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Real-Time PCR for Diagnosis and Follow-Up of Toxoplasma Reactivation after Allogeneic Stem Cell Transplantation Using Fluorescence Resonance Energy Transfer Hybridization Probes

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Toxoplasma reactivation is a life-threatening complication of allogeneic stem cell transplantation. A poor prognosis is probably linked to a difficult diagnosis, based on the detection of evidence of parasites in tissue. We developed a real-time PCR test using fluorescence resonance energy transfer hybridization probes to detect and quantify *Toxoplasma gondii* DNA in serum. This PCR test gave reproducible quantitative results over a dynamic range of from 0.75×10^6 to 0.75 parasites per PCR mixture. Serial samples from four patients with toxoplasma reactivation were evaluated. Three patients had several consecutive PCR-positive samples which corresponded to ≤0.75 parasites. These three patients became PCR negative during trimethoprim-sulfamethoxazole therapy but never developed clinically apparent toxoplasmosis. In contrast, one patient had an increasing PCR signal, from 1 to 396 parasites in 12 days, and developed cerebral symptoms. The parasite count decreased to 5 parasites in 3 days after pyrimethamine-clindamycin treatment. Real-time quantitative PCR is useful for diagnosis and follow-up of toxoplasma reactivation.

Toxoplasmosis is a worldwide infectious disease caused by the protozoan *Toxoplasma gondii*. In humans, the parasite exists in two stages: the tachyzoite stage and the bradyzoite stage (7). Tachyzoites are responsible for the acute infection, and their differentiation into bradyzoites correlates with the onset of protective immunity. Bradyzoites are located within cysts, which are believed to persist for life. These quiescent stages are able to reconvert into active tachyzoites when the immune system fails. This reactivation is thought to be the main source of cerebral or disseminated toxoplasmosis in immunocompromised individuals.

In allogeneic stem cell transplant recipients, the infection is usually life-threatening and is often diagnosed postmortem (6, 13, 15), probably because the diagnosis is difficult, being based on the detection of evidence of parasites in tissue. Serological tests are of little help in these patients and can be used to say only that the patient, if positive, is at risk of reactivation as he or she harbors some toxoplasma cysts. Therefore, empiric treatment is often initiated on the basis of clinical suspicion and cerebral computed tomography (CT) scan abnormalities. This treatment, based on pyrimethamine and sulfadiazine or clindamycin, can have multiple side effects. Efficacy cannot be clinically assessed for several days. The risk is then of missing other diagnoses.

A positive PCR result with blood is expected to improve the prognosis because treatment can be initiated when the parasite burden is still low and allows avoidance of invasive procedures such as brain biopsy (2, 9, 10–12, 14). Quantitative PCR gives precious clues that support the choice of specific treatments for patients harboring several pathogens. Therefore, we have developed a quantitative PCR for the diagnosis of *T. gondii* infection based on the LightCycler technology (16). This technology relies on hybridization of amplicon-specific probes with adjacent fluorophores capable of fluorescence resonance energy transfer when they bind to the target sequence. This technology provides a real-time measure of the amplification product. We subsequently analyzed serum samples from allogeneic stem cell transplant recipients.

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MATERIALS AND METHODS

Development of LC-PCR. The LightCycler PCR (LC-PCR) test was targeted at the *T. gondii* B1 gene (4). The B1 gene amplicon comprises a 126-bp fragment. The primers used for amplification were the 23-mer 5′-GGAGGACTGGCAA CCTGGTGTCG-3′ (sense) and the 25-mer 5′-TTGTTTCACCCGGACCGTT TAGCAG-3′ (antisense). Two hybridization probes (Tib MolBiol, Berlin, Germany) were designed that recognize adjacent internal sequences within the target B1 gene sequence. One (5′-ACGGGCGAGTAGCACCTGAGGAGAT-3′) was labeled at the 5′ end with LC-Red 640 and was phosphorylated at the 3′ end to prevent probe elongation by the *Taq* DNA polymerase. The other one (5′-CG GAAATAGAAAGCCATGAGGCACTCC-3′) was labeled at the 3′ end with fluorescein.

T. gondii DNA (RH strain) was extracted by a conventional phenol-chloroform procedure from purified parasites obtained from ascitic fluids of inoculated mice and was quantified by UV spectrophotometry. Because some mouse DNA can contaminate the *T. gondii* DNA, we measured the mouse DNA in the total DNA extracted using a real-time quantitative PCR assay that we have developed for other purposes. This TaqMan PCR assay is targeted at a single copy of the mouse GALT gene (GenBank access number M96265). Therefore, we extracted the mouse DNA from the total DNA for further measurements. We estimated that one parasite corresponded to 0.1 pg of DNA, assuming that the haploid *T. gondii* genome size is 80 Mb (5) and that the B1 gene, the target for the LC-PCR test, is repeated 35 times (4). After extraction, one 10-fold serial dilution of *T. gondii* DNA was made, with parasite concentrations ranging from 0.75×10^6 to 0.75 parasites per 10 μl. This series of 10-fold dilutions was included in each amplification run.

