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Comparison of PCR–Enzyme-Linked Immunosorbent Assay and Real-Time PCR Assay for Diagnosis of an Unusual Case of Cerebral Toxoplasmosis in a Stem Cell Transplant Recipient

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A PCR–enzyme-linked immunosorbent assay and a real-time PCR assay were compared for diagnosis and follow-up of cerebral toxoplasmosis in a stem cell transplant recipient. The sensitivity of detection was similar for both assays but was higher when the assays were performed on buffy coat rather than on whole blood or serum.

CASE REPORT

A 90-kg 43-year-old male patient was diagnosed with an imatinib- and interferon-resistant chronic myeloid leukemia in June 1999. On 31 January 2002, an unrelated cord blood cell transplantation was performed. The patient's serology for toxoplasmosis was positive before transplantation. The conditioning regimen included cyclophosphamide (120 mg/kg of body weight), total body irradiation (12 Gy), and a total dose of 10 mg of antithymocyte immunoglobulins per kg. The initial graft-versus-host disease (GVHD) prophylaxis regimen consisted of cyclosporine and methylprednisolone (1 mg/kg/day). The patient remained afebrile while receiving intravenous tazocillin, amikacin, acyclovir, and fluconazole and oral amphotericin B. Acute digestive GVHD occurred on day 34 after transplantation and was successfully treated with mycophenolate mofetil, methylprednisolone (2 mg/kg/day), and cyclosporine (3 mg/kg/day).

On days 40, 41, and 42 posttransplantation, the patient presented low-grade fever (38°C), progressive headache, and photophobia. No meningeal signs or seizures were detected. On day 43 posttransplantation, discrete right hemiparesis was noted. Complete blood counts showed 9.9 g of hemoglobin/dl, 1.6×10^9 leukocytes/liter, 1.17×10^9 neutrophils/liter, 0.2×10^9 lymphocytes/liter with no detectable CD4 or CD8 lymphocytes, and 0.2×10^9 monocytes/liter. A computed tomography (CT) scan revealed several peripheral non-ring-enhanced hypodense lesions in the brain, highly suggestive of cerebral aspergillosis, although a chest CT scan performed on the same day was normal. Magnetic resonance imaging confirmed the presence of multiple non-ring-enhanced low-signal zones, spread through the hemispheres and the posterior fossa. No involvement of the basal ganglia or mass lesions or perilesional edema were observed. In the absence of well-documented diagnosis, an empirical treatment combining broad-spectrum antibiotics, voriconazole plus caspofungin and sulfadiazine (6 g/day) plus pyrimethamine (75 mg/day), was initiated on day 43. Forty-eight hours after the initiation of this treatment, the patient became afebrile; photophobia and headache almost disappeared. Viral (cytomegalovirus, herpes simplex virus types 1 and 2, varicella-zoster virus, Epstein-Barr virus, human herpesvirus 6 and 8, and JC virus), *Cryptococcus neoformans*, bacterial, and mycobacterial infections were ruled out in blood and cerebrospinal fluid (CSF) specimens. Conventional PCR–enzyme-linked immunosorbent assay (ELISA) for *Toxoplasma gondii* was positive with both blood and CSF samples drawn on day 43. The diagnosis of cerebral toxoplasmosis was considered highly probable, and anti-*Toxoplasma* treatment was continued at the same doses for 4 months. Thereafter, the patient received sulfadiazine (4 g/day) and pyrimethamine (50 mg/day) as continuous maintenance treatment. Sequential cerebral imaging showed progressive improvement with a scar appearance on day 167. The patient died on day 228 posttransplantation from uncontrolled GVHD and posttransplantation Epstein-Barr virus-induced lymphoma without evidence of recurrent toxoplasmosis. No autopsy was performed.

Sequential follow-up on blood, serum, and CSF samples. The availