Real-time quantitative PCR in cerebral toxoplasmosis diagnosis of Brazilian human immunodeficiency virus-infected patients

Rafael T. Mesquita,¹ Ângela P. Ziegler,² Roberto M. Hiramoto,¹ Jose E. Vidal^{2,3} and Vera L. Pereira-Chioccolla¹

Correspondence

Vera L. Pereira-Chioccolla
pchioccolla@ial.sp.gov.br

¹Laboratório de Parasitologia, Instituto Adolfo Lutz, Sao Paulo, SP, Brazil

²Departamento de Neurologia, Instituto de Infectologia Emílio Ribas, Sao Paulo, SP, Brazil

³Serviço de Extensão ao Atendimento de Pacientes HIV/AIDS, Divisão de Moléstias Infecciosas e Parasitárias, Hospital das Clínicas da Faculdade de Medicina da Universidade de Sao Paulo, Sao Paulo, SP, Brazil

Cerebral toxoplasmosis is the most common cerebral mass lesion in AIDS patients in Brazil, and results in high mortality and morbidity, despite free access to HAART (highly active antiretroviral treatment). Molecular diagnosis based on conventional PCR (cnPCR) or real-time quantitative PCR (qrtPCR) has been indispensable for definitive diagnosis. We report here the evaluation of qrtPCR with blood and cerebrospinal fluid (CSF) samples from AIDS patients in Brazil. This prospective study was conducted for 2 years, analysing DNA samples extracted from 149 AIDS patients (98 blood and 51 CSF samples) with confirmed clinical and radiological diagnosis. The laboratory diagnosis included cnPCR (with the B22/B23 primer set) and indirect immunofluorescence (IF). For qrtPCR, two primer sets were simultaneously designed based on described genes and using a 6-carboxyfluorescein dye-labelled TaqMan MGB (minor groove binder) probe. One was B1Tg, which amplified a sequence from the B1 gene. The other was the RETg, which amplified a PCR product of the 529 bp sequence. The overall cnPCR and qrtPCR results were: positive results were observed in 33.6 % (50) patients. The sensitivities were 98 % for cnPCR (B22/B23), and 86 and 98 % for qrtPCR (B1Tg and RETg, respectively). Negative reactions were observed in 66.4 % patients. The specificities were 97 % for cnPCR and qrtPCR (B1Tg), and 88.8 % for RETg. These data show that RETg PCR is highly sensitive as it amplifies a repeat region with many copies; however, its specificity is lower than the other markers. However, B1Tg PCR had good specificity, but lower sensitivity. Among the patients, 20 had blood and CSF collected simultaneously. Thus, their results permitted us to analyse and compare molecular, serological and clinical diagnosis for a better understanding of the different scenarios of laboratorial and clinical diagnosis. For nine patients with confirmed cerebral toxoplasmosis diagnosis, four scenarios were observed: (i) and (ii) negative molecular diagnosis for CSF and positive for blood with variable IF titres for the sera and CSF (negative or positive); (iii) positive molecular diagnosis with CSF and negative with blood; and (iv) positive molecular diagnosis in both samples. In the latter two situations, normally the IF titres in sera and CSF are variable. Other opportunistic infections were shown in 11 patients. Despite the IF titres in sera and CSF being variable, all of them had negative molecular diagnosis for both samples. qrtPCR allows for a rapid identification of *Toxoplasma gondii* DNA in patient samples; in a minority of cases discrepancies occur with the cnPCR.

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INTRODUCTION

Toxoplasma gondii is a widely distributed apicomplexan parasite of great medical importance infecting all mammalian cells. During acute infection, *T. gondii* differentiates into tissue cysts that reside in the muscles and brain. Most primary infections are asymptomatic, but some symp-

Abbreviations: cnPCR, conventional PCR; CSF, cerebrospinal fluid; FAM, 6-carboxyfluorescein; HAART, highly active antiretroviral treatment; HIV, human immunodeficiency virus; IF, indirect immunofluorescence; NPV, negative predictive value; PPV, positive predictive value; qrtPCR, real-time quantitative PCR.

tomatic chronic forms exist as ocular and disseminated infections (Dubey, 1996, 1998; Montoya & Liesenfeld, 2004; Hill *et al.*, 2005). However, symptomatic forms are frequent when primary infection occurs during pregnancy, which can result in severe neonatal malformations and ocular complications in the fetus. In human immunodeficiency virus (HIV)-infected patients, low levels of CD4⁺ T cells allow reactivation of the latent infection, parasite proliferation and result in symptomatic disease such as cerebral toxoplasmosis, a life-threatening condition without timely diagnosis and treatment (Luft & Remington, 1992; Cohen, 1999; Montoya & Liesenfeld, 2004; Hoffmann, 2005).

Cerebral toxoplasmosis remains a prevalent disorder of the central nervous system, particularly among severely immunosuppressed HIV-infected patients in the absence of antibiotic prophylaxis (Antinori *et al.*, 2004). Its incidence among HIV-infected individuals directly correlates with the prevalence of anti-*T. gondii* antibodies among the general population. It represents the most common cerebral mass lesion in AIDS patients and is the third-most-frequent condition associated with AIDS in Brazil, still accounting for high mortality and morbidity, despite free access to highly active antiretroviral treatment (HAART) (Vidal *et al.*, 2005).

