Molecular Diagnosis

PCR detection of Toxoplasma gondii DNA in CSF for the differential diagnosis of AIDS-related focal brain lesions

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To evaluate whether the detection of Toxoplasma gondii DNA in CSF could contribute to the differential diagnosis of AIDS-related focal brain lesions, CSF samples from 88 HIV-infected patients (56 with focal brain lesions and 32 without) were tested prospectively by a nested PCR for the B1 gene of T. gondii. The assay had a detection limit of 10 trophozoite equivalents. Six of 18 patients with toxoplasmic encephalitis, but none of the 70 patients with other disorders, were PCR-positive (33.3% sensitivity and 100% specificity). Considering only those patients with cerebral toxoplasmosis from whom CSF was collected before or during the first week of antitoxoplasmic therapy, sensitivity rose to 50%. This was higher than the sensitivity in patients whose CSF was collected after the first week of treatment (odds ratio (OR) of 7.0; 95% CI: 0.46–218.2). The administration of antitoxoplasmic prophylaxis did not affect the PCR results. Patients with a poor response to therapy had a higher probability of detectable T. gondii DNA in their CSF (OR of 5.0; 95% CI: 0.37–86.6). All patients with other central nervous system disorders were PCR-negative. Despite the moderate sensitivity, the high specificity and positive predictive value (100%) make this assay a useful tool in the differential diagnosis of AIDS-related focal brain lesions as part of a series of CSF and neuroradiological examinations.

Introduction

Toxoplasmic encephalitis (TE) is still the most common disorder causing focal brain lesions in AIDS patients [1]. Currently, the diagnosis is often made presumptively, following guidelines formulated by the Centers for Disease Control [2] based on clinical experience during the 1980s. The introduction of prophylaxis protocols with protective activity against cerebral toxoplasmosis [3,4] may have reduced the incidence of TE among the disorders causing focal brain lesions. However, the incidence of primary brain lymphoma is increasing in this population [1] and empirical treatment with antitoxoplasmic drugs may therefore be inadequate and cause a highly undesirable diagnostic delay. Clearly, a rapid, non-invasive and more reliable diagnostic method is required. PCR-based assays have now been developed to detect Toxoplasma gondii DNA in biological fluids, including amniotic fluid, blood and bronchoalveolar lavage fluid [5–7], and the detection of T. gondii DNA in CSF by PCR has been shown to be a promising method for the diagnosis of T. gondii encephalitis in AIDS patients [8–12]. These reports, based on retrospective examination of CSF, show a highly variable sensitivity of 11–67% [8,12]. The work in the present paper aimed to assess the sensitivity and specificity of a nested PCR for detecting T. gondii DNA in CSF samples collected prospectively for the differential diagnosis of TE in HIV-infected patients with focal brain lesions.

Patients and methods

Patients

HIV-infected patients (n = 88) were evaluated prospectively as part of a validation cohort study on the predictive role of different variables in the differential diagnosis of focal brain lesions. A focal brain lesion was found in 56 patients by computed tomography (CT) or magnetic resonance imaging (MRI). TE was diagnosed by presumptive criteria (neuroradiological
signs, seropositivity for *T. gondii* and response to antitoxoplasma treatment) or by histopathological examination. Diagnostic criteria for other disorders included a suggestive brain MRI picture and detection of JCV-DNA from CSF or brain histology for progressive multifocal leukoencephalopathy (PML) [13], and the overall histopathological picture for primary brain lymphoma and remaining disorders. CSF samples from 32 HIV-infected patients without focal brain lesions were also examined. Demographic, epidemiological, immunological and radiological features were recorded for each patient. CSF (2 ml) was drawn by lumbar puncture, when not contra-indicated, after written informed consent. CSF was examined for cryptococcal antigen, HIV-p24 antigen, and for EBV-DNA [14] and JCV-DNA [13] by nested PCR. Serum IgG and IgM anti-*T. gondii* were detected with an automated quantitative ELFA method (bioMérieux, Lyon, France).

