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Quantitative real-time polymerase chain reaction for the accurate detection of *Toxoplasma gondii* in amniotic fluid

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Abstract

Infection with *Toxoplasma gondii* during pregnancy is often asymptomatic and may cause severe fetal damage. A quantitative TaqMan minor groove binder real-time polymerase chain reaction (PCR) assay was developed for the specific and sensitive detection of the previously described 529-bp repeat element occurring up to 200 to 300 times in *T. gondii* genome. The qualitative and quantitative detection limits determined were 6 and 20 marker copies (1/30 to 1/50 of 1 parasite) per PCR, respectively. In addition to standard PCR cycling conditions, 3 different fast PCR protocols were evaluated to minimize run time. A higher variability but no loss of specificity was observed. For the evaluation of clinical applicability, a total of 135 amniotic fluid samples were analyzed targeting both 529-bp and B1 gene. The sensitivity and specificity were 88.0% and 100.0% for B1, and 100.0% and 98.2% for 529-bp PCR assay (positive predictive value and negative predictive value: 100.0% and 97.4%, and 92.6% and 100.0%, respectively). Our results demonstrated an increased sensitivity of the 529-bp PCR assay even in a faster protocol.

Keywords: *Toxoplasma gondii*; Congenital toxoplasmosis; Fast real-time PCR; Quantitative PCR; B1; 529-bp repeat element

1. Introduction

The parasite *Toxoplasma gondii* infects up to a 3rd of the world's population (Montoya and Liesenfeld, 2004). It is an obligate intracellular protozoan belonging to the phylum Apicomplexa, subclass Coccidia. Infection acquired during pregnancy can lead to maternal–fetal transmission. Connatal toxoplasmosis is associated with a wide spectrum of clinical signs and symptoms, such as retinochoroiditis, intracerebral calcification, hydrocephalus, and mental retardation, which may be present at birth or develop later in life. (Petersen, 2007; Wilson et al., 1980). In immunocompromised individuals, reactivation of latent *Toxoplasma* infection can also be life threatening by causing encephalitis (Israelski and Remington, 1993). Diagnosis of infection with *T. gondii* is performed indirectly by serologic methods, such as enzyme immunoassays, indirect immunofluorescence test, and, more precisely, the Sabin–Feldman dye test (SFT) (Sabin and Feldman, 1948), by antibody detection (mouse inoculation and tissue culture), or directly by detection of specific genomic sequences using polymerase chain reaction (PCR) diagnostic. Serologic tests are of limited effect in detecting acute infection because peak concentrations of specific immunoglobulins are found weeks after parasitemia has subsided. Furthermore, reactivation of the disorder in infected individuals and immunocompromised patients is rarely accompanied by significant titer rise. Mouse inoculation or tissue cultures for direct identification of *T. gondii* are time consuming and impractical and show only moderate sensitivity.

Consequently, there was a great focus on establishing reliable PCR assays for direct detection of *T. gondii* genomic

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sequences in biologic samples. Maximal assay sensitivity is essential in amniotic fluid samples after amniocentesis because of further prenatal management. In recent years, a variety of specific gene targets has been described such as single-copy genes (e.g., SAG1, SAG2, SAG3, and GRA4) or multicopy gene sequences (e.g., B1). Quality and sensitivity of a given PCR is highly dependent on the target sequence used (Switaj et al., 2005). A recently described 529-bp repetitive sequence of unknown function, which occurs in 200 to 300 copies per genome, seems to be a promising target for sensitive diagnostic PCR assays (Edvinsson et al., 2006; Homan et al., 2000). In addition, substantial progress has been made in the field of quantitative real-time PCR (qPCR) techniques with the development of fast cycling PCR devices and specific reagents, which provide a powerful tool to perform PCR with highly accurate results.

The present study investigated a real-time TaqMan minor groove binder (MGB) qPCR assay for the quantitative detection of *T. gondii* that is both highly sensitive and reliable. We analyzed samples from amniotic fluid to verify the clinical applicability of the newly developed qPCR assay. In addition to universal cycling PCR conditions, we tested 3 different less time-consuming real-time qPCR protocols to minimize total PCR run time and to evaluate the optimal time–efficacy ratio. Specificity, sensitivity, and the linear range of quantification of different time protocols were compared.

