Preliminary evaluation of one conventional nested and two real-time PCR assays for the detection of *Toxoplasma gondii* in immunocompromised patients

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Toxoplasma reactivation is a serious complication in patients receiving allogenic stem cell transplantation. Real-time PCR assays allow a rapid diagnosis of toxoplasma infection; however, no comparative data are available on the performance of real-time PCR protocols under routine conditions. Therefore, the aim of this study was to amplify *Toxoplasma gondii* DNA from routine samples of allogenic stem cell recipients using two real-time PCR assays on a LightCycler, and using conventional nested PCR. Conventional nested PCR revealed *T. gondii* DNA in 16 samples. Only 12 of the 16 samples yielded a positive result in both real-time PCRs. The accuracy of the conventional PCR results was demonstrated by direct sequencing. Amplification and detection of the amplicon was completed in only 1 h using the real-time PCR assays. Thus, real-time PCR substantially accelerates the detection of *T. gondii* DNA in the majority of positive specimens; however, conventional nested PCR is required for detection of *T. gondii* DNA in some samples.

Introduction

Toxoplasma reactivation represents a rare but potentially life-threatening complication in bone marrow transplant recipients and other immunocompromised patients (Mele et al., 2002). Rapid diagnosis of toxoplasmosis is crucial in patients with impaired immune function, as early treatment may improve the clinical outcome. Classical serological diagnosis of toxoplasmosis relies on the detection of anti-toxoplasma immunoglobulin, but serology may be unreliable in immunodeficient individuals who fail to produce significant titres of specific antibodies. Over the past decade, PCR assays have allowed the sensitive detection of *Toxoplasma gondii* DNA in clinical specimens. Molecular diagnosis of toxoplasmosis can be accelerated by performing real-time PCR protocols (Bell & Ranford-Cartwright, 2002). These newly developed real-time assays allow amplification and simultaneous detection of DNA in 1 h. However, no comparative data are available on the performance of conventional and real-time PCR assays for the detection of *T. gondii* DNA under routine conditions. Therefore, the aim of this study was to amplify *T. gondii* DNA from routine samples of allogenic transplant recipients by applying two real-time PCR protocols on a LightCycler (LC), and a conventional nested PCR.

Abbreviation: LC, LightCycler.

Methods

Over a period of 18 months, 1000 routine specimens (83 % EDTA blood, 4 % bronchoalveolar fluid, 6 % cerebrospinal fluid, 2 % pleural effusion, 2 % bone marrow aspirate, 1 % lymph node biopsies and 2 % tissue biopsies) were collected from allogenic stem cell recipients. Extracted DNA was amplified with a conventional nested PCR targeting the *T. gondii* B1 gene (Roth et al., 1992). DNA preparations that were found to be positive in the conventional nested PCR were amplified with two LC real-time PCR protocols targeting the *T. gondii* B1 gene (Costa et al., 2000) and the *T. gondii* 529 bp repeat region (Reischl et al., 2003). In the conventional nested PCR (Roth et al., 1992) the outer primers P3 (5′-CCTCAAGCGGTATTGTCG-3′) and P7b (5′-CTTTTTAAA GCGTGCCTGTCG-3′) and the inner primers P7 (5′-TAAAGCGTG TCAGTTCACT-3′) and P8 (5′-GGAATGCTTCGGGTCACTA-3′) were used. The amplification mixture (25 μl) contained 200 μM dNTPs (Promega), 3 mM MgCl2, Taq DNA polymerase (Roche Diagnostics) and 0·2 μM of each primer. The amplification mixture contained 5 μl extracted DNA in the first round of the nested PCR, and 0·5 μl of the PCR product was amplified in the second round for 40 cycles (20 s denaturation at 94 °C, 20 s annealing at 55 °C and 20 s extension at 74 °C). The amplicon (190 bp) was detected by agarose gel electrophoresis and ethidium bromide staining.

For the LC PCR targeting the *T. gondii* B1 gene (Costa et al., 2000) primers B1 sense (5′-GGAGGACTGCAACCCTGGTGTCG-3′) and B1 antisense (5′-TTGTTTCCACCGGACCTTTAGCA-3′) and probes Toxi1 (5′-CGGAAAATAGAAAGCCATGAGGCACTCC–fluorescein-3′) and Tox2 (5′-Red 640–ACGGCCGATAGCACCCTGAAGGAGAT–Ph-3′) were used. The reaction mixture (20 μl; Master Hybridization Probes kit; Roche Diagnostics) contained 0·5 μM of each primer, 0·2 μM of each probe, 5 mM MgCl2 and 5 μl extracted DNA.
Amplification was performed in a LightCycler (Roche Diagnostics) for 50 cycles: 5 s denaturation at 95 °C, 10 s annealing at 60 °C and 15 s extension at 72 °C, with an overall ramp rate of 20 °Cs⁻¹. For the LC PCR targeting the T. gondii 529 bp repeat region (Reischl et al., 2003) primers Tox9 (5’-AGGAGAGATATCAGGACTGTAG-3’) and 10as (5’-GGGTCGTCTCGTCTGGATCG-3’) and probes Tox3 (5’-GAGTCGGAGAGGAGAAGATGTT–fluorescein-3’) and Tox4 (5’-Red 640–CCGGGTTCGTTTCTTCTCTC-Ph–3’) were used. The reaction mixture (20 μl) contained 0.5 μM of each primer, 0.2 μM each probe, 3 mM MgCl₂ and 5 μl extracted DNA. Amplification was performed for 50 cycles: 10 s denaturation at 95 °C, 10 s annealing at 60 °C and 20 s extension at 72 °C, with an overall ramp rate of 20 °Cs⁻¹.

To evaluate the sensitivity of the conventional nested PCR assay, the PCR product of a sample containing T. gondii DNA was sequenced and cloned into a plasmid vector (TOPO TA cloning kit; Invitrogen). Toxoplasma-free EDTA blood was spiked with the plasmid. Anti-toxoplasma antibodies were detected in serum by immunosorbent agglutination assay (ISAGA; Toxo Multi Tool; Innogenetics), immunofluorescence test (IFT; Toxo-Spot IP; bioMérieux) and ELISA (IgG/IgM kit; VirionSerion).

All PCR assays were performed according to quality standards for nucleic acid amplification techniques published by the German Society for Hygiene and Microbiology (Roth et al., 2001). This included physical separation of working areas for the preparation of amplification reaction mixtures, for specimen preparation, for preparation of positive controls and for the amplification and detection of nucleic acids. In all rooms the equipment was labelled to indicate clearly the working area to which it belonged. Only plugged pipette tips were used to prevent contamination by aerosols. All areas were equipped with flow benches. Moreover, the risk of contamination was reduced by a unidirectional workflow ensuring that pre- and post-amplification procedures were not performed alternately. To inactivate nucleic acids, working surfaces were cleaned with sodium hypochlorite solutions and the risk of contamination was reduced by a physical separation of working areas for the preparation of amplification reaction mixtures, for specimen preparation, for preparation of positive controls and for the amplification and detection of nucleic acids. In all rooms the equipment was labelled to indicate clearly the working area to which it belonged. Only plugged pipette tips were used to prevent contamination by aerosols. All areas were equipped with flow benches. Moreover, the risk of contamination was reduced by a unidirectional workflow ensuring that pre- and post-amplification procedures were not performed alternately. To inactivate nucleic acids, working surfaces were cleaned with sodium hypochlorite solutions and flow benches were irradiated with UV light. The following controls were performed in each run. As a negative control for specimen processing, DNA was extracted from a sample that did not contain the target sequence and the DNA extract was subjected to amplification and detection. As a negative control for amplification, reaction mixtures containing reagents only, but not nucleic acids, were amplified (in the case of the nested PCR, in both rounds). As a positive control for extraction and amplification, DNA of a sample containing the target sequence in low copy number was extracted and amplified. Finally, as a control for inhibition, a fragment of the human β-actin gene was amplified in each sample. Samples with discrepant results in the three PCR assays were analysed in duplicate.

