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PCR Assay Using Cerebrospinal Fluid for Diagnosis of Cerebral Toxoplasmosis in Brazilian AIDS patients

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Highly active antiretroviral therapy has decreased the incidence of opportunistic infections in the central nervous system in AIDS patients. However, neurological abnormalities still remain important causes of mortality and morbidity in developing countries. In Brazil, cerebral toxoplasmosis is the most common cerebral mass lesion in AIDS patients. For these reasons, early, inexpensive, and sensitive diagnostic tests must be evaluated. The aim of this study was to evaluate PCR, using cerebrospinal fluid (CSF) samples to detect *Toxoplasma gondii* DNA, and to determine if the association of PCR with immunological assays can contribute to a timely diagnosis. We studied two sample groups. First, we analyzed stored CSF samples from 29 newborns and from 39 adults with AIDS without a definitive diagnosis of toxoplasmosis. The goal of this step was to standardize the methodology with a simple and economical procedure to recover the *T. gondii* DNA. Next, we prospectively evaluated CSF samples from 12 AIDS patients with a first episode of cerebral toxoplasmosis and 18 AIDS patients with other neurological opportunistic diseases and without previous cerebral toxoplasmosis. In all PCR samples, an indirect immunofluorescent assay and an enzyme-linked immunosorbent assay were performed. Samples from all patients with cerebral toxoplasmosis presented positive PCR results (sensitivity, 100%), and a sample from one of the 18 AIDS patients with other neurological diseases also presented positive PCR results (specificity, 94.4%). These findings suggest the clinical utility of PCR in the diagnosis of cerebral toxoplasmosis in developing countries.

The worldwide human population is constantly exposed to and infected by *Toxoplasma gondii*. Normally, human infections occur from the ingestion of raw or undercooked meat that is infected with cysts, contaminated water, or foods contaminated with oocysts or from transplacental transmission from a mother who acquired her infection during gestation (15, 21). The chronic asymptomatic form exists in 80 to 90% of infected people. Only 10 to 20% of cases of *T. gondii* infection in adults and children are symptomatic (15, 21, 25). In Brazil, the serological prevalence of *T. gondii* infection is high, ranging from 50 to 80% in the adult population (5).

Toxoplasmosis is a serious and often life-threatening disease in immunodeficient patients. Moreover, the incidence of cerebral toxoplasmosis among human immunodeficiency virus-infected individuals directly correlates with the prevalence of anti-*T. gondii* antibodies among the general human immunodeficiency virus-infected population. Frequently, the disease results in the reactivation of latent chronic infections from cysts present in the brain, eyes, heart, and muscles. Cerebral toxoplasmosis represents the most common cerebral mass lesion in AIDS patients and is the third-most-frequent condition associated with AIDS in Brazil (5, 14, 23, 24, 31, 32).

The definitive diagnosis of cerebral toxoplasmosis requires demonstration of tachyzoites in brain biopsy or necropsy. The presumptive diagnosis is based on positive tests for anti-*T. gondii* antibodies, on suggestive clinical signs and symptoms of central nervous system (CNS) dysfunction, and on typical lesions in the brain, detected by computed tomography or by magnetic resonance imaging scan (5, 14, 18, 29, 30).

In recent years, PCR and other methods have been used together to detect *T. gondii* in various biological specimens, including amniotic fluid (22, 25), aqueous humor (26), cerebrospinal fluid (CSF), and blood (16, 17, 27, 28, 37). At the same time, several DNA targets were used for this purpose, and sensitivity has proved to be high, principally in the diagnosis of congenital and ocular toxoplasmosis.

In this study, we report the results of a PCR study of CSF samples from Brazilian AIDS patients with cerebral toxoplasmosis.