**DNA preparation and amplification**

DNA was prepared from CSF by a modification of a published method [15]. In brief, a 100-μl sample of CSF was boiled for 15 min, precipitated in 2.5 vol of ethanol 95% v/v at −20°C for 1 h and centrifuged at 15 000 g for 30 min. The pellet was washed in ethanol 70% v/v, centrifuged at 15 000 g for 5 min, dried under vacuum, dissolved in 15 μl of distilled water and boiled for 10 min. Portions (10 μl) were used for DNA amplification. This extraction method was compared with a more rapid method involving heating 10 μl of unextracted CSF at 95°C for 10 min. In both cases, nested PCR was performed with primers derived from the 35-fold repetitive DNA region B1 of *T. gondii* [16]. Each 50-μl reaction mixture contained 50 mM KCl, 10 mM Tris-HCl, 2.5 mM MgCl₂, 400 μM of each dNTP, 2.5 U of Taq polymerase (Perkin Elmer) and 50 pmols of each outer primer in the first round and of each inner primer in the second round. After the first PCR, 2.5 μl of the amplification end-product were transferred to the second round reaction buffer and amplified with the same conditions. Each amplification round comprised 35 cycles of 50 s at 93°C, 50 s at 46°C and 50 s at 72°C. Amplification products were detected by electrophoresis on agarose 2% w/v gels, followed by staining with ethidium bromide 0.5 μg/ml and UV transillumination. Positive samples showed a 97-bp DNA fragment after the second amplification round.

The detection limit of the PCR test was assessed with 10-fold dilutions of a *T. gondii* standard. *T. gondii* tachyzoites were obtained from the peritoneal cavity of Swiss-Webster mice on day 3 after inoculation, purified by differential centrifugation and suspended in phosphate-buffered saline containing formalin 1% v/v. DNA from purified tachyzoites was extracted by phenol:chloroform treatment, quantified spectrophotometrically [17] and amplified, as described above, in a PCR-negative CSF sample—shown previously to be free of PCR inhibitors (see below)—from an HIV-positive patient.

**Semiquantitative analysis of PCR results**

To estimate *T. gondii* concentrations in CSF, a limiting dilution analysis was performed on PCR-positive samples, with serial dilutions of up to 50-fold in sterile bi-distilled water. Diluted samples were amplified in parallel with a diluted standard. The detection limit for the diluted standard was assumed to correspond with the detection limit for the diluted sample and was used to estimate the concentration of *T. gondii* DNA in the undiluted sample.

**PCR inhibitors**

To investigate the presence of PCR inhibitors, negative CSF samples from patients with presumptive TE were re-amplified after adding 50 pg of *T. gondii* DNA. As parallel controls, CSF samples from TE-free patients were amplified after adding the same quantity of *T. gondii* DNA. To avoid sample contamination, PCR steps were performed in separate rooms, with the use of positive displacement pipettes and frequent changing of gloves. All amplification reactions were performed at least twice.

**Statistical analysis**

All continuous variables were compared by Student's *t* test. Discrete variables were analysed by Fisher's exact test, with measures of risk expressed as a crude odds ratio (OR). Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were all calculated by appropriate formulas with contingency tables [18]; 95% confidence interval (CI) values were estimated for all analyses; p-values of <0.05 were taken as significant, and all p-values were two-tailed.

**Results**

**Diagnoses**

TE was diagnosed in 18 of 56 patients with a focal brain lesion (group A), with 16 presumptive and two histological diagnoses. The mean CD4 lymphocyte count in this group was 50 cells/μl (95% CI: 27–74). All these patients had *T. gondii*-specific IgG serum antibodies with a mean titre of 222 (95% CI: 81–363). Six (33%) of these 18 cases were receiving anti-Toxoplasma prophylaxis at the time of TE diagnosis. Therapy led to a clinical and neuroradiological improvement in 16 patients (89%). The mean time between the start of anti-toxoplastic therapy and the lumbar puncture was 10.7 days (95% CI: 3.5–17.9). Lumbar puncture was performed before therapy or during the first week of treatment in 10 cases (mean time from start of therapy: 0.7 days; 95% CI: 0.25–
1.65) and >1 week after the start of therapy in eight cases (mean time: 23.2 days; 95% CI: 11.8–34.7) (Table 1).

