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A promising diagnostic tool for toxoplasmic encephalitis: tachyzoite/bradyzoite stage-specific RT-PCR

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SUMMARY

Objectives: To determine the diagnostic accuracy, technical benefit, and clinical application of the duplex reverse transcription-PCR (duplex RT-PCR) assay specific to bradyzoite (BAG1) and tachyzoite (SAG1) genes, for diagnosing toxoplasmic encephalitis (TE) in HIV-infected patients, using the US Centers for Disease Control and Prevention (CDC) recommended diagnostic criteria as the reference standard.
Methods: Advanced HIV-infected individuals with central nervous system opportunistic infections were enrolled in a prospective study, performed from July 2007 to January 2009; patients were classified as TE- or non-TE subjects in accordance with the CDC recommended criteria. Blood and cerebrospinal fluid samples were assayed by duplex RT-PCR to detect tachyzoite, bradyzoite, both, or none.
Results: A total of 61 advanced AIDS patients were included in the study, eight with TE and 53 as non-TE subjects. The duplex RT-PCR assay showed high diagnostic accuracy, with 100% specificity and positive predictive value, as well as 87.5% sensitivity. Its efficacy reached 98.3%. This diagnostic method was rapid, needed only moderately skilled technicians, and was four times cheaper than procedures used in the CDC diagnostic recommendations. It worked very well for blood samples, even after drug treatment had been started.
Conclusions: The duplex RT-PCR assay is simple and rapid, and provides high efficacy with lower costs than the reference standard procedures. This is a promising alternative diagnostic tool for TE in HIV/AIDS individuals, especially in resource-limited settings.

1. Introduction

Toxoplasmic encephalitis (TE), a life-threatening disease, is the most common focal brain lesion in HIV/AIDS individuals. Although the incidence decreased from 4.0–20.5 to 3.9 cases/100 patient-years as a result of widely advocated prophylaxis, it has further decreased dramatically to 1.0 case/100 person-years since the introduction of highly active antiretroviral therapy (HAART).[1–5] It has been found that approximately 20–40% of patients who are co-infected with HIV and *Toxoplasma gondii* develop TE, and 97% of these cases are as a result of reactivation of a chronic quiescent infection when CD4 cell counts are <100 cells/mm[3,6–9] In such patients, dormant bradyzoites change into rapidly dividing and active tachyzoites, causing severe tissue destruction and inflammation. Hence, the bradyzoite/tachyzoite stage conversion reaction plays a crucial role as the first step in the event, before severe clinical manifestations subsequently occur.

The definite diagnosis of TE as recommended by the US Centers for Disease Control and Prevention (CDC), requires the identification of the organism in a clinical sample. This necessitates a brain biopsy by stereotactic computed tomography (CT)-guided needle biopsy, and Toxoplasma tachyzoites are then demonstrated by hematoxylin and eosin (H&E) staining, for which the sensitivity may be increased by immunohistochemistry techniques.[10] The patient's condition and the limitation of experienced pathologists often make these processes impracticable. For the presumptive diagnosis of clinically suspected TE cases, the CDC recommended criteria should thus be applied, i.e., (1) recent onset of clinical features consistent with generalized and focal neurological abnormalities; (2) suggestive radiological characteristics on brain imaging of the lesions; and (3) evidence of chronic *T. gondii* infection or successful response to anti-Toxoplasma therapy.[8] By using these presumptive diagnostic criteria, the positive predictive value is achievable in approximately 80%.[11] The CDC criteria are thus widely used as the reference

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standard to diagnose TE. On brain imaging, most TE lesions usually appear as multiple ring-enhanced nodular lesions,[12] however solitary lesions are evident in 14% of cases and mimic primary cerebral lymphoma, which more commonly presents as a single mass than toxoplasmosis.[13] To differentiate between the two, highly sophisticated radiological procedures are needed; however these are costly and their availability is limited, especially in resource-poor settings. Therefore their diagnostic value is decreased.

We recently developed a duplex reverse transcription PCR (duplex RT-PCR) assay based on the stage-specific tachyzoite (SAG1) and bradyzoite (BAG 1) genes for the detection of bradyzoite/tachyzoite stage conversion.[14] It was found that the duplex RT-PCR could distinguish between the chronic and acute stages of Toxoplasma infection. In addition, it was an easy, sensitive, and reproducible method. We therefore conducted a prospective study to evaluate the diagnostic sensitivity and specificity of the developed duplex RT-PCR assay in HIV/AIDS patients with TE. Technical benefits and clinical application were also analyzed and compared to the CDC recommended diagnostic criteria as the reference standard.

2. Materials and methods

2.1. Patients and selection criteria

This prospective study was carried out with the approval of the Ethics Review Committee for Research in Human Subjects, Ministry of Public Health, Thailand (Ref. No. 33/2550). After obtaining informed consent, patients from a tertiary hospital setting, the Bamrasnaradura Infectious Disease Institute, Department of Disease Control, Ministry of Public Health, were enrolled consecutively from July 2007 to January 2009. The selection criteria were: (1) age $\geq$18 years, (2) both sexes, (3) advanced HIV with CD4 counts $<$200 cells/mm^3, and (4) neurological abnormalities at the time of enrollment. Patients were included whether this was a first episode of TE or a relapsed case, and whether or not they had received prophylaxis. Once the study subject was enrolled, 200 μl of whole blood and cerebrospinal fluid (CSF) samples were collected on the day of admission; the samples were mixed with 1 ml TRIzol and then kept at −80 °C until use.