LC-PCRs were set up in a final volume of 20 μl with the DNA Master Hybridization Probes Kit (Roche Molecular Biochemicals, Meylan, France), each primer at a concentration of 0.5 μM, each probe at a concentration of 0.25 μM, and 10 μl of extracted DNA sample. A hot-start procedure was systematically used by the addition of an anti-*Taq* DNA polymerase antibody (Clontech, Paris, France) to the amplification reaction mixture. Carryover was prevented by using heat-labile uracil-DNA-glycosylase (UNG; Roche Molecular Biochemicals, Meylan, France). The reaction mixture was initially incubated for 1 min at room temperature to allow the UNG to act. This incubation was followed by a 2-min step at 95°C to denature the DNA, to inactivate the UNG, and to free the *Taq* DNA polymerase from the anti-*Taq* DNA polymerase antibody. Amplifica-

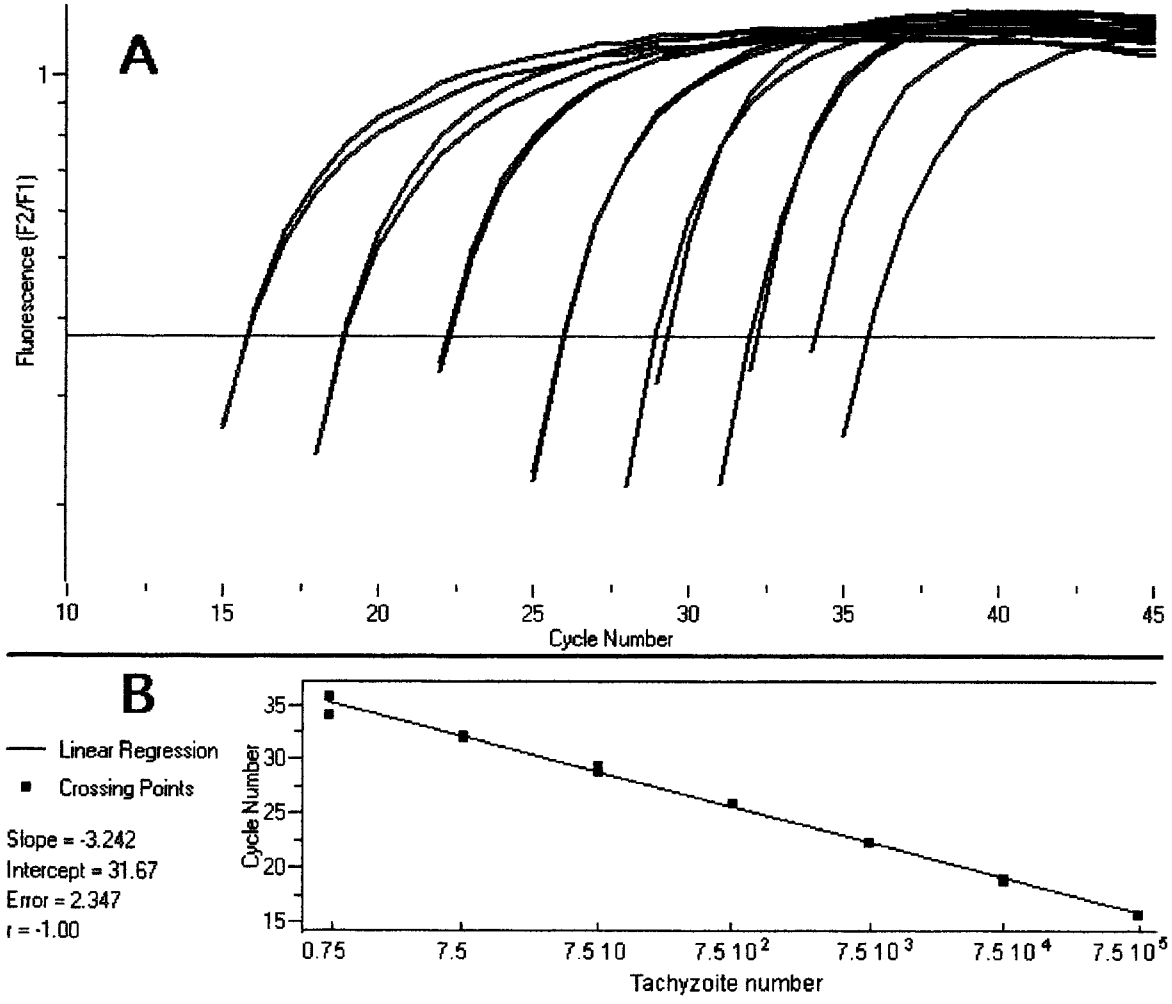

FIG. 1. Real-time quantitative LC-PCR test using fluorescence energy transfer. (A) Duplicate amplification plots obtained for *T. gondii* DNA dilutions from 0.75×10^6 (right) to 0.75 parasite in 10 μl (left). Each slope corresponds to a particular input target quantity. (B) Plot of the duplicate Cp against the input target quantity (common log scale) showing the linearity of the results. The computer-calculated correlation coefficient is 1.

tion was performed for 50 cycles of denaturation (95°C for 5 s; ramp rate, 20°C/s), annealing (60°C for 10 s; ramp rate, 20°C/s), and extension (72°C for 15 s; ramp rate, 20°C/s).

The amplification was carried out in a LightCycler Instrument (Roche Molecular Biochemicals), and a single fluorescence reading for each sample was taken at the annealing step. Quantitative results were expressed by determination of the threshold of detection, or the crossing point (Cp), which marked the cycle when the fluorescence of a given sample significantly exceeded the baseline signal. They were expressed as a fractional cycle number. Then, the Cp's were plotted against the known concentration of the parasite to obtain the standard curve. The parasite count for a given clinical sample was calculated by interpolation from this standard curve.

Patients. Several serum samples were obtained from four allogeneic stem cell recipients known to be toxoplasma PCR positive by our routine test (3, 8). Serum samples were collected in Vacutainer SST tubes (Becton Dickinson) at least once a week, frozen within 4 h of receipt in the laboratory, and stored at −20°C. After thawing, DNA from 200 μl of serum was extracted with the High Pure PCR Template Preparation kit (Roche Molecular Biochemicals) according to the manufacturer's recommendations. PCR amplification was performed with 10 μl of the eluate. The PCR results were secondarily compared to the clinical data in the medical records.

RESULTS