Of the 38 patients with a focal brain lesion without TE (group B), 13 (34%) had Toxoplasma-specific IgG and the mean CD4 count was 43 cells/µl (95% CI: 18–68). Fourteen (37%) patients from group B were given empirical anti-Toxoplasma treatment, with a mean time from the start of therapy to lumbar puncture of 14.5 days (95% CI: 6.6–22.3). The mean time from the start of therapy to lumbar puncture of TE patients (group A) and patients without TE (group B) was comparable (p at test = 0.45). Of group B patients, 18 cases were diagnosed as primary brain lymphoma, 17 as PML, one as CMV encephalitis, one as mectoma and one as brain vasculitis.

The 32 patients without focal brain lesions (group C) had a mean CD4 cell count of 15 cells/µl (95% CI: 11–20) and 12 (37%) had Toxoplasma-specific IgG. HIV-encephalopathy was diagnosed in eight cases, one as CMV encephalitis, one as non-Hodgkin's lymphoma without central nervous system involvement in 14 cases.

**Table 1.** Clinical details for 18 patients with toxoplasmic encephalitis

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex/Age</th>
<th>CD4 count (µl)</th>
<th>T. gondii-specific IgG titre</th>
<th>Anti-T. gondii prophylaxis</th>
<th>CT/MRI</th>
<th>Length of therapy (days) before LP</th>
<th>Therapy</th>
<th>Clinical response</th>
<th>T. gondii DNA concentration in CSF*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M/31</td>
<td>150</td>
<td>103</td>
<td>No</td>
<td>Multiple</td>
<td>0</td>
<td>S/P</td>
<td>Early</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>F/35</td>
<td>48</td>
<td>10</td>
<td>No</td>
<td>Single</td>
<td>0</td>
<td>S/P</td>
<td>Early + (&gt;500)</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>F/36</td>
<td>60</td>
<td>84</td>
<td>Yes</td>
<td>Single</td>
<td>0</td>
<td>S/P</td>
<td>Early + (100)</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>M/55</td>
<td>49</td>
<td>101</td>
<td>No</td>
<td>Single</td>
<td>0</td>
<td>S/P</td>
<td>Early</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>M/32</td>
<td>30</td>
<td>143</td>
<td>No</td>
<td>Multiple</td>
<td>2</td>
<td>S/P</td>
<td>Early</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>F/33</td>
<td>31</td>
<td>34</td>
<td>Yes</td>
<td>Single</td>
<td>0</td>
<td>S/P</td>
<td>Early</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>M/36</td>
<td>40</td>
<td>75</td>
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<td>Multiple</td>
<td>0</td>
<td>S/P</td>
<td>Early</td>
<td>–</td>
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<tr>
<td>8</td>
<td>M/35</td>
<td>35</td>
<td>234</td>
<td>No</td>
<td>Multiple</td>
<td>4</td>
<td>C/P</td>
<td>Early + (200)</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>M/30</td>
<td>30</td>
<td>128</td>
<td>No</td>
<td>Multiple</td>
<td>0</td>
<td>P/A</td>
<td>Died + (100)</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>M/30</td>
<td>35</td>
<td>536</td>
<td>No</td>
<td>Single</td>
<td>26</td>
<td>S/P</td>
<td>Late</td>
<td>–</td>
</tr>
<tr>
<td>11</td>
<td>F/33</td>
<td>169</td>
<td>172</td>
<td>Yes</td>
<td>Multiple</td>
<td>43</td>
<td>P/A</td>
<td>Died + (&gt;500)</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td>M/32</td>
<td>98</td>
<td>1200</td>
<td>No</td>
<td>Multiple</td>
<td>13</td>
<td>S/P</td>
<td>Early</td>
<td>–</td>
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<tr>
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<td>0</td>
<td>27</td>
<td>No</td>
<td>Multiple</td>
<td>11</td>
<td>C/P</td>
<td>Early</td>
<td>–</td>
</tr>
<tr>
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<td>120</td>
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<td>Single</td>
<td>35</td>
<td>S/P</td>
<td>Late</td>
<td>–</td>
</tr>
<tr>
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<td>M/37</td>
<td>5</td>
<td>324</td>
<td>No</td>
<td>Multiple</td>
<td>37</td>
<td>S/P</td>
<td>Early</td>
<td>–</td>
</tr>
<tr>
<td>16</td>
<td>M/35</td>
<td>72</td>
<td>490</td>
<td>No</td>
<td>Multiple</td>
<td>9</td>
<td>S/P</td>
<td>Early</td>
<td>–</td>
</tr>
<tr>
<td>17</td>
<td>M/49</td>
<td>5</td>
<td>154</td>
<td>No</td>
<td>Multiple</td>
<td>1</td>
<td>S/P</td>
<td>Late + (200)</td>
<td>–</td>
</tr>
<tr>
<td>18</td>
<td>F/30</td>
<td>35</td>
<td>225</td>
<td>Yes</td>
<td>Single</td>
<td>12</td>
<td>C/P</td>
<td>Early</td>
<td>–</td>
</tr>
</tbody>
</table>