MATERIALS AND METHODS

CSF samples. For the present study, we used two different groups of samples. For group I, we retrospectively analyzed samples stored in the Department of Parasitology at the Instituto Adolfo Lutz during the period from January 2002 to October 2002. We received 68 CSF samples from different hospitals located in São Paulo, Brazil. These samples were from 29 newborns and from 39 adult AIDS adult patients who presented serious neurological alterations and were suspected of having a disease caused by *T. gondii*. After immunoglobulin M (IgM) or IgG evaluation by indirect immunofluorescence assay (IF) and enzyme-linked immunosorbent assay (ELISA), the samples were stored at −20°C. Next, they were assayed by PCR. For group II, we prospectively studied CSF samples from 30 AIDS patients with neurological involvement who were admitted to the Instituto de Infectologia Emilio Ribas, São Paulo, Brazil, between April and July 2003. The first episode of cerebral toxoplasmosis was confirmed in 12 patients by immunological, clinical, radiological, and imaging diagnostic methods. These criteria were established by the Centers for Disease Control and Prevention (9, 10) and included the following: (i) the recent onset of a consistent focal neurological abnormality with intracranial disease or reduced level of consciousness, (ii) a lesion having a mass effect evidenced by brain imaging (on computed tomography or magnetic resonance imaging) or a lesion with radiographic appearance enhanced by the injection of contrast medium, and (iii) a positive test

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TABLE 1. CSF sample results by IF, ELISA, and PCR

Patient population	Total no. of samples	CSF sample results by:					
		IF		ELISA		PCR	
		Positive	Negative	Positive	Negative	Positive	Negative
Group I							
Newborns	29	20	9	17	12	20	9
Adults with AIDS	39	22	17	22	17	24	15
Group II							
Adult AIDS patients with cerebral toxoplasmosis	12	12	0	12	0	12	0
Adult AIDS patients with other neurological disease	18	4	14	4	14	1	17

for anti-*T. gondii* antibodies in serum or a successful response to treatment for cerebral toxoplasmosis. CSF samples were collected before or until the third day of anti-toxoplasmosis therapy. Of the 18 patients with noncerebral toxoplasmosis, 11 patients were diagnosed with cryptococcal meningitis, defined by the presence of *Cryptococcus neoformans* in CSF culture. Three patients presented with progressive multifocal leukoencephalopathy, defined by a positive PCR to JC virus in CSF and a typical magnetic resonance imaging pattern. Two patients suffered from cerebrovascular disease, which was defined as acute onset of consistent clinical features and characteristic neuroimaging studies. Finally, two patients had tuberculosis meningitis, defined by a CSF culture positive for *Mycobacterium tuberculosis*. Samples from patients with a previous history of cerebral toxoplasmosis and patients with more than one opportunistic CNS infection were excluded. No patient had been receiving highly active antiretroviral therapy (HAART). The institutional review boards of the ethics committees of the Instituto de Infectologia Emilio Ribas and Instituto Adolfo Lutz approved this study. All patients in group II gave informed written consent to participate in this protocol.

Mice and parasites. *T. gondii* tachyzoites (strain RH) were maintained in Swiss mice by intraperitoneal inoculation. Every 2 or 3 days after infection, the peritoneal fluids from infected mice were collected in phosphate-buffered saline (PBS), pooled, and centrifuged at $1,000 \times g$ for 10 min. The parasite pellets were washed twice, counted, and suspended in PBS at concentration of 2×10^7 cells/ml.

Antigens and immunological tests. For IF, the tachyzoites were incubated in 2% buffered formalin for 30 min at 37°C, washed twice in PBS at $1,000 \times g$ for 10 min, and fixed on glass slides. For ELISA, the tachyzoites were frozen and heated. The crude antigen was dissolved in PBS, and the protein concentration was determined. The IF and ELISA were carried out as previously described (8). CSF samples were used in serial dilutions and assayed in duplicate. The presence of IgM antibodies in newborns' samples from group I was investigated by both assays.

DNA purification. DNA molecules were extracted from whole CSF samples sent to the laboratory. Samples were centrifuged for 10 min at $3,000 \times g$. CSF supernatants were used in immunological tests for anti-*T. gondii* antibody determination. Packed cells were washed twice in PBS to prevent the action of any *Taq* polymerase inhibitor. Whole cells were lysed by incubation for 5 min at 100°C in 50 μl of ultrapure water containing 20 μg of RNase/ml. As a positive control, DNA was extracted from the tachyzoites of infected mice. The cell pellets were digested with proteinase K (100 μg/ml) in 50 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2% sodium dodecyl sulfate and incubated for 2 h at 56°C. DNA was extracted by the phenol–chloroform-isoamylalcohol method and precipitated with isopropanol (42). After being washed with 70% ethanol for 10 min at $5,000 \times g$, the DNA pellet was dissolved in ultrapure water containing 20 μg of RNase/ml. The DNA concentrations were determined at an optical density of 260 nm.

