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The utility of cerebrospinal fluid for the molecular diagnosis of toxoplasmic encephalitis ☆

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ABSTRACT

The aim of this study was to assess the efficacy of nested polymerase chain reaction (PCR) and the loop-mediated isothermal amplification (LAMP) assay, which were developed to detect and identify toxoplasma parasites in human cerebrospinal fluid (CSF). Nested PCR was performed using primers generated by Dr. L.D. Sibley to target the 18S rDNA instead of the conventionally used primers which target the B1 gene. We also designed *Toxoplasma gondii*–specific LAMP primers targeting both genes. In vitro detection sensitivity was evaluated using 10-fold serially diluted genomic DNA purified from RH tachyzoites, and clinical sensitivity and specificity were evaluated using clinical CSF samples from 16 patients with toxoplasmic encephalitis (TE) and from 12 patients with other diseases. The 18S rDNA nested PCR showed the highest detection sensitivity limit with a minimum of 1.0×10^{-8} ng/μL. However, sensitivity and specificity of nested PCR with clinical specimens were 50% and 100%, respectively. The sensitivity of molecular diagnosis of TE is not sufficient; therefore, patients clinically suspected of having TE should be treated promptly. Our molecular diagnostic tool would restrictively facilitate a definitive diagnosis of TE at an early stage in approximately 50% of patients.

1. Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite that possesses the capability to infect a variety of warm-blooded animals, including humans. Although most of its primary infections are known to be asymptomatic, it is speculated that up to one-third of the human population has been persistently or latently infected by this protozoan (Hill and Dubey, 2002). Among such cases, the lives of immunocompromised patients, especially those with acquired immunodeficiency syndrome (AIDS) and organ transplant recipients, could be threatened by reactivation of a latent *T. gondii* infection, which can cause potentially fatal encephalitis (Dubey, 2008; Montoya and Liesenfeld, 2004). The diagnosis of toxoplasmic encephalitis (TE) is clinically defined by the following 3 criteria: a) the recent onset of a focal neurologic abnormality that is consistent with intracranial disease or an impaired level of consciousness; b) lesions presenting with a mass effect or a radiographic appearance that is enhanced by the injection of a contrast medium in brain imaging (computed tomography [CT] or nuclear magnetic resonance); and c) the presence of *T. gondii*–specific serum antibody or a successful response to specific therapeutics for toxoplasmosis (CDC, 1993. However, serology is not a sufficiently sensitive marker for the diagnosis of TE because a rise in the titer of IgG antibody occurs in only approximately 30% of cases (Derouin et al., 1991), and IgM antibody is seldom found (Luft et al., 1984). Even the intrathecal production of antibody to *T. gondii* is found in only 50% of TE-presenting AIDS patients (Potasman et al., 1988).

Recently, DNA-based diagnostic tools, including nested polymerase chain reaction (PCR) and real-time PCR, have become powerful tools for the detection of *T. gondii* DNA in various clinical specimens (Alfonso et al., 2008; Mesquita et al., 2010) Although several DNA-based diagnostic test kits are commercially available, the majority have not been validated in prospective large clinical trials and have consequently lacked reliability. Although a few well-conducted trials have been performed that compared the efficacy of different types of tests, the lack of clinical data has limited their routine use in many settings. For the diagnosis of TE, the low sensitivity of these tests has led to most of the clinical cases being diagnosed empirically by determining the effective response to *T. gondii*–specific chemotherapy.

The loop-mediated isothermal amplification (LAMP) assay has proved useful and efficacious for the definitive diagnosis of infectious

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Table 1
Primers and PCR cycle conditions used for the amplification and detection of *Toxoplasma gondii* 18S rDNA.

