal infection were diagnosed for these patients. In this group too, blood samples were taken before therapy and between days 7 and 10 of therapy. Group C consisted of nine patients who had a history of confirmed cerebral toxoplasmosis more than 6 months prior to the study and who were treated successfully. Group D consisted of 20 HIV-infected patients with no clinical evidence for toxoplasmosis. For patients in groups C and D only one blood sample was examined.

Methods. *T. gondii* tachyzoites were grown in mouse ascites after intraperitoneal injection. Tachyzoites in mouse ascites were washed and resuspended in water at a concentration of 2×10^3 tachyzoites/μl (courtesy of H. M. Seitz, Institute for Parasitology, University of Bonn). One microliter of suspended *T. gondii* tachyzoites was mixed with 200 μl of human blood from a healthy volunteer, and the blood sample was diluted with distilled water at the following dilutions: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$, and $1:10^6$. In a second dilution series 1 μl of the suspended *T. gondii* tachyzoites was diluted with 200 μl of human blood from a healthy volunteer and the blood sample was diluted with the same blood at the following dilutions: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$, and $1:10^6$. Additionally, blood samples from mice with intraperitoneal *T. gondii* infection were examined.

DNA was prepared from 200 μl of the blood samples by using a QIAamp blood kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions.

Amplifications were done in 50-μl reaction mixtures under the following conditions: 25 pmol of each primer, 200 μM concentrations of each dNTP, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer, Norwalk, Conn.). Between 0.1 and 1.0 μg of genomic

DNA and 40 μl of mineral oil were used. Reactions were run in a Perkin-Elmer thermocycler by using a step cycle program. After initial denaturation of the DNA at 94°C for 5 min, 35 cycles were run: 94°C for 30 s, 65°C for 40 s, and 72°C for 40 s, with a 10-min 72°C extension after the 35 cycles.

The primer pairs B5 (5′-TGAAGAGAGGAAACAGGTGGTCG-3′) and B6 (5′-CCGCCTCCTTCGTCCGTCGTA-3′) and T1 (5′-ATGGTCCGGCCGGT GTATGATATGCGAT-3′) and T2 (5′-TCCCTACGTGGTGCCGCAGTTGC T-3′) were used to amplify 131- and 191-bp DNA fragments of the repetitive B1 gene and of the $TGR1_E$ sequence of *T. gondii*, respectively (3, 4, 27). A 10-μl aliquot from each reaction mixture was run on a 1.5% electrophoresis-grade agarose gel in 1× TAE buffer (0.04 M Tris, 0.001 M EDTA) with ethidium bromide (0.5 mg/ml) to visualize the amplified PCR products under UV illumination.

Two internal oligonucleotides (5′-TACTGGTTG TGTGTCGTTATG-3′ and 5′-GCAAGAGAAGTATTTGAGGTC-3′) were used for Southern blot hybridization by previously described methods (10, 11). Briefly, after gel electrophoresis, PCR products were denatured and neutralized by soaking the gel in denaturation buffer (0.4 N NaOH, 0.6 M NaCl) and then in neutralization buffer (1.5 M NaCl, 0.5 M Tris, pH 7.5) for 30 min each. The amplified DNA fragments were transferred to positively charged nylon membranes (Hybond-N+; Amersham, Buckinghamshire, United Kingdom) by using a vacuum blotter (Appligene, Illkirch, France) at 55 mbar for 1 h with 20× SSPE (3 M NaCl, 0.2 M $NaH_2PO_4H_2O$, 1 M EDTA) and were coupled to the membranes by using a UV cross-linker (Appligene) at 0.12 J/qcm. Membranes were washed in 2× SSC (300 mM NaCl, 30 mM Na_3citrate) and prehybridized for 1 h at 42°C with hybridization buffer (Amersham). The internal oligonucleotides were 3′ labeled with fluorescein-11-dUTP by using terminal deoxynucleotidyl transferase (Amersham), and 15 pmol of the labeled probes was added to the hybridization buffer. Hybridization was carried out overnight at 42°C. The blots were washed twice in 2× SSC–0.1% sodium dodecyl sulfate (SDS) and in 1× SSC–0.1% SDS at 46°C. As described in detail elsewhere (10, 11) bound fluorescein-11-dUTP-labeled oligonucleotides were visualized with an enhanced chemiluminescence detection kit (Amersham) in accordance with manufacturer's instructions.

PCR tests were considered positive if amplification of correct DNA fragments occurred with both primer pairs and if both amplified DNA fragments hybridized with the appropriate internal probes during Southern blot hybridization.

Procedures for avoiding contamination were strictly followed. DNA extraction, preparation of reaction media, and amplification and analysis were physically separated and performed in three different rooms. Positive-displacement tips were used for all manipulations. Positive controls (DNA extracted from *T. gondii* tachyzoites) and negative controls containing reaction mixtures without DNA were always tested.

RESULTS

Two DNA fragments of the correct sizes were detected up to a dilution of $1:10^5$ on ethidium bromide-stained gels when DNA from the blood samples spiked with *T. gondii* tachyzoites diluted with water or with blood were used as the target for PCR amplification with the two primer pairs. These two fragments hybridized with the two specific internal probes during Southern blot hybridization. So our PCR assay was able to detect 20 tachyzoites in 100 μl of blood. Extracted DNA from the dilution of $1:10^4$ was used as the positive control in all further amplifications. Correct DNA fragments were also amplified from the blood samples of intraperitoneally infected mice.

