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Usefulness and limitations of polymerase chain reaction in the etiologic diagnosis of neurotoxoplasmosis in immunocompromised patients

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ABSTRACT

The objective of the present study was to assess the performance and the best indication of the polymerase chain reaction (PCR) for the detection of *Toxoplasmosis gondii* DNA in cerebrospinal fluid (CSF) from patients with suspected neurotoxoplasmosis. CSF samples were collected from 79 patients for amplification of the *T. gondii* genome (gene B1) by two PCR techniques (nested and real time). Twenty-seven of the 79 patients were classified as probable cases of neurotoxoplasmosis on the basis of clinical criteria, neuroimaging and therapeutic response. PCR showed high sensitivity (86.6%) when performed in CSF samples which were collected up to the seventh day of specific toxoplasmosis treatment. There was no positive test after 1 week of treatment. These results suggest the usefulness of PCR for the diagnosis of cerebral toxoplasmosis, and support the first week as the window for the best performance of toxoplasmosis PCR in CSF.

1. Introduction

Among AIDS patients, toxoplasmosis usually occurs in individuals with CD4+ T lymphocyte counts <100/μl by reactivation of chronic infection [20]. The clinical signs and symptoms of neurotoxoplasmosis in immunosuppressed patients can be acute or subacute and a definite diagnosis of toxoplasmosis requires the identification of *Toxoplasma gondii* in tissue biopsies and eventually in the cerebrospinal fluid (CSF) [19]. In practice, the diagnosis of neurotoxoplasmosis is usually presumptive and is based on a set of clinical and neuroimaging data, in addition to the response to specific treatment against the agent [2]. The differential diagnosis of neurotoxoplasmosis includes primary lymphoma of the central nervous system (CNS), cytomegalovirus encephalitis, abscesses and other granulomatous diseases such as tuberculosis, syphilis, *Cryptococcus* infection, histoplasmosis, cysticercosis, and Chagas disease [17,25]. Imaging exams, i.e., computed tomography (CT) and magnetic resonance (MRI), typically demonstrate multiple granulomatous lesions but do not differentiate toxoplasmosis from other lesions such as abscesses and lymphoma [13,25]. The methods for the detection of IgG and IgM antibodies are of little use, although the presence of high IgG titers is common in patients with a diagnosis of neurotoxoplasmosis. However, the absence of antibodies does not rule out the diagnosis in a definitive manner [19,23,27]. In this respect, molecular biology and direct detection methods such as the polymerase chain reaction (PCR) represent a good alternative for the etiologic diagnosis of the disease or for decision making in cases with an unfavorable course despite specific treatment [1,11,17]. The objective of the present study was to assess the performance and the best indication of PCR for *T. gondii* detection in patients with suspected CNS neurotoxoplasmosis.

2. Materials and methods

2.1. Patients

Inclusion criteria: immunosuppressed patients older than 18 years suspected of having neurotoxoplasmosis based on history, physical examination and CNS images, who were seen at a reference hospital in the Northeast of São Paulo State, Brazil. Patients included were from January 2010 to February 2012. Exclusion criteria were: patients with severe intracranial hypertension, coagulopathy or any condition that would contraindicate lumbar or occipital puncture. All selected

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patients gave written informed consent to participate. A 1 ml CSF sample was then obtained from each subject and submitted to PCR. Amplification was performed according to two protocols, i.e., nested PCR and real time PCR, with all samples being tested simultaneously. The target of both reactions was amplification of the B1 gene of *T. gondii*, which is the region of the most conserved DNA of the parasite [5,14].

The criteria were those established by the Centers for Disease Control and Prevention [6] as follows: (i) the onset of a focal neurologic abnormality consistent with intracranial disease or reduced level of consciousness, (ii) a lesion having a mass effect demonstrated by neuroimaging (CT or MRI) or a lesion with radiographic appearance enhanced by injection of contrast medium, and (iii) a positive test for anti-*T. gondii* in serum or a response to specific treatment for toxoplasmosis. The study was conducted at the Clinical Hospital of Ribeirão Preto and approved by the Ethics Committee.

2.2. DNA extraction

DNA was extracted from the CSF samples using a commercial kit (Qiamp Blood kit, Qiagen Inc., Valencia, CA, USA) according to manufacturer specifications.

2.3. Nested PCR

In the first reaction step, the following external primers described by Burg et al. [5] were used: 5-GGAACTGCATCCGTTCATGAG-3 and 5-TCTTTAAAGCGTTCGTGGTC-3 (Invitrogen — USA), which correspond to positions 757 to 776 and 887 to 868 bp, respectively, in the *T. gondii* DNA genome. A 1 μl aliquot of each product amplified in the first reaction was submitted to a second amplification step using the internal primers 5-TGCATAGGTTGCAGTCACTG-3 and 5-GGCGACCAATCTGCGAATACACC-3, which correspond to positions 694 to 714 and 853 to 831 bp, respectively, in the *T. gondii* DNA genome [14]. DNA extracted from a *T. gondii* suspension in mouse ascitic fluid was used as the positive control and deionized water was used as a negative control. The samples containing *T. gondii* DNA with a final amplified product of 97 bp were visualized on 0.2% agarose gel under ultraviolet light. The lower detection limit of this assay was 1 parasite/ml.