PCR. The amplifications were carried out with a kit purchased from Amersham-Pharmacia Biotech. The PCR beads were composed of 1.5 U of *Taq* DNA polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM of each deoxynucleoside triphosphate, and stabilizers such as bovine serum albumin. Each reaction was performed by the addition of 10 μl of each DNA template and 50 pmol of each primer in a final volume of 25 μl. The primer pair used was B22 and B23, which amplified a 115-bp sequence in a specific repetitive region of the B1 gene (6, 7). The amplifications were performed in an automated thermal cycler (Progene) and consisted of one initial cycle of denaturation for 5 min at 95°C and 35 cycles each consisting of denaturation at 95°C for 1 min, annealing at 62°C for 30 s, and extension at 72°C for 1 min. The procedure was completed by a final cycle extension for 10 min. Each amplification run contained two negative controls (ultrapure water and toxoplasmosis-negative CSF sample) and a positive control. After the thermal cycles, the amplicons were electrophoresed in a 2% agarose gel and stained with ethidium bromide. The DNA fragments were visualized under UV illumination (42). To standardize the procedure, different DNA concentrations and thermal cycles were tested (data not shown).

RESULTS

The first step of our study was to establish a simple and economical procedure to recover most of the DNA present in CSF samples. The samples were centrifuged to recover all cells and washed to remove any *Taq* polymerase inhibitor. Heat and hydrolysis lysed the pellet cells. Under the conditions described in Materials and Methods, the primer pair from the B1 gene amplified a 115-bp sequence from the DNA template.

The next step was to investigate the presence of *T. gondii* DNA by PCR in 68 CSF samples from group I patients. The samples were from newborns and adults with serious neurological symptoms but without a definitive clinical diagnosis. Among these samples, 42 presented antibodies against *T. gondii*, including IgM or IgG detected by IF and 39 samples with antibodies detected by ELISA. *T. gondii* was detected by PCR in 44 CSF samples. IF was negative with 26 samples and ELISA was negative with 29 samples. PCR was negative with 24 CSF samples. Samples from all patients with cerebral toxoplasmosis from group II presented IgG antibodies against *T. gondii* determined by ELISA, IF, and positive PCR. On the other hand, of 18 samples from AIDS patients without cerebral toxoplasmosis, 4 (22.2%) samples presented IgG antibodies against *T. gondii* and 1 (5.6%) was positive for *T. gondii* by PCR. A summary of these results, including IF, ELISA, and PCR methodologies, is shown in Table 1.

As the definitive diagnosis was not reached for group I patients, PCR sensibility and specificity were evaluated using the IF as a "gold standard" for detecting *T. gondii* infection, as established previously (8). The values obtained are shown in Table 2. The 42 reagent samples determined to be positive by IF were also positive by PCR, presenting a sensitivity of 100%. Among the 26 nonreagent CSF samples, 24 had negative PCR results. In this case, two samples from AIDS patients had positive PCR results, resulting in a specificity of 92.3%.

PCR sensibility and specificity in group II samples were evaluated with Centers for Disease Control and Prevention criteria as the gold standard (9, 10); PCR sensibility and specificity are described in detail in Materials and Methods. The values obtained are shown in Table 3. Samples obtained from

TABLE 2. Group I infection analysis of PCR assay sensitivity and specificity, considering IF results

CSF sample source	No. of patients	PCR result		No. of positive results by IF
		Positive[a]	Negative[b]	
Newborns	29	20 (100%)	9 (100%)	20
Adult AIDS patients	39	24 (100%)	15 (88.2%)	22
Total	68	44 (100%)	24 (92.3%)	42

[a] The values in parentheses are percent sensitivity.
[b] The values in parentheses are percent specificity.

all 12 AIDS patients with cerebral toxoplasmosis presented positive PCR results, giving a sensitivity of 100%. A sample from 1 of the 18 AIDS patients with other neurological diseases presented a positive PCR. These data resulted in a specificity of 94.4%.

DISCUSSION

The introduction of HAART resulted in the decline of incidence of opportunistic infections in the CNS (41). However, cerebral toxoplasmosis still results in high levels of morbidity and mortality in developing countries (33, 44, 45). In addition, atypical forms of cerebral toxoplasmosis have been described in the HAART era (20). These data show the importance of evaluating a sensitive, inexpensive, and rapid diagnostic test.