Development of LC-PCR. Figure 1 shows the amplification of the purified *T. gondii* DNA in 10-fold serial dilutions with fluorescence plotted against cycle number. The higher the initial copy number, the earlier the fluorescent signal appears. As the initial template concentration decreases, the curves are shifted to the right. The sensitivity of the system is such that a single parasite can be distinguished from the background noise. The intra-assay coefficient of variation was below 1% for the high-concentration DNA (Cp, 24.24 ± 0.07) and 1.6% for the low-concentration DNA (Cp, 33.74 ± 0.55) by using commercial *T. gondii* DNA (Advanced Biotechnologies Inc., Columbia, Md.) tested 10 times at two different concentrations in the same run. Reproducibility was estimated by testing the 10-fold dilution 10 times in independent runs. The interassay coefficients of variation were 6.4, 12.3, 13.8, and 36% for samples with 10^3, 10^2, 10, and 1 parasite(s), respectively.

Quantitative analysis of clinical samples. Real-time quantitative analysis by the LC-PCR was carried out with DNA extracted from serum samples from four patients (Table 1). All samples positive by our routine PCR test (3, 8) were also positive by the quantitative PCR test. Moreover, the serum samples were also proved to be free of PCR inhibitors since the internal control for our competitive PCR test was correctly amplified each time (3, 8).

Patients 613, 685, and 696 did not develop full-blown toxo-

plasmosis (Table 1). The first and last samples were PCR negative. These patients were febrile at the time of detection of the first PCR-positive sample but chest X ray, fundoscopic examination, and cerebral CT scan were normal. The quantified PCR signal corresponded to less than 0.75 parasite. For these three patients the positive PCR signal disappeared in 22, 9, and 11 days, respectively, after trimethoprim-sulfamethoxazole was given orally at doses of 160 mg of trimethoprim and 800 mg of sulfamethoxazole three times weekly.

In contrast, patient 708 (Table 1) had encephalitic symptoms compatible with toxoplasmosis, and this raised several therapeutic issues. Because of known allergy to sulfonamide, aerosolized pentamidine instead of trimethoprim-sulfamethoxazole was given for *Pneumocystis carinii* prophylaxis. On day 61, the patient was febrile and agitated. The fever was not documented, and he was empirically given antibiotics. A cerebral CT scan was normal, and the behavior troubles were attributed to steroid therapy (2 mg/kg of body weight/day). A couple of days later, abdominal pain developed and ultrasonic examination showed signs of cholecystitis. A cholecystectomy was performed on day 70. After surgery, the patient did not recover consciousness. A new cerebral CT scan showed numerous cerebral lesions compatible with herpes, toxoplasma encephalitis, or metabolite disorders. Pyrimethamine (100 mg/day) and clindamycin (1.2 g/day) were given on day 75. The patient died on day 91 without recovery of consciousness. An autopsy was not performed.