CE, contrast enhancement; LP, lumbar puncture; S/P, sulphadiazine/pyrimethamine; C/P, clindamycin/pyrimethamine; P/A, pyrimethamine/atoquavone.

*Semiquantitative estimate obtained by PCR and expressed as the number of tachizoites/µl.

**Table 2.** Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of T. gondii DNA detection in CSF according to time of lumbar puncture after the start of anti-Toxoplasma therapy

<table>
<thead>
<tr>
<th>Time*</th>
<th>PCR+/number of patients with TE</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity† (95% CI)</th>
<th>PPV (95% CI)</th>
<th>NPV (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1 week</td>
<td>5/10</td>
<td>50% (20.1–79.9)</td>
<td>100% (93.5–100)</td>
<td>100% (46.3–100)</td>
<td>93.3% (84.5–97.5)</td>
</tr>
<tr>
<td>&gt;1 week</td>
<td>1/8</td>
<td>12.5% (0.7–53.3)</td>
<td>100% (93.5–100)</td>
<td>100% (5.5–100)</td>
<td>90.9% (81.6–96)</td>
</tr>
<tr>
<td>All patients</td>
<td>6/18</td>
<td>33.3% (14.4–58.8)</td>
<td>100% (93.5–100)</td>
<td>100% (51.7–100)</td>
<td>85.4% (75.4–91.9)</td>
</tr>
</tbody>
</table>

TE, toxoplasmic encephalitis.

*Time from start of anti-Toxoplasma therapy to lumbar puncture.

†All 70 patients without toxoplamosis were PCR-negative.

**PCR results**

The nested PCR was able to detect 50 pg/ml of T. gondii DNA, corresponding to c. 10 tachizoites in 10 µl of clinical sample. T. gondii DNA was detected in six of 18 CSF samples from group A patients, but in none from groups B or C, with an overall sensitivity of 33% and a specificity of 100% (Table 2). No difference was observed between the two methods of DNA preparation. Previous anti-Toxoplasma prophylaxis, the anti-T. gondii IgG titre, nor the number of brain lesions and the presence of contrast enhancement did not correlate with the PCR results. In five of 10 patients for whom there was <1 week from the start of therapy to lumbar puncture, Toxoplasma DNA was detected in CSF (50% sensitivity), whereas only one of eight patients in whom a lumbar puncture performed after the first week of therapy was PCR-positive for T. gondii DNA (12.5% sensitivity) (Table 2). TE patients whose lumbar puncture was performed during the first week of therapy, had a non-significant increased risk of having Toxoplasma DNA detected in CSF than those with a delayed lumbar puncture (OR = 7.0; 95% CI: 0.46–218.2; p = 0.15).

Considering the outcome of anti-Toxoplasma therapy,
13 of 18 patients had a prompt response to specific therapy during the first 2 weeks ('early responders'). Of these 13 patients, three had a PCR-positive CSF. Of the remaining five patients, two were ‘non-responders’—both diagnosed definitively as TE at autopsy and PCR-positive—and three patients (one of whom had detectable *T. gondii* DNA) responded to therapy after 2 weeks ('slow-responders'). Patients with a PCR-positive CSF had a non-significant increased risk of a delayed response or a poor outcome than PCR-negative patients (OR = 5.0; 95% CI: 0.37–86.6; p = 0.26).

Semiquantitative analysis of PCR-positive results for TE patients showed trophozoites equivalents/μl of CSF ranging between 100 and 500.

The presence of *Taq* polymerase inhibitor(s) was detected in only one of nine PCR-negative CSF samples from patients with TE. Attempts to remove inhibitors by dilution, standard phenol:chloroform DNA extraction, binding of DNA to silica particles and proteinase K digestion were unsuccessful (results not shown).