The use of molecular diagnosis is particularly appropriate for such patients, since it does not depend on the immunological status of the host. Molecular diagnosis based on conventional PCR (cnPCR) has become an indispensable tool for toxoplasmosis diagnosis (Bretagne, 2003; Vidal *et al.*, 2004; Colombo *et al.*, 2005; Bastien *et al.*, 2008). However, we have reported some difficulties in its use in laboratory practice. Parasite levels in blood and, principally, cerebrospinal fluid (CSF) are very poor in some patients. Consequently, a reduced amplification of cnPCR products is seen in the agarose gels resulting in false readings due to difficulty in interpreting the cnPCR product.

In recent years, real-time quantitative PCR (qrtPCR) has been introduced for molecular diagnosis of diverse diseases and has been used frequently in medical centres (Espy *et al.*, 2006; Bastien *et al.*, 2008). Several studies have demonstrated the applicability of qrtPCR for toxoplasmosis diagnosis, and others compared the different targets (Costa *et al.*, 2000; Buchbinder *et al.*, 2003; Hierl *et al.*, 2004; Edvinsson *et al.*, 2006; Brenier-Pinchart *et al.*, 2007). Among them, two targets are more frequently used because of their high sensitivity and specificity. One is the 529 bp sequence, which has 200–300 copies in the genome of *T. gondii* (RE) (Homan *et al.*, 2000). The other is the B1 gene that has 35 copies in the genome and is conserved in different parasite strains (B1) (Burg *et al.*, 1989). The sensitivity, accuracy and comparison of the targets, RE and B1 gene, for qrtPCR have been well analysed (Costa *et al.*, 2000; Hierl *et al.*, 2004; Reischl *et al.*, 2003; Chabbert *et al.*, 2004; Cassaing *et al.*, 2006; Calderaro *et al.*, 2006;

Edvinsson *et al.*, 2006). The majority of these studies aimed to compare the sensitivities of both DNA regions were conducted using European clinical samples; however, Brazilian samples are poorly studied. Recently, we have shown that targets for both B1 and RE in cnPCR were highly sensitive for *T. gondii* determination although B22/B23 (for B1) was the best (Pereira-Chioccia *et al.*, 2009). In order to investigate the capacity of qrtPCR for determining the molecular diagnosis of cerebral toxoplasmosis, we have reported here the evaluation of qrtPCR of blood and CSF samples from AIDS patients in Brazil. We investigated two primer sets from regions highly repetitive in the *T. gondii* genome (B1 and RE), and the results allowed for the discussion of the different cerebral toxoplasmosis diagnoses that occur in laboratory practice

METHODS

Clinical samples. This prospective study was conducted for 2 years (June 2007 to May 2009). Samples received by our laboratory from patients suspected of having cerebral toxoplasmosis were tested during routine diagnosis. From each patient, 3 ml CSF and/or 5 ml peripheral blood with EDTA were collected for DNA extraction. Sera for serological diagnosis were obtained from 5 ml blood. No patient had received HAART before the blood sample collection. The tests included cnPCR using the primer pair B22/B23, which amplified a 115 bp sequence of B1 gene (Burg *et al.*, 1989) and indirect immunofluorescence (IF). Simultaneously two qrtPCR protocols were tested. Sensitivity and specificity were evaluated from the analysis of DNA from 149 AIDS patients (98 blood and 51 CSF samples). Cerebral toxoplasmosis was confirmed in 50 patients (36 blood and 14 CSF samples) by clinical and radiological diagnosis. The case definition for toxoplasmosis included the following criteria: (i) progressive neurological deficits; (ii) contrast-enhancing mass lesion(s) on computed tomography scans; (iii) a successful response within 2 weeks to specific treatment (Portegies *et al.*, 2004; Vidal *et al.*, 2005). Other diseases such as cryptococcal meningoencephalitis, progressive multifocal leukoencephalopathy, HIV-associated cognitive motor disorder, pulmonary tuberculosis, bacterial pneumonia, cerebral tuberculosis, chronic renal deficiency, hepatitis C, cytomegalovirus encephalitis, epilepsy, histoplasmosis and oral candidiasis were confirmed in 99 patients (62 blood and 37 CSF samples). The ethics committees of both institutions approved this study.

***T. gondii* DNA.** The RH strain was grown and maintained in Swiss mice by intraperitoneal inoculation. Tachyzoites were harvested from the peritoneal cavities in PBS, pooled, and then centrifuged and washed twice, with centrifugation at 2000 g for 10 min. The parasite pellets were used for DNA extraction. For IF antigens, the parasite pellets were washed twice, counted and resuspended in PBS at a concentration of 2×10^7 cells ml⁻¹. The tachyzoites were incubated in 2% buffered formalin for 30 min at 37 °C, washed twice in PBS, with centrifugation at 1000 g for 10 min and then fixed on glass slides.

IF. IF was carried out as previously described (Colombo *et al.*, 2005) and was used to determine the presence or absence of anti-*T. gondii* IgG and IgM antibodies. Serial dilutions of the samples were used and samples were assayed in duplicate. The sera were diluted from 1:4 to 1:4096, and the cut-off was considered to be 1:16. The CSF samples were used and diluted to 1:64. Dilutions more than 1:4 were considered reactive.