This study evaluated the usefulness of a PCR assay with B1 gene sequences as primers to detect *T. gondii* DNA in CSF samples from Brazilian patients with cerebral toxoplasmosis. We chose these primers because they were able to amplify and detect the DNA of a single organism directly from a crude cell lysate or 10 parasites in the presence of 100,000 human leukocytes (6).

We found PCR sensitivity to be higher than other studies have. Data from most previously published studies showed moderate sensitivity and high specificity (13, 16, 28, 35, 36, 39, 43). Our data, studying Brazilian AIDS patients with neurological involvement, presented a sensitivity of 100% in both groups and specificity of 92 and 94% for groups I and II, respectively. Two samples in group I presented false-positive results, and a sample in group II from one patient with cryptococcal meningitis had PCR results that were positive for toxoplasmosis.

TABLE 3. Group II disease analysis of PCR sensitivity and specificity, considering chemotherapy response and clinical and radiological diagnoses

PCR result	No. of patients	Adult AIDS patients with:	
		Cerebral toxoplasmosis	Other neurological diseases
Positive	13	12 (100%)[a]	1
Negative	17	0	17 (94.4%)[b]
Total	30	12	18

[a] The values in parentheses are percent sensitivity.
[b] The values in parentheses are percent specificity.

Variations in technical procedure between different laboratories can affect PCR results (11). We believe that some conditions contributed to raise sensitivity with our PCR assay. The first step was to establish a simple procedure to recover most of the DNA present in the samples. CSF collection is invasive, principally in children; normally, laboratories use small volumes (sometimes only 0.1 ml) from newborns to diagnose different infections. The CSF contains few cells, and the DNA molecules are easily released with water and heat. However, when the DNA molecules were extracted by the phenol–chloroform-isoamylalcohol method, DNA concentrations were very low and no or a small amount of DNA amplification was observed (data not shown). The second step was to determine whether any *Taq* polymerase inhibitor could change the results. PCRs were performed with a mixture of a negative DNA sample with positive DNA (from a positive control) as a template. The results were positive, showing that no substance present in DNA samples inhibits the reaction.

After procedure standardization with samples from group I patients, the second step was to collect CSF samples from group II patients. Knowledge of the specific treatment and the sample collection during the first week of specific therapy is an important tool for PCR sensitivity (34, 40). In agreement, we only collected CSF samples from patients who had received anti-toxoplasmosis treatment up to day 3. The high sensitivity found here could also be explained by the presence of severe neurological involvement in most of the patients studied and by the high prevalence of toxoplasmosis in Brazil (12, 32).

The presumptive diagnosis of cerebral toxoplasmosis in AIDS patients consists of the presence of less than 200 $CD4^+$ T lymphocytes/μl, anti-*T. gondii* IgG antibodies in the serum, consistent clinical features, characteristic neuroimaging studies, and a positive response to empirical specific therapy (10). Failure to respond to therapy is indicated by the persistence or worsening of either clinical symptomatology or the mass lesions observed by diagnostic stereotactic biopsy. In addition, negative toxoplasmosis serology and a single lesion on radiographic imaging are sufficient to perform a biopsy (2, 19). In recent years, brain biopsies have been replaced by molecular approaches, including the use of PCR in focal brain lesion diagnosis, for AIDS patients. This minimally invasive approach has been evaluated principally to detect Epstein-Barr virus DNA or JC virus DNA, as a useful tool for the rapid diagnosis of primary CNS lymphoma or progressive multifocal leukoencephalopathy, respectively (3, 4). We believe that the data presented here emphasize the applicability of these approaches.

The IF and ELISA detected anti-*T. gondii* antibodies in samples from 4 of 18 patients without cerebral toxoplasmosis (group II). This finding could be explained, in part, by the high prevalence of toxoplasmosis in the Brazilian population. The sensitivity to both serological methods was also high in samples from this group. These findings suggest and confirm other studies (1, 37, 38) in which the association of both PCR and serological methods can be a good tool for early diagnosis and timely therapy.

In conclusion, this study demonstrates the importance of including PCR in cerebral toxoplasmosis diagnosis in AIDS patients, because neurological problems caused by opportunist diseases still remain a serious problem in developing countries.

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