2. Materials and methods

2.1. Patients, clinical isolates, and DNA extraction

A total of 135 amniotic fluid samples from women with primary infection during pregnancy were retrospectively investigated in this study by the toxoplasmosis reference laboratory in the Department of Pediatrics and Adolescent Medicine, Medical University of Vienna, Austria. The acute *Toxoplasma* infection has been diagnosed routinely by the nationwide prenatal serologic screening in Austria. Women with "definite" and "probable" infection status were included according to the Lebech criteria (Lebech et al., 1996). Serologic diagnosis of primary maternal infection during pregnancy was "definite" in case of seroconversion (both samples taken after conception) or confirmed connatal infection in offspring. "Probable" infection was defined as significant rise of immunoglobulin G (IgG) titers or high IgG titers plus the presence of IgM and low avidity index. For confirmation, the SFT was applied (World Health Organization gold standard test for the serodiagnosis of *Toxoplasma* infection) (Reiter-Owona et al., 1999; Sabin and Feldman, 1948). The dye test enables antibody detection at very low concentrations and offers discrimination between acute and preconceptional infection superior to commercial test systems (Lebech et al., 1996). *Toxoplasma* IgM antibodies were determined by the immunosorbent agglutination assay (ISAGA; Bio Mérieux, France). The ISAGA IgM is suitable for the diagnosis and screening of acute *Toxoplasma* infection in pregnant women (Carlier et al., 1980; Naot and Remington, 1980). Furthermore, the anti-*Toxoplasma* IgG binding capacity (avidity index) (Labsystems, Finland) was additionally applied to assist in discriminating acute from latent phases of *T. gondii* infection (Flori et al., 2008; Jenum et al., 1997) and to further determine the time frame in which infection may have occurred within a 2-month window (Beghetto et al., 2003).

For routine PCR diagnostics, amniotic fluid of these women at risk were analyzed for B1 gene target (Gratzl et al., 1998). In addition, for the development of the new qPCR assay, DNA from peritoneal lavage fluid of mice infected with *T. gondii* was extracted using QIAamp DNA Mini Kit (QIAGEN, Valencia, CA) according to the manufactures protocol and was analyzed immediately or stored at −20 °C.

2.2. Assay development

The published DNA gene sequences, AF146527 and AF487550, were aligned with Vector NTI® 10.0 software (InforMax, Oxford, United Kingdom). Both primers and the TaqMan MGB probe were designed from the derived consensus sequence using Primer Express® software (Applied Biosystems, Foster City, CA). The designed primers amplify an 81-bp fragment of the 529-bp repeat element (RE) of the *T. gondii* genome. The primers were designated ToxoRE_f (5′-CAC AGA AGG GAC AGA AGT CGA A-3′), ToxoRE_r (5′-CAG TCC TGA TAT CTC TCC TCC AAG A-3′), and the probe ToxoRE_p (FAM-5′-CTA CAG ACG CGA TGC C-3′-NFQ–MGB; FAM, 6-carboxy-fluorescein; NFQ–MGB, nonfluorescent quencher plus attached MGB). Polymerase chain reaction was performed on a Mastercycler® ep realplex (Eppendorf, Hamburg, Germany) using 4 different time protocols (Table 1).

2.2.1. Quantitative real-time PCR

The optimized reaction mixture composition was 2.5 μL of sample DNA dilution, 200 nmol/L ToxoRE_f, 200 nmol/L ToxoRE_r, 100 nmol/L ToxoRE_p, 12.5 μL of RealMasterMix™ Probe Supermix (Eppendorf), and PCR grade water to a final volume of 25 μL. SYBR green PCR was used in assay optimization for temperature gradient and melting curve analysis: reaction mixture composition was 2.5 μL of sample DNA dilution, 200 nmol/L ToxoRE_f, 200 nmol/L ToxoRE_r, 12.5 μL of RealMasterMix™ SYBR Supermix (Eppendorf), and PCR grade water to a final volume of 25 μL. All reactions were performed in triplicates in 3 independent experiments. A SYBR green assay was used to optimize annealing temperature of the primer pair by a temperature gradient. Melting curve analysis was performed to verify the correct product size and did not result in formation of side products or primer dimers. Optimized primer and probe concentrations were determined by a matrix approach (Applied Biosystems, 2005). Polymerase chain reaction was performed from DNAs of 5 samples. All PCR products were checked by agarose gel electrophoresis