DNA purification. DNA from blood samples was extracted using a PureLink genomic DNA kit (Invitrogen) according to the manufacturer's instructions. Previously, blood samples were centrifuged at 2500 g for 10 min and washed with PBS. The supernatants with plasma were discarded. In order to lyse the erythrocytes, the packed cells were mixed with a three times volume of a buffer containing 150 mM ammonium chloride, 1 mM potassium bicarbonate, 0.1 mM EDTA, pH 7.3, incubated for 15 min at room temperature under mild shaking and centrifuged for 10 min at 3000 g. The blood pellets, containing only nuclei cells were digested with proteinase K (20 µg ml⁻¹) in 50 mM Tris/HCl, 25 mM EDTA, pH 8.0, 2% SDS and incubated for 30 min at 56 °C. DNA pellets were dissolved in ultrapure water. As a positive control, DNA was extracted from the tachyzoite pellets using the kit. DNA from CSF samples was extracted as described previously (Vidal *et al.*, 2004). Samples were centrifuged for 10 min at 3000 g. CSF supernatants were used in serological tests for anti-*T. gondii* antibody determination. After washing the packed cells twice in PBS, whole cells were lysed by incubation for 5 min at 100 °C in 50 µl ultrapure water. DNA purity was determined by the ratio of the optical density at 260 and 280 nm in a NanoDrop ND100 (Thermo Scientific) spectrophotometer.

Primer selection. The primer pairs used for cnPCR were: (1) B22/B23 (5'-AACGGGCGAGTAGCACCTGAGGAGA-3' and 5'-TGG-GTCTACGTCGATGGCATGACAAC-3'), which amplified a 115 bp sequence from a specific repetitive region of the B1 gene (Burg *et al.*, 1989); (2) β1/β2 (5'-ACCACCAACTTCATCCACGTTCAACC-3' and 5'-CTTCTGACACAACCTGTGTTCACTAGC-3') (Lee *et al.*, 2001), which amplified a 140 bp fragment of the human β-globulin gene, and was used to assay all samples to control the course of extraction and check for PCR inhibitors. The reactions using the human marker were run simultaneously with the same temperature protocol for the B22/B23 primer and in the same PCR machine. For qRT-PCR, two primer sets were selected. One directed to B1: B1Tg-F (forward) 5'-CAAGCAGCGTATTGTCGAGTAGAT-3'; B1Tg-R (reverse) 5'-GCG-TCTCTTTTCATCCACATTTT-3'; and B1Tg-TM [TaqMan probe, 6-carboxyfluorescein (FAM) dye-labelled] 5'-FAM-CAGAAAGGAA-CTGCATCCGTT-3'). The second was directed to RE: RETg-F (forward) 5'-GGCGAGGGTGAGGATGAG-3'; RETg-R (reverse) 5'-CATCTTCTCCCTCTCCGACTCT-3'; and RETg-M (TaqMan probe, FAM dye-labelled) 5'-FAM-TCGCTTCCCAACCACG-3'). Both primer sets had NFQ (non-fluorescent quencher) as a reporter quencher and the location of the sequences in the *T. gondii* genome is shown in Fig. 1. The primer design was carried out with Primer Express software from Applied Biosystems.

cnPCR. The reactions were carried out in a LongGene thermal cycler in a final volume of 25 µl. The DNA samples (5 µl for blood or 10 µl for CSF), or the controls, and 25 pmol each primer were added to reagents from a kit purchased from Promega (GoTaq Green master mix). The PCR mix (12.5 µl) was composed of 1 unit *Taq* DNA polymerase, 10 mM Tris/HCl pH 8.5, 50 mM KCl, 1.5 mM MgCl₂ and 200 mM each dNTP. The amplification run contained two negative controls (ultrapure water and DNA from a sample from a toxoplasmosis-negative patient) and one positive control (DNA extracted from the RH strain). The thermal cycling conditions were: 1 initial denaturation cycle at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 62 °C for 1 min and extension at 72 °C for 1 min. The procedure was completed with a final extension cycle at 72 °C for 5 min. The PCR products were electrophoresed in 2% agarose gel and stained with ethidium bromide. The stained DNA fragments were visualized under UV illumination. The images were analysed by a MiniBIS gel imager and documentation system (BioSystematica). The size of fragments was determined based on comparison with a 100 bp ladder.

qRT-PCR. The reactions were performed with an Applied Biosystems 7500 real-time PCR system in a final volume of 20 µl. The clinical samples or control DNA (3 µl) were added to a reaction mixture containing 10 µl 2× TaqMan Universal PCR master mix, and 1 µl 'assay mix' that included (i) the forward primer (18 µM), (ii) the reverse primer (18 µM) and (iii) the TaqMan FAM dye-labelled (5 µM) MGB (minor groove binder) probe. Amplification runs contained two negative controls and one positive control, as used for cnPCR. The thermal cycling conditions included a 2 min 50 °C step for optimal AmpliErase UNG activity and denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Reactions were previously standardized using serial *T. gondii* DNA dilutions as template DNA. The cycle threshold value, indicative of the quantity of target gene at which the fluorescence exceeds a preset threshold, was automatically determined by the equipment.