Patients were assigned to the TE group when they were diagnosed as having TE based on the CDC criteria, i.e., (1) signs and symptoms of central nervous system (CNS) dysfunction, (2) suggestive lesions on CT or magnetic resonance imaging (MRI), and (3) seropositivity for *T. gondii* antibody or a response to anti-Toxoplasma therapy. The remaining patients, who had neurological abnormalities but were not suspected of having TE and who were without suggestive brain imaging features, as well as being negative for *T. gondii* antibody, were assigned to the non-TE group.

2.2. CDC recommended diagnostic criteria

To apply the CDC diagnostic criteria for assignment to the TE or non-TE groups, all of the study subjects underwent three examinations. The first was a physical examination, performed by three infectious disease specialists looking for any of the following: (1) generalized cerebral dysfunction, (2) focal signs and symptoms in the CNS, and (3) psychiatric abnormalities. Presenting neurological abnormalities suggestive of TE include headache, fever, alteration of consciousness, confusion, cognitive impairment, hemiparesis, facial nerve palsy, and convulsions. The second examination involved brain imaging studies, CT or MRI, evaluating features typical of TE; this was performed by qualified radiologists. Features include multiple nodular, ring-enhanced lesions with edema and mass effect, as well as lesions occurring in the basal ganglia, thalamus, and corticomedullary junction.[12] The third examination involved the detection of Toxoplasma IgG antibody by a gold standard dye test at the reference laboratory unit, the Department of Protozoology, Faculty of Tropical Medicine, Mahidol University. Pyrimethamine and sulfadiazine plus folinic acid were given to suspected TE cases who showed, at the least, either clinical features or laboratory evidence to strengthen the diagnosis.[10]

2.3. Duplex RT-PCR assay

In addition to the clinical examination, all blood and CSF samples were individually subjected to a duplex RT-PCR assay to determine whether they contained bradyzoites, or tachyzoites, or both stages, or none. This was carried out by a single specialist who was blinded to whether the samples came from TE or non-TE cases. The assay comprised three main steps: nucleic acid extraction, reverse transcription, and duplex polymerase chain reaction.

Total RNA from collected blood and CSF samples was extracted using TRIzol reagent (Invitrogen), as previously prescribed.[14] All samples were individually homogenized in 1 ml TRIzol reagent and the mixture squeezed several times through 20-gauge needles to thoroughly break up the cells, resulting in a higher RNA yield. Once extracted, the RNA concentration was measured, RNA integrity checked, and the sample was then stored at −80 °C until use.

The reverse transcription process was carried out starting with the generation of cDNA from the extracted RNA. A 20-μl reaction mixture containing 0.5 μg oligo(dT)-primers and 200 U of RevertAid™ M-MuL V Reverse Transcriptase (Fermentas) was incubated at 42 °C for 1 h; the reverse transcriptase enzyme was then stopped by increasing the temperature to 70 °C for 10 min. Five microliters of the generated cDNA were used for the subsequent duplex-PCR reactions.

Two sets of primers were used in this study, as described by Mahittikorn and colleagues.[14] First, specific to the tachyzoite (SAG1) gene, the primers 5′-GCT GTA ACA TTG AGC TCC TTG ATT CCT G-3′ and 5′-CCG GAA CAG TAC TGA TTG TTG TCT TGA G-3′ were used to generate a 355-bp product. Second, specific to the bradyzoite (BAG1) gene, the primers 5′-AGT CGA CAA CGG AGC CAT CGT TAT C-3′ and 5′-ACC TTG ATC GTG ACA CGT AGA ACG C-3′ were used to generate a 200-bp product and a 637-bp product, amplified from cDNA and gDNA, respectively.

The optimized PCR conditions were: a final volume of 25 μl, containing 1.0 U Taq DNA polymerase (Fermentas), 2.5 μl of 10 nM Tris–HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 0.18 μM of SAG1 and 0.22 μM BAG1 primers. The temperature profile (MasterCycler-Gradient, Eppendorf) was: a single denaturation step at 95 °C for 1 min; 40 cycles of 95 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min; and a final extension step at 72 °C for 10 min. All experiments were done in triplicate and included negative and positive controls. The PCR products were finally separated by size on a 1.8% agarose gel and documented with G:BOX HR (Syngene).

2.4. Data collection and statistical analysis

The following variables were collected from the patient case record forms (CRF) and recorded: sex, age, duration of HIV infection, the most recent CD4 counts before samples were taken, history of treatment for opportunistic infections (OIs), drugs received including HAART, clinical manifestations, and OIs diagnosed by clinicians at the Bamrasnaradura Infectious Disease Institute according to the standard criteria for each OI. Three CDC recommended criteria were used for the diagnosis of TE.[8] For the results of the duplex RT-PCR, the patient was considered positive when two or more of three tested samples were positive.

Qualitative variables were described using frequencies and percentages, while mean and range were used for quantitative