Target		PCR Primers	Nucleotide position	Cycle condition
L37415	First PCR	5′-CCA TGC ATG TCT AAG TAT AAG G-3′	48–70	60 s at 94 °C
		5′-GTT ACC CGT CAC TGC CAC-3′	359–342	60 s at 42 °C
	Second PCR	5′-CTA AGT ATA AGC TTT TAT ACG GC-3′	58–80	60 s at 72 °C
		5′-TGC CAC GGT AGT CCA ATA C-3′	348–330	40 cycles (each)

diseases including other protozoan diseases, such as *Plasmodium* spp. (Han et al., 2007; Poon et al., 2006) and *Trypanosoma* spp. (Njiru et al., 2008a,b). Regarding *T. gondii*, although Lau et al. (2010) reported that the LAMP assay was useful for the diagnosis of acute toxoplasmosis using human blood specimens, no reports, to the best of our knowledge, have demonstrated the usefulness of the LAMP assay to diagnose TE using human cerebrospinal fluid (CSF). This study is the first to determine whether the recently developed molecular tools are useful for making a proper diagnosis of TE.

2. Materials and methods

2.1. Standard DNA and parasite preparation

Tachyzoites of the RH strain of *T. gondii* were obtained by in vitro culture as described previously (Sibley et al., 1994). Harvested tachyzoites were suspended in phosphate-buffered saline, and the suspension was subjected to 3 cycles of freezing in liquid nitrogen followed by thawing. Genomic DNA was extracted from the lysate using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.2. Nested PCR

Dr. L.D. Sibley (Washington University School of Medicine) kindly provided us with *T. gondii* 18S rDNA–specific nested PCR primers that were designed on the basis of sequence information (unpublished data). Nested PCR for amplification of the *T. gondii* B1 gene was performed as described previously (Schoondermark-van de Ven et al., 1993). The primers and specific PCR running conditions are summarized in Table 1.

PCR reactions were performed using the *TaKaRa Ex Taq* Kit (TAKARA, Kyoto, Japan) in a thermal cycler according to the manufacturer's instructions. The nested PCR products were electrophoresed on 1.2% agarose gels, stained with ethidium bromide, and visualized using an LAS-3000 Chemiluminescence Image Analyzer (Fujifilm, Tokyo, Japan).

2.3. DNA sequencing of the nested PCR products

Each nested PCR product with expected size was cloned into the pCR 2.1-TOPO TA cloning plasmids (Invitrogen, Carlsbad, CA, USA) and transformed into TOP10 *E. coli* (Invitrogen) according to the manufacturer's instructions. Positive clones were selected on Luria-Bertani plates containing 100 μg/mL ampicillin. After plasmid purification using the Qiagen Plasmid Mini Kit (Qiagen), the amplified DNA products were verified by DNA sequencing using the ABI PRISM BigDye Terminator Cycle Sequencing Kit with T3 or T7 primers (Applied Biosystems, Foster, CA, USA) and loaded onto an ABI PRISM 310 DNA Sequencer.

2.4. Design of LAMP primers and assay

The oligonucleotide LAMP primers for the detection of the *T. gondii* genes were designed on the basis of the 18S rDNA sequence (GenBank accession no. L37415) and B1 gene (GenBank accession no. AF179871.1). Nine candidate sets of *T. gondii*–specific LAMP primers were designed within the consensus regions of each gene using online LAMP primer design software (PrimerExplorer 4, http://primerexplorer.jp/index.html; Eiken Chemical, Tokyo, Japan) (Table 2).

LAMP reactions were performed using the Loopamp DNA Amplification Reagent Kit (Eiken Chemical) in a thermal cycler (TAKARA) according to the manufacturer's instructions. The LAMP reactions were performed for 60 min at 61–67 °C and inactivated for 2 min at 95 °C. The LAMP products were analyzed as described above for the nested PCR products.

2.5. Determination of the detection sensitivity of nested PCR and LAMP

To compare the sensitivity of the nested PCR and LAMP assays, the same batch of purified genomic DNA extracted from *T. gondii* RH tachyzoites was used in the amplifications. Ten-fold serially diluted genomic DNA samples, adjusted from 10 ng/μL to 1.0×10^{-8} ng/μL, were made into *T. gondii* DNA standard solutions and used to determine the detection threshold of the nested PCR and LAMP assays. For the LAMP assays, which required an optimal temperature for the reactions, several temperatures ranging from 61 °C to 67 °C were tested. Each standardized positive control was tested in triplicate.