Data analysis. All divergent results were repeated at least twice. Clinical and laboratory (IF and cnPCR) diagnoses were used to establish sensitivity, specificity, positive predictive values (PPVs) and negative predictive values (NPVs), and were calculated as: (i) per cent of sensitivity – ratio of true positives/true positives + false negatives × 100; (ii) per cent of specificity – ratio of true negatives/true negatives + false positives × 100; (iii) PPV – ratio of true positives/(true positives + false positives); and (iv) NPV – ratio of true negatives/(true negatives + false negatives).

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(a) 601 gcaaatgaaaaggattcatttttcgcagtagacaccaggagttggattttgtagagcgtctct
    661 cttcaagcagcgtattgtcagtagatcgaaaggaaactgcatccgttcatgagtataag
    721 aaaaaatgtgggaatgaaagagacgctaatgtgtttgcataggttgagtcactgacga
    781 gctccctctctgctggcgaaagtgaattcatgagtagtctgtgcaactttgggtgtattcg

(b) 181 cacagaaggagacagaagtcgaaggggactacagacgcgatgccgctcctccagccgtctt
    241 ggaggagagatatcaggactgtagatgaaggcgagggtaggagtagaggggtggcgtggt
    301 tggaagcgaagagtcggagagggagaagatgtttccggcttggtgcttttctctgga
    361 ggggtgaaaaagagacaccggaatgcgatccagacgagacgacgcttctcgtggtgat
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Fig. 1. Details of the primer sets used in qRT-PCR. (a) *T. gondii* B1 gene partial sequence 2214 bp (GenBank accession no. AF179871). The B1Tg primer sequences are located between nucleotides 664 and 747. The forward (B1Tg-F) and reverse (B1Tg-R) primers are shown in bold and underlined. The location of the FAM dye-labelled TaqMan probe (B1Tg-M) is shown in bold, italic and boxed text. (b) *T. gondii* repeat region linear 529 bp DNA (GenBank accession no. AF146527). RETg is located between nucleotides 270 and 334. The forward (RETg-F) and reverse (RETg-R) primers are shown in bold and underlined. The location of the FAM dye-labelled TaqMan probe (RETg-M) is shown in bold, italic and boxed text.

RESULTS

The objective of this study was to investigate two primer sets for qrtPCR and to compare them with cnPCR in routine molecular diagnosis. We used the B22/B23 primer set in cnPCR, which has been highly evaluated in our laboratory (Vidal *et al.*, 2004; Colombo *et al.*, 2005). For qrtPCR we chose two primer sets from two described genes. One was the B1Tg set, which amplified a (partial) sequence from B1 gene (GenBank accession number AF179871) (Burg *et al.*, 1989) with 35 copies in the genome. The other was the RETg set, which amplified a sequence from the 529 bp sequence (GenBank accession number AF146527) (Homan *et al.*, 2000) that has 200–300 copies in the genome (Fig. 1).

The overall cnPCR and qrtPCR results of the 149 DNA samples were evaluated to determine the sensitivity, specificity, PPV and NPV for each individual primer set. Among the 98 DNA samples extracted from blood, positive results and sensitivity were observed in 35 (97.2%), 32 (88.9%) and 35 (97.2%) samples for B22/B23, B1Tg and RETg, respectively. The sensitivities for the 51 CSF samples were 78.6% (11) for B1Tg, and 100% (14) for B22/B23 and RETg. When the 149 samples were analysed together, the sensitivities were 98.0% (49) for B22/B23 and RETg, and 86.0% (43) for B1Tg. According to the specificity, in blood, the reactions using the primer sets B22/B23, B1Tg and RETg were negative in 61 (98.4%), 61 (98.4%) and 55 (88.7%) samples, respectively. The results for the CSF samples were 35 (97.2%) in B22/B23 and RETg, and 33 (91.7%) in B1Tg. The specificities of all 149 samples were 97.0% (96) for B22/B23 and B1Tg, and 88.8% (88) for RETg. Despite the high sensitivity of RETg (98%), its specificity (88.8%) and NPV (0.900) were lower than B22/B23 and B1Tg (0.970). However, B1Tg had the lowest

sensitivity (86.0%) and PPV (0.877). Table 1 shows the results of each individual primer set in detail.

Among the patients, 20 had blood and CSF collected simultaneously. Thus, their results permitted us to analyse and compare the molecular, serological and clinical information of the same patient. Nine patients were diagnosed as having cerebral toxoplasmosis but the laboratorial diagnosis differed, and four situations can be seen in Table 2. The first and second cases had negative molecular diagnosis for CSF and positive for blood. IF titres can be variable in CSF, as shown for case 1 (negative) and case 2 (positive). Thus a negative serological profile does not exclude the presence of the active infection, principally when the serological diagnosis is made using CSF, since its value in CSF is limited because the sensitivity and specificity are around 60 to 70% (Collazos, 2003). The third case had a positive molecular diagnosis for CSF and a negative diagnosis for blood. The fourth case had a positive molecular diagnosis for both samples. IF titres in sera and CSF can be variable, high as in case 3, or low as in case 4.