The retrospective analysis of nine serum specimens by LC-PCR showed that the first PCR-positive serum was retrieved on day 61 after transplantation, when psychiatric troubles and fever occurred. LC-PCR showed that the number of parasites increased from 1 per 200 μl of serum on day 61, to 4 per 200 μl of serum on day 69, and to 397 per 200 μl of serum on day 75, when antitoxoplasma therapy was started. Then, the parasite number declined sharply in 3 days to six parasites per 200 μl of serum and then remained positive below the last point of the standard curve from day 82 until death. On day 75 only the cerebrospinal fluid (CSF) was available, and the results of cytologic and biochemical analyses of the CSF were normal. The number of parasites was 4,240 per 200 μl of CSF, 10 times higher than the number in serum. In addition, a PCR with CSF for detection of herpes simplex virus was negative.

DISCUSSION

The real-time quantitative LC-PCR system that we have developed for determination of the concentration of *T. gondii* DNA in serum gives reproducible quantitative results over a wide dynamic range and has a fast turnaround time of less than 2 h, including the DNA extraction steps. The risk of a false-positive result due to contamination with previously amplified products, the main concern in PCR, is dramatically reduced since there is no need to open the tube at the end of the amplification. Furthermore, the enzymatic method of prevention of contamination, based on the use of dUTP instead of dTTP in the reagent mixture and the systematic use of the enzyme UNG, increases the safety of the test.

The most important observation in this study is that the parasite count could be determined and correlated with the clinical symptoms and treatment. In the three PCR-positive patients who did not develop full-blown toxoplasmosis, the parasite count was always low and PCR became negative when trimethoprim-sulfamethoxazole was given. This finding might be frequent in allogeneic bone marrow transplant recipients. Indeed, in a recent prospective study we observed that 12.5% (3 of 24) of *Toxoplasma*-seropositive allogeneic stem cell trans-

TABLE 1. Main features, pretransplant toxoplasma serological status of the recipient and donor, antitoxoplasma treatment, and outcomes for four allogeneic stem cell transplant recipients with toxoplasma PCR-positive blood samples studied by quantitative LC-PCR test[a]

Patient no.	Sex, age (yr), underlying disease	Stem cell transplant type	Toxoplasma serological status	Graft-versus-host disease	Main symptom(s)	No. of samples tested (no. of PCR-positive samples)	Time (day) of first and last PCR-positive blood samples	Antitoxoplasma drug treatment	Outcome (cause of death)
613	M, 47, aplastic anemia	Familial mismatch	R+, D+	Acute II	Fever	6 (4)	21–41	TMP-SMZ on days 19–37; Pyr-Clin from day 38 until death	Death on day 180 (bacterial sepsis)
685	F, 11, sickle-cell disease	HLA-identical sibling	R+, D−	None	Fever	9 (7)	12–38	TMP-SMZ on day 29, continued	Alive
696	M, 40, lymphoma	HLA-identical sibling	R+, D+	Acute I	Fever, enlarged lymph nodes	6 (4)	34–45	TMP-SMZ on day 34, continued	Alive
708	M, 47, lymphoma	Unrelated	R+, D−	Acute III	Fever, behavior trouble, diffuse lesions on cerebral CT scan	9 (8)	61[b] until death	Aerosolized pentamidine on days 50–70; Pyr-Clin on day 75 until death	Death on day 109 (coma of uncertain cause)

[a] Abbreviations and symbols: AML, acute myeloid leukemia; TMP-SMZ, trimethoprim-sulfamethoxazole; Pyr-Clin, pyrimethamine-clindamycin; R, recipient; D, donor; +, positive; −, negative.
[b] PCR-positive CSF on day 75.

plant recipients had transient PCR-positive test results but did not develop full-blown toxoplasmosis (1). In contrast, for the patient in the present study who developed cerebral symptoms, the parasite count increased until antitoxoplasma therapy was started. Then, the parasite count decreased sharply. Because no autopsy was performed, the cause of death was not ascertained, but we can conclude that the antitoxoplasma therapy was efficient in reducing the parasite load.

The real-time quantitative LC-PCR assay developed in this study can be used not only to detect the presence of *T. gondii* DNA but also to provide precise evaluations of the parasite load in immunocompromised patients. This PCR test should be useful for the monitoring of treatment efficacy and should help provide an understanding of the pathogenesis of toxoplasma reactivation.

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