Table 2
LAMP primers used in the present study.

Name		LAMP Primers (sequence)
B1-1	B3	5′-GGT GGT CGA CTT CAT GGG A-3′
	F3	5′-AGA AGA AGG GCT GAC TCG A-3′
	BIP	5′-TTC CGC CTC CTT CGT CCG TCC TCT TGC AGT AAG GGT GCC-3′
	FIP	5′-CAC GTC TGG GAA GAA CTC CGT TCA GAT GTG CTA AAG GCG TCA-3′
B1-2	B3	5′-ACG TGA CAG TGA AGA GAG GA -3′
	F3	5′-CAG ATG TGC TAA AGG CGT CA-3′
	BIP	5′-TGT TCG CTG TCT GTC TAG GGC AGG TGG TCG ACT TCA TGG GA-3′
	FIP	5′-AGG CGG AAC CAA CGG AAA TCC TTG CTG TTC TGT CCT ATC GC-3′
B1-3	B3	5′-GGC GGA CCT CTC TTG TCT-3′
	F3	5′-CGT GGA TTT CCG TTG GTT CC-3′
	BIP	5′-ATC GTC CCA TGA AGT CGA CCA CTG CTG GAT CTC TTC CCT TGA-3′
	FIP	5′-GTA AGG GTG CCC CCT CCT TCG TCC GTC GTA A-3′
18S-1	B3	5′-CGC GAT CCG TTC GGT-3′
	F3	5′-AGA TTA AGC CAT GCA TGT CT-3′
	BIP	5′-GCA CAT GCC TCT TCC CTC ACC TGA GGA CCA CCG-3′
	FIP	5′-GGT TAT CCA TGT AGT AAA GAC CTT TTA TAC GGC TAA ACT GCG-3′
18S-2	B3	5′-TCC AAT ACA GTA CCG TCG A-3′
	F3	5′-ACA TGG ATA ACC GTG GTA A-3′
	BIP	5′-TGG TCC TCA GGT GAT TCA TAG TAA GCT GAT AGG TCA GAA ACT TGA A-3′
	FIP	5′-GTA TCT AAT AAA CAC TGC CCT TCC AGG TAA TTC TAT GGC TAA TAC ATG CG-3′
18S-3	B3	5′-CCC TAA TTC CCC GTT ACC-3′
	F3	5′-ACA TGG ATA ACC GTG GTA A-3′
	BIP	5′-CGA ACG GAT CGC GTT GAC TTA ATA CAG TAC CGT CGA AAG C-3′
	FIP	5′-GGT GGG TTG GTT CTG TAT CTA ATA ATG GCT AAT ACA TGC GCA CAT-3′
18S-4	B3	5′-CGC GAT CCG TTC GGT-3′
	F3	5′-AGA TTA AGC CAT GCA TGT CT-3′
	BIP	5′-CAT GCG CAC ATG CCT CTT CAC CTG AGG ACC ACC GG-3′
	FIP	5′-GGT TAT CCA TGT AGT AAA GAC CCT AAA CTG CGA ATG GCT CA-3′
18S-5	B3	5′-CCC TAA TTC CCC GTT ACC-3′
	F3	5′-ACA TGG ATA ACC GTG GTA A-3′
	BIP	5′-CGA ACG GAT CGC GTT GAC TTA ATA CAG TAC CGT CGA AAG C-3′
	FIP	5′-GGT GGG TTG GTT CTG TAT CTA TGC GCA CAT GCC TCT TC-3′
18S-6	B3	5′-CTA ATT CCC CGT TAC CCG-3′
	F3	5′-CTA ATA CAT GCG CAC ATG C-3′
	BIP	5′-CGG TCT GCG ACG GAT CAT TCC ACG GTA GTC CAA TAC AGT ACC-3′
	FIP	5′-TAT GAA TCA CCT GAG GAC CAG GGC AGT GTT TAT TAG ATA CAG AAC-3′