The other 11 patients had other neurological opportunistic infections and cases 5, 6, 7, 8 illustrate the negative. Although all samples had negative molecular diagnosis, IF titres in sera and CSF were variable: negative (case 5), high (case 6) or low (case 7) titres. Negative IF for CSF and positive for sera were also shown (case 8).

DISCUSSION

In recent years, great strides have been made in developing laboratory techniques for diagnosing infectious diseases including toxoplasmosis. Nevertheless, the diagnosis of cerebral toxoplasmosis in HIV-infected patients still presents difficulties. In different clinical centres, specific

Table 1. Molecular diagnosis for cerebral toxoplasmosis in 149 AIDS patients employing primer sets B22/B23 (cnPCR), and B1Tg and RETg (qrtPCR): sensitivity, specificity, PPVs and NPVs

The PPV, NPV, sensitivity and specificity for each primer set were determined considering the clinical diagnosis described in Methods.

Sample/primer set	No. of samples		Sensitivity (%)	Specificity (%)	PPV	NPV
	Positive	Negative				
Blood						
B22/B23 (cnPCR)	35	61	97.2	98.4	0.973	0.984
B1Tg (qrtPCR)	32	61	88.9	98.4	0.900	0.984
RETg (qrtPCR)	35	55	97.2	88.7	0.973	0.898
CSF						
B22/B23 (cnPCR)	14	35	100	97.2	1.0	0.973
B1Tg (qrtPCR)	11	35	78.6	97.2	0.823	0.973
RETg (qrtPCR)	14	33	100	91.7	1.0	0.924
Total						
B22/B23 (cnPCR)	49	96	98.0	97.0	0.980	0.970
B1Tg (qrtPCR)	43	96	86.0	97.0	0.877	0.970
RETg (qrtPCR)	49	88	98.0	88.8	0.980	0.900

Table 2. Clinical and laboratory diagnosis determined in patients with AIDS and cerebral toxoplasmosis (cases 1 to 4) or other opportunistic infections (cases 5 to 8)

Blood and CSF samples were collected simultaneously.

Case	Sample	Primer set			IF*	Clinical diagnosis
		qrtPCR		cnPCR		
		B1Tg	RETg	B22/B23		
1	CSF	—	—	—	—	Cerebral toxoplasmosis
	Blood	+	+	+	1:1024	
2	CSF	—	—	—	1:8	Cerebral toxoplasmosis
	Blood	+	+	+	1:4000	
3	CSF	+	+	+	1:32	Cerebral toxoplasmosis
	Blood	—	—	—	1:4000	
4	CSF	+	+	+	1:8	Cerebral toxoplasmosis
	Blood	+	+	+	1:256	
5	CSF	—	—	—	—	Cerebral tuberculosis
	Blood	—	—	—	—	
6	CSF	—	—	—	1:16	Cytomegalovirus encephalitis
	Blood	—	—	—	1:4000	
7	CSF	—	—	—	1:4	Epilepsy
	Blood	—	—	—	1:256	
8	CSF	—	—	—	—	Progressive multifocal leukoencephalopathy
	Blood	—	—	—	1:1024	

*Sera and CSF were considered reactive when fluorescent tachyzoites were shown in dilutions of more than 1:16 and 1:4, respectively.

treatment is usually initiated according to a presumptive diagnosis, which is based on clinical and radiological findings (Luft & Remington, 1992; Vidal *et al.*, 2005). However, the importance of additional laboratory methods, principally molecular ones, is incontestable. Thus, the evaluation of such methods for diagnosis seems to be important. As a result, several European studies have demonstrated qrtPCR applicability in toxoplasmosis diagnosis (Costa *et al.*, 2000; Buchbinder *et al.*, 2003; Hierl *et al.*, 2004; Edvinsson *et al.*, 2006; Brenier-Pinchart *et al.*, 2007). As similar findings may be expected for Brazilian patients, the present study investigated the diagnostic value of qrtPCR, in this population, using primer sets constructed to amplify products of the B1 and RE regions known to be highly repetitive in the *T. gondii* genome.

As a laboratory control, we used cnPCR (with B22/B23 primers), which is rated highly and has been used routinely for molecular diagnosis in our laboratory (Vidal *et al.*, 2004; Colombo *et al.*, 2005). For qrtPCR evaluation, we analysed the B1Tg primer set that amplified a sequence from the B1 gene partial sequence (GenBank accession number AF179871) (Burg *et al.*, 1989), and RETg that amplified part of the 529 bp sequence (GenBank accession number AF146527) (Homan *et al.*, 2000). The primer sets chosen were joined to the procedure checkerboard as DNA extraction, cnPCR and qrtPCR, aiming at optimal working conditions. In order to improve the sensitivity and specificity, the clinical samples were processed rapidly within 48 h of collection to prevent *Taq* polymerase

inhibition. Many components found in biological samples such as blood and CSF inhibit *Taq* polymerase, modifying PCR results (Al-Soud & Rådström, 1998). As the amount of human chromosomal DNA and the pathogen can vary from sample to sample, and interfere in the results, all samples were quantified after extraction. Therefore, the DNA extraction and PCR inhibitors were evaluated by a $\beta 1$ – $\beta 2$ marker that amplified a PCR fragment of human β -globulin gene. Positive amplifications showed that no substance present in the DNA samples inhibited the reaction. The clinical centres were informed to collect blood or CSF from the suspected patients before or during the first 3 days of the specific therapy (Colombo *et al.*, 2005). Previous studies reported that anti-toxoplasmic therapy decreases diagnostic sensitivity, especially if samples were collected after the first week of treatment (Cingolani *et al.*, 1996; Gianotti *et al.*, 1997).

Molecular diagnosis using CSF or peripheral blood samples is a useful tool for early minimally invasive diagnosis of cerebral toxoplasmosis (Bretagne, 2003; Bastien, 2002; Colombo *et al.*, 2005). However, results should be always interpreted in association with serological, clinical and radiological information (Pereira-Chioccola *et al.*, 2009).

This prospective study analysed a considerable number of clinical samples (149 patients with confirmed clinical and radiological diagnosis). Among them, 20 patients had two samples collected simultaneously, one from blood and another from CSF, allowing for better understanding of

the different scenarios of laboratory and clinical diagnosis. This small group of patients serves to illustrate different situations. The results of the patients with cerebral toxoplasmosis allowed for concluding that divergent situations can be observed in laboratory and clinical practice of this opportunistic infection. Some patients may have a negative molecular diagnosis for CSF and positive for blood, and the anti-*T. gondii* antibodies may be variable. Others can present with a positive molecular diagnosis for CSF and a negative one for blood; or positive molecular diagnosis from both samples with the frequent presence of high titres of anti-*T. gondii* antibodies. However, a small proportion of these patients had negative serological diagnoses (Meira *et al.*, 2008). According to a recent Brazilian study, patients with four or more brain lesions present greater positivity in molecular diagnosis (Correia *et al.*, 2010).

The patients with other opportunistic diseases had negative molecular diagnosis for both clinical samples despite the fact that some of them presented anti-*T. gondii* antibodies for sera or CSF. The analysis of the differential diagnosis of expansive brain lesions in HIV patients is extremely significant because it determines the effectiveness of specific treatment. The majority of the samples analysed in this study were negative (66.4%). cnPCR employing the B22/B23 primer set presents a high specificity, as repeatedly shown before, including for Brazilian samples (Bretagne, 2003; Vidal *et al.*, 2004; Colombo *et al.*, 2005; Okay *et al.*, 2009; Correia *et al.*, 2010), and has been used in different laboratories. The same high specificity was observed in qrtPCR using the B1Tgt set whose target region is the B1 gene as for B22/B23. These data confirmed those from other studies (Raffi *et al.*, 1997; Bretagne, 2003; Reischl *et al.*, 2003; Nagy *et al.*, 2006; Okay *et al.*, 2009; Correia *et al.*, 2010), but RETg, whose target region is the 529 bp fragment, presented lower specificity (88.8%) and NPV (0.908) than two other sets from the B1 gene.

Among all studied samples, 33.6% (50) were positive. cnPCR sensitivity was 98.0%, as shown before in samples from our laboratory (Colombo *et al.*, 2005; Vidal *et al.*, 2005) and others (Contini *et al.*, 2005; Okay *et al.*, 2009). A similar high sensitivity was shown when the RETg set was used. These data were extensively confirmed by published studies (Homan *et al.*, 2000; Costa *et al.*, 2000; Calderaro *et al.*, 2006; Cassaing *et al.*, 2006). As RETg PCR amplified a region in the *T. gondii* genome 10 times more repetitive than the B1 gene, the arithmetic mean of the samples cycle threshold value was lower (27.11) than that shown for B1Tg (32.52) (data not shown). In addition, B1Tg had the lowest sensitivity (86.0%), as has been shown in other studies (Bretagne, 2003; Reischl *et al.*, 2003; Cassaing *et al.*, 2006; Correia *et al.*, 2010).

As this study analysed a considerable number of samples under the same laboratory conditions, only varying the primer sets and technology (cnPCR and qrtPCR), we can

conclude that RETg PCR was highly sensitive as it amplified a repeat region with many copies. However, its specificity was lower than that of the other markers. Around 10% of the negative samples can present positive results when this primer is used (NPV=0.900), principally in DNA extracted from blood samples. Similar results were shown recently by Wahab *et al.* (2010). However, B1Tg had good specificity, but lower sensitivity (86%) compared with the other markers. Negative results were seen in approximately 12% of the samples from patients with cerebral toxoplasmosis (PPV=0.877).

From our point of view, even though the number of copies of the DNA target in the genome of the micro-organism is an essential factor that affects sensitivity (Bastien *et al.*, 2008), the specificity is also extremely important. qrtPCR allows for rapid identification of *T. gondii* DNA in patient samples; in a minority of cases discrepancies occur with the cnPCR.

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REFERENCES

- Al-Soud, W. A. & Rådström, P. (1998). Capacity of nine thermostable DNA polymerases to mediate DNA amplification in the presence of PCR-inhibiting samples. *Appl Environ Microbiol* **6**, 3748–3753.
- Antinori, A., Larussa, D., Cingolani, A., Lorenzini, P., Bossolasco, S., Finazzi, M. G., Bongiovanni, M., Guaraldi, G., Grisetti, S. & other authors (2004). Prevalence, associated factors, and prognostic determinants of AIDS-related toxoplasmic encephalitis in the era of advanced highly active antiretroviral therapy. *Clin Infect Dis* **39**, 1681–1691.
- Bastien, P. (2002). Molecular diagnosis of toxoplasmosis. *Trans R Soc Trop Med Hyg* **96**, S205–S215.
- Bastien, P., Procop, G. W. & Reischl, U. (2008). Quantitative real-time PCR is not more sensitive than conventional PCR. *J Clin Microbiol* **46**, 1897–1900.
- Brenier-Pinchart, M. P., Morand-Bui, V., Fricker-Hidalgo, H., Equy, V., Marlu, R. & Pelloux, H. (2007). Adapting a conventional PCR assay for *Toxoplasma gondii* detection to real-time quantitative PCR including a competitive internal control. *Parasite* **14**, 149–154.
- Bretagne, S. (2003). Molecular diagnostics in clinical parasitology and mycology: limits of the current polymerase chain reaction (PCR) assays and interest of the real-time PCR assays. *Clin Microbiol Infect* **9**, 505–511.
- Buchbinder, S., Blatz, R. & Rodloff, A. C. (2003). Comparison of real-time PCR detection methods for B1 and P30 genes of *Toxoplasma gondii*. *Diagn Microbiol Infect Dis* **45**, 269–271.
- Burg, J. L., Grover, C. M., Pouletty, P. & Boothroyd, J. C. (1989). Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction. *J Clin Microbiol* **27**, 1787–1792.
- Calderaro, A., Piccolo, G., Gorrini, C., Peruzzi, S., Zerbini, L., Bommezzadri, S., Dettori, G. & Chezzi, C. (2006). Comparison

between two real-time PCR assays and a nested-PCR for the detection of *Toxoplasma gondii*. *Acta Biomed* 77, 75–80.

Cassaing, S., Bessieres, M. H., Berry, A., Berrebi, A., Fabre, R. & Magnaval, J. F. (2006). Comparison between two amplification sets for molecular diagnosis of toxoplasmosis by real-time PCR. *J Clin Microbiol* 44, 720–724.

Chabbert, E., Lachaud, L., Crobu, L. & Bastien, P. (2004). Comparison of two widely used PCR primer systems for detection of *Toxoplasma* in amniotic fluid, blood and tissues. *J Clin Microbiol* 42, 1719–1722.

Cingolani, A., De Luca, A., Ammassari, A., Murri, R., Linzalone, A., Grillo, R. & Antinori, A. (1996). PCR detection of *Toxoplasma gondii* DNA in CSF for the differential diagnosis of AIDS-related focal brain lesions. *J Med Microbiol* 45, 472–476.

Cohen, B. A. (1999). Neurological manifestations of toxoplasmosis in AIDS. *Semin Neurol* 19, 201–211.

Collazos, J. (2003). Opportunistic infections of the CNS in patients with AIDS. *CNS Drugs* 17, 869–887.

Colombo, F. A., Vidal, J. E., Penalva de Oliveira, A. C., Hernandez, A. V., Bonasser-Filho, F., Nogueira, R. S., Focaccia, R. & Pereira-Chiocola, V. L. (2005). Diagnosis of cerebral toxoplasmosis in AIDS patients in Brazil: importance of molecular and immunological methods using peripheral blood samples. *J Clin Microbiol* 43, 5044–5047.

Contini, C., Seraceni, S., Cultrera, R., Incorvaia, C., Sebastiani, A. & Picot, S. (2005). Evaluation of a real-time PCR-based assay using the lightcycler system for detection of *Toxoplasma gondii* bradyzoite genes in blood specimens from patients with toxoplasmic retinochoroiditis. *Int J Parasitol* 35, 275–283.

Correia, C. C., Melo, H. R. L. & Costa, V. M. A. (2010). Influence of neurotoxoplasmosis characteristics on real-time PCR sensitivity among AIDS patients in Brazil. *Trans R Soc Trop Med Hyg* 104, 24–28.

Costa, J. M., Pautas, C., Ernault, P., Foulet, F., Cordonnier, C. & Bretagne, S. (2000). Real-time PCR for diagnosis and follow-up of *Toxoplasma* reactivation after allogeneic stem cell transplantation using fluorescence resonance energy transfer hybridization probes. *J Clin Microbiol* 38, 2929–2932.

Dubey, J. P. (1996). *Toxoplasma gondii*. In *Medical Microbiology*, 5th edn. Edited by S. Baron, R. C. Peake, D. A. James, M. Susman, C. A. Kennedy, M. J. D. Singleton & S. Schuenke. Galveston, TX: University of Texas Medical Branch.

Dubey, J. P. (1998). Advances in the life cycle of *Toxoplasma gondii*. *Int J Parasitol* 28, 1019–1024.

Edvinsson, B., Lappalainen, M. & Evengard, B. on behalf of the ESCMID Study Group for Toxoplasmosis (2006). Real-time PCR targeting a 529-bp repeat element for diagnosis of toxoplasmosis. *Clin Microbiol Infect* 12, 131–136.