The two DNA fragments of the correct sizes could be amplified with the two primer pairs from 5 of the 20 blood samples from the patients of group A who suffered from confirmed cerebral toxoplasmosis. All these amplified DNA fragments hybridized with the two specific internal probes during Southern blot hybridization. With DNA from blood samples from these patients taken after 7 to 10 days of antiparasitic therapy as the target, PCR results became negative in all five cases. No amplification was seen with DNA from blood samples from the other 15 patients of group A before therapy as well as between days 7 and 10 of therapy. With DNA from blood of patients with suspected cerebral toxoplasmosis for whom the diagnosis was not confirmed (group B), no amplification of DNA fragments was seen. Also, no amplification was observed with DNA from blood from patients with a history of cerebral toxoplasmosis more than 6 months prior to the study (group C) or from the 20 patients with no clinical evidence for toxoplasmosis (group D) (Table 1).

TABLE 1. Diagnosis by PCR for 89 blood samples from 59 HIV-infected patients

Group (*n*)	No. of samples with indicated result at:			
	Day 0		Day 7 to 10	
	Positive	Negative	Positive	Negative
A (20)	5	15	0	20
B (10)	0	10	0	10
C (9)	0	9	ND[a]	ND
D (20)	0	20	ND	ND

[a] ND, not done.

In no case did amplification of DNA fragments occur with only one primer pair. If correct DNA fragments were produced, they were amplified with both primer pairs in all five cases of positive PCR results and all amplified fragments hybridized with the specific internal probes during Southern blot hybridization.

DISCUSSION

Cerebral toxoplasmosis is a major cause of morbidity and mortality in HIV-infected patients, and methods for direct detection of the parasites are time consuming and of low sensitivity (6, 15, 16, 20). So diagnosis depends heavily on the clinical presentation of the patients together with computerized tomography or magnetic resonance imaging scans, and often therapy is given without definitive diagnosis following the occurrence of clinical improvement.

PCR has been used to detect *T. gondii* in various biological samples of HIV-infected patients (1, 3, 5, 6, 9, 13, 14, 15, 16, 18, 20). Most of these studies were done by examining cerebrospinal fluid. The sensitivities were between 40 and 50% but the specificities were usually nearly 100% in many of these studies (6, 8, 21, 22, 23, 25). Other materials such as brain tissue, liver tissue, bronchoalveolar lavage, and lymph nodes were less frequently used (1, 12, 26, 28), and, up to now, the diagnostic value of PCR for the detection of *T. gondii* in blood samples from HIV-infected patients has been evaluated only for small groups of HIV-infected patients (6, 7, 9, 14, 17, 19).

The results presented here show that PCR testing of blood from HIV-infected patients is of limited value for the diagnosis of cerebral toxoplasmosis. Although our PCR assay is very sensitive and was able to detect only 20 tachyzoites of *T. gondii* in 100 μl of blood, only 5 of our 20 patients with confirmed cerebral toxoplasmosis had positive PCR results, representing a sensitivity of only 25%. On the other hand no false-positive results were obtained, so the specificity was very high (100%). Consequently, this technique may be useful for discriminating between cerebral toxoplasmosis and other brain diseases which may be mistaken as cerebral toxoplasmosis. Similar results have been reported in previously published studies which used blood as the target for the detection of *T. gondii* DNA by PCR. Sensitivities were usually between 10 and 30% (6, 9, 14, 19), and only Dupouy-Camet et al. reported positive results for 9 of 13 patients (69%) with confirmed cerebral toxoplasmosis by using PCR (7). Two very small studies from the United Kingdom, with only 7 and 5 HIV-infected patients, also showed higher sensitivities of 43 and 40%, respectively (17, 18).

All samples from patients with a history of cerebral toxoplasmosis more than 6 months prior to the study were negative in our PCR assay, and PCR results for the five patients with

initial positive results became negative under antiparasitic therapy. Similar results from other studies, in which PCR results also became negative after antiparasitic therapy, have been reported (19). This indicates that DNA of *T. gondii* can be detected only during active disease and not after successful therapy. In our opinion, cerebral toxoplasmosis in HIV-infected patients is only a local reactivation of the disease in the brain, which is not followed by systemic parasitemia in most patients. This may be the reason why PCR testing of blood is not a very sensitive technique to diagnose cerebral toxoplasmosis in HIV-infected patients.

Studies of many cases of extracerebral or disseminated toxoplasmosis in AIDS patients have been published in the last few years (1, 2, 19, 20, 24). Maybe for these patients PCR will be a more sensitive technique to diagnose this disease. In our study no cases of extracerebral or disseminated toxoplasmosis were included, but Khalifa et al. reported that of seven patients with pulmonary toxoplasmosis, parasite DNA was detected by PCR in the blood of five (19).

In conclusion, PCR testing of blood samples from HIV-infected patients with suspected cerebral toxoplasmosis is of limited diagnostic value but may be useful for discriminating between cerebral toxoplasmosis and other brain diseases as well as for the diagnosis of extracerebral and disseminated toxoplasmosis.

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