**Discussion**

TE is still the major cause of focal brain lesions in AIDS patients but primary anti-*T. gondii* prophylaxis reduces TE episodes [3, 4] and a reduced incidence of cerebral toxoplasmosis can be expected in a population receiving prophylaxis. Prompt differential diagnosis between primary brain lymphoma and TE represents a major clinical challenge in AIDS patients with focal brain lesions—particularly in *T. gondii* seronegative patients or in seropositive patients receiving anti-Toxoplasma prophylaxis—as this could avoid the diagnostic delay caused by empirical anti-Toxoplasma treatment courses [19–21].

The present study assessed the diagnostic efficiency of a nested PCR for detection of the *T. gondii* B1 gene in a prospective series of CSF samples from HIV-infected patients with and without focal brain lesions. The overall diagnostic sensitivity of 33.3% in the 18 patients with TE rose to 50% when only the patients in whom the lumbar puncture was performed before or during the first week of therapy were considered. Lumbar puncture performed before starting anti-Toxoplasma therapy or during the first week was the most important factor for the successful detection of *T. gondii* DNA, confirming that the parasite is probably cleared from CSF after a few days of therapy [10]. The only case in this series with detectable *T. gondii* DNA in CSF after the first week of therapy (lumbar puncture performed at day 43) was a patient with a TE episode about 6 months previously and a relapse at the time of lumbar puncture who failed to respond to treatment with atovaquone and died 3 weeks after lumbar puncture (TE was confirmed at autopsy).

Previous retrospective studies have reported variable diagnostic sensitivity of PCR [8–12]. However, sensitivity was not related to the primers used; indeed, PCR assays in which the target was part of a rDNA repetitive gene [11] or the 35-times repeated genomic B1 gene [8–10, 12] showed a diagnostic sensitivity that never exceeded 67%, with a lower detection limit ranging from one [10] to 10 parasites [12]. One study with an identical nested PCR assay showed a sensitivity of 100% (11 of 11 patients) when CSF was collected during the first week of anti-Toxoplasma therapy [10]. The discrepancy with the findings of the present study cannot be explained by the slightly lower detection limit of the previous study (one parasite equivalent versus 10) because patients with a PCR-positive CSF generally show a high parasite burden (Table 1). One possible explanation is that the previous study [10] may have contained patients with more severe clinical features who were diagnosed retrospectively, as can be argued indirectly by the fact that five of the 11 patients in the earlier study were diagnosed at autopsy. Although the sample size in the present study was too small to reach definitive conclusions, the finding that a higher proportion of the TE patients who had a slow response or were non-responders to anti-Toxoplasma therapy were PCR-positive, suggests that there may be a correlation between results of PCR analysis and clinical outcome. No interference of anti-Toxoplasma prophylaxis with the detection of parasite DNA in CSF by PCR was found.

In agreement with previous retrospective studies, PCR detection of *T. gondii* DNA in CSF had a high specificity and positive predictive value. Controls included patients with and without focal brain disorders, but no patients without TE had detectable *T. gondii* DNA in CSF. Therefore, the assay is a helpful tool for the differential diagnosis of focal brain lesions, particularly when performed in combination with assays for EBV or JCV DNA in CSF—as markers of primary brain lymphoma and PML, respectively [13, 14]—or new neuroradiological approaches such as positron emission tomography [22]. Patients with enhancing lesions and a doubtful picture—especially when tests for EBV DNA in CSF are negative or anti-Toxoplasma prophylaxis has been administered—may represent the ideal target population for the assay. Prospective data for a cohort of patients with focal brain lesions showed that the conditional probability of cerebral toxoplasmosis in the presence of enhancing brain lesions in patients seropositive for *T. gondii* at the start of prophylaxis rose from 59% to 99% when *T. gondii* DNA was detectable in CSF (A. Antinori, unpublished observations).
In conclusion, nested PCR with CSF is a moderately sensitive, but highly specific assay for the diagnosis of TE in AIDS patients with focal brain lesions. The assay shows a higher sensitivity when performed at the beginning of the disease and can be included in the diagnostic algorithm for HIV-infected patients with a focal brain lesion, as part of a series of CSF and neuroradiological examinations.

This work was supported by VIII Progetto di Ricerche sull’AIDS (1995). Grant 9305-30 from the Istituto Superiore di Sanità, Rome, Italy.

References