Results and Discussion

Over the 18-month study, 16 samples obtained from eight allogenic transplant recipients were shown to contain T. gondii DNA by the nested PCR protocol (Table 1). Twelve of these samples yielded a positive result following amplification with each of the three PCR protocols. The remaining four samples were negative in at least one of the real-time PCRs. PCR products from two discordant samples were sequenced, which confirmed the accuracy of the conventional PCR. PCR inhibitors were not detected when a fragment of the human β-actin gene was amplified (Murray et al., 1990). To investigate the analytical sensitivity of the nested PCR assay, toxoplasma-free EDTA blood was spiked with a plasmid containing the PCR positive control at different concentrations (10⁷ to 10⁹ copies ml⁻¹). DNA was extracted and amplified. Amplicon analysis (190 bp) on an agarose gel indicated a sensitivity of the nested PCR in the range of 1–10 copies (ml blood)⁻¹. Amplification of DNA from a diluted T. gondii-positive blood sample using each of the three PCR assays indicated similar sensitivities of the

Table 1. Detection of T. gondii DNA in patient samples with real-time and conventional nested PCR assays

<table>
<thead>
<tr>
<th>Patient</th>
<th>Specimen</th>
<th>LightCycler repetitive DNA fragment</th>
<th>LightCycler B1 gene</th>
<th>Nested PCR B1 gene</th>
<th>β-actin PCR</th>
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<tbody>
<tr>
<td>1</td>
<td>EDTA blood</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>BAL</td>
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<td>2</td>
<td>EDTA blood</td>
<td>–</td>
<td>–</td>
<td>+*</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>EDTA blood</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<td>+</td>
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<tr>
<td></td>
<td>EDTA blood</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>Lung tissue</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>CSF</td>
<td>+</td>
<td>–</td>
<td>+*</td>
<td>+</td>
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</table>

*amplicon specificity demonstrated by sequencing.
three assays (data not shown). Over the entire study, 1000 routine DNA extracts were amplified using the nested PCR, in which no non-specific amplicons were detected. Amplification of DNA extracted from 20 randomly selected routine EDTA-blood samples using either of the two LC assays did not generate a fluorescence signal. Amplification and detection of the amplicon was completed within 1 h using the real-time PCR assays, compared to 3-5 h using the conventional nested PCR plus subsequent gel electrophoretic detection of the amplicon. The clinical data of three patients whose specimens showed discrepant PCR results are summarized in Table 2.

Our observation that the LC assays failed to detect \textit{T. gondii} DNA in some specimens confirms the results of a recent study on the performance of LC technology in human samples (Teo et al., 2002). Several parameters were identified to have an adverse effect in the LC assays, including abstraction of PCR reagents on glass capillaries, primer-dimer formation and non-specific product generation (Teo et al., 2002). Also, the amount of human chromosomal DNA in the specimen was shown to influence the amplification efficiency of the target DNA. In our study, the LC assay targeting the \textit{T. gondii} 529 bp repeat region (200–300 copies per parasite) (Homan et al., 2000) revealed false-negative results in only two samples, whereas the LC assay targeting the \textit{T. gondii} B1 gene (35 copies per parasite) (Burg et al., 1989) revealed false-negative results in four samples. Therefore, it appears likely that the discrepancies in PCR results are partly caused by different copy numbers of the target sequences. This is in agreement with a recent study on \textit{T. gondii}-positive amniotic fluids, which showed that amplification of a sequence within the \textit{T. gondii} 529 bp repeat region is at least 10 times more sensitive than targeting the \textit{T. gondii} B1 gene (Reischl et al., 2003). The three patients for whom the conventional PCR and the LC assays gave discrepant results had non-specific symptoms compatible with toxoplasma reactivation.

In conclusion, real-time PCR allows rapid identification of \textit{T. gondii} DNA in patient samples. However, discrepancies between real-time PCR and conventional nested PCR results occur in a minority of routine samples. Therefore, real-time and conventional PCR protocols for \textit{T. gondii} should be optimized and carefully evaluated in patient samples before they are implemented as routine methods.

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**References**