Espy, M. J., Uhl, J. R., Sloan, L. M., Buckwalter, S. P., Jones, M. F., Vetter, E. A., Yao, J. D., Wengenack, N. L., Rosenblatt, J. E. & other authors (2006). Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clin Microbiol Rev* 19, 165–256.

Gianotti, N., Cinque, P., Castagna, A., Novati, R., Moro, M. & Lazzarin, A. (1997). Diagnosis of toxoplasmic encephalitis in HIV-infected patients. *AIDS* 11, 1529–1530.

Hierl, T., Reischl, U., Lang, P., Hebart, H., Stark, M., Kyme, P. & Autenrieth, I. B. (2004). Preliminary evaluation of one conventional nested and two real-time PCR assays for the detection of *Toxoplasma gondii* in immunocompromised patients. *J Med Microbiol* 53, 629–632.

Hill, D. E., Chirukandoth, S. & Dubey, J. P. (2005). Biology and epidemiology of *Toxoplasma gondii* in man and animals. *Anim Health Res Rev* 6, 41–61.

Hoffmann, C. (2005). Opportunistic infections. In *HIV Medicine*. Edited by C. Hoffmann, J. K. Rockstroh & B. S. Kamps. Paris: Flying Publisher. www.HIVMedicine.com (in Spanish).

Homan, W. L., Vercammen, M., De Braekeleer, J. & Verschueren, H. (2000). Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in *Toxoplasma gondii*, and its use for diagnostic and quantitative PCR. *Int J Parasitol* 30, 69–75.

Lee, C. N., Cavanagh, H. M., Lo, S. T. & Ng, C. S. (2001). Human papillomavirus infection in non-neoplastic uterine cervical disease in Hong Kong. *Br J Biomed Sci* 58, 85–91.

Luft, B. J. & Remington, J. S. (1992). Toxoplasmic encephalitis. *Clin Infect Dis* 15, 211–222.

Meira, C. S., Costa-Silva, T. A., Vidal, J. E., Ferreira, I. M. R., Hiramoto, R. M. & Pereira-Chiocola, V. L. (2008). Use of the serum reactivity against *Toxoplasma gondii* excreted–secreted antigen in cerebral toxoplasmosis diagnosis in HIV-infected patients. *J Med Microbiol* 57, 845–850.

Montoya, J. G. & Liesenfeld, O. (2004). Toxoplasmosis. *Lancet* 363, 1965–1976.

Nagy, B., Ban, Z., Beke, A., Nagy, G. R., Lazar, L., Papp, C., Toth-Pal, E. & Papp, Z. (2006). Detection of *Toxoplasma gondii* from amniotic fluid, a comparison of four different molecular biological methods. *Clin Chim Acta* 368, 131–137.

Okay, T. S., Yamamoto, L., Oliveira, L. C., Manuli, E. R., Andrade, H. F., Jr & Del Negro, G. M. B. (2009). Significant performance variation among PCR systems in diagnosing congenital toxoplasmosis in So Paulo, Brazil: analysis of 467 amniotic fluid samples. *Clinics* 64, 171–176.

Pereira-Chiocola, V. L., Vidal, J. E. & Su, C. (2009). Cerebral toxoplasmosis in HIV-infected patients. *Future Microbiol* 4, 1363–1379.

Portegies, P., Solod, L., Cinque, P., Chaudhuri, A., Begovac, J., Everall, I., Weber, T., Bojar, M., Martinez-Martin, P. & Kennedy, G. E. (2004). Guidelines for the diagnosis and management of neurological complications of HIV infection. *Eur J Neurol* 11, 297–304.

Raffi, F., Aboulker, J. P., Michelet, C., Reliquet, V., Pelloux, H., Huart, A., Poizot-Martin, I., Morlat, P., Dupas, B. & other authors (1997). A prospective study of criteria for the diagnosis of toxoplasmic encephalitis in 186 AIDS patients. *AIDS* 11, 177–184.

Reischl, U., Bretagne, S., Kruger, D., Ernault, P. & Costa, J. M. (2003). Comparison of two DNA targets for the diagnosis of toxoplasmosis by real-time PCR using fluorescence resonance energy transfer hybridization probes. *BMC Infect Dis* 3, 7.

Vidal, J. E., Colombo, F. A., Penalva de Oliveira, A. C., Focaccia, R. & Pereira-Chiocola, V. L. (2004). PCR assay using cerebrospinal fluid for diagnosis of cerebral toxoplasmosis in Brazilian AIDS patients. *J Clin Microbiol* 42, 4765–4768.

Vidal, J. E., Hernandez, A. V., Penalva de Oliveira, A. C., Dauar, R. F., Barboza, S. P. & Focaccia, R. (2005). Cerebral toxoplasmosis in HIV-positive patients in Brazil: clinical features and predictors of treatment response in the HAART era. *AIDS Patient Care STDS* 19, 626–634.

Wahab, T., Edvinsson, B., Palm, D. & Lindh, J. (2010). Comparison of the AF146527 and B1 repeated elements, two real-time PCR targets used for detection of *Toxoplasma gondii*. *J Clin Microbiol* 48, 591–592.