2.4. Real time PCR — Sybr Green dye

Real time PCR was based on the fluorescent dye Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen — Carlsbad, CA, USA) and was carried out using the Rotor-Gene 3000 instrument (Corbett, Australia) and the Rotor-Gene software version 6.0. The primers used were 5-GGAACTGCATCCGTTCATGAG-3 and 5-TCTTTAAAGCGTTCGTGGTC-3, which amplify the same 97 bp region of the B1 gene. The reaction was carried out for 2 min at 50 °C and for 2 min at 95 °C for the initial activation of Taq polymerase, followed by 40 denaturation cycles at 95 °C for 25 s, annealing at 55 °C for 30 s, and denaturation at 72 °C for 30 s [5]. At the end of amplification, the temperature was reduced to 60 °C and then gradually increased to 95 °C (0.2 °C/s). Samples with a dissociation temperature of 84.5 °C to 84.8 °C were defined as positive and the detection limit of this assay was 1 parasite/ml.

2.5. Serologic diagnosis

The exams for the serologic diagnosis of toxoplasmosis and for the investigation of infection with acquired human immunodeficiency virus (HIV) in cases suspected of toxoplasmosis in the CNS were carried out according to the routine of the serology laboratory of the hospital. Serum and CSF samples were submitted to an automated qualitative test that permits the detection of anti-toxoplasma IgM/IgG by Enzyme Linked Fluorescent Assay (ELFA) according to manufacturer specifications (VIDAS TOXO IgM/IgG — Biomérieux).

2.6. Neuroimaging exams

All patients with suspected neurotoxoplasmosis were submitted to CT and/or MRI exams performed by specialists. The imaging exams were decisive for the diagnostic suspicion and/or case definition, in addition to clinical signs and symptoms and response to specific treatment.

2.7. Statistical analysis

The PCR results were analyzed for the 79 CSF samples (total group) and compared to the definition of toxoplasmosis in the CNS of immunosuppressed patients. In cases suspected of neurotoxoplasmosis, the PCR results were analyzed considering the duration of specific treatment at the time of CSF collection. Data were analyzed in 2×2 tables for the calculation of PCR sensitivity and specificity for the 27 patients with cerebral toxoplasmosis and also for a subgroup with a time of treatment of seven days or less. The Chi-square test was used to analyze the clinical manifestations and the presence or absence of the disease. The Fisher test was used for the analysis of serology and ELISA exams and the Mann–Whitney test was used to compare the numerical means between CD4+ T cell count and CSF protein.

3. Results

Seventy-eight of the 79 patients studied were HIV infected. The non-infected patient was being treated for an autoimmune disease with high corticoid doses and had developed clinical and tomographic signs and symptoms compatible with neurotoxoplasmosis, which responded to the specific treatment instituted for the disease. The CSF of this patient was analyzed and found to be positive for toxoplasmosis.

Of the 79 patients, 24 (30%) were females and 55 (70%) were males, with a mean age of 43 years (range: 16–75 years). Table 1 compares the results for patients with cerebral toxoplasmosis and patients for whom this diagnosis was excluded.

Twenty-seven of the 79 patients were classified as probable cases of neurotoxoplasmosis on the basis of clinical criteria, neuroimaging and therapeutic response. In two of these cases, the diagnosis was confirmed after death by anatomopathological exams which revealed *T. gondii* cysts in brain tissue. Further confirmation was obtained by immunohistochemistry using specific monoclonal anti-toxoplasma antibodies. Of the 27 probable patients with neurotoxoplasmosis, 13 showed positive PCR for the detection of *T. gondii* DNA and one of them was not HIV infected. In all cases of positive PCR, CSF had been collected up to the seventh day after the beginning of specific treatment for the disease, as shown in Table 2.

Clinical, laboratory and radiological parameters (CT and MRI) were used to monitor the clinical and therapeutic outcomes of these patients. Most of the 27 patients with probable cerebral toxoplasmosis improved

Table 1
Comparison of the clinical manifestations and complementary exams of patients with neurotoxoplasmosis and of patients with other diagnoses.

Clinical signs and symptoms	Toxoplasmosis (27)	Other diagnoses (52)	X^2test $p < 0,05$
Fever	63% (17/27)	48% (25/52)	1.58
Headache	52% (14/27)	46% (24/52)	0.63
Vomiting	44% (12/27)	27% (14/52)	0.12
Paresis	37% (10/27)	25% (13/52)	0.57
Seizures	30% (8/27)	30% (16/52)	0.66
Exams	**Toxoplasmosis (27)**	**Other diagnoses (52)**	**p Value**
Blood Toxo.-reagent IgG	100 (27/27)	36,6 (32/52)	$p < 0.001$
CD4+ T lymphocytes	28 (25/27)	147 (39/52)	$p < 0.001$
CSF protein	82 (27/27)	47 (47/52)	$p = 0.04$
CSF cellularity/mm^3	9.65 (22/27)	3,66 (27/52)	$p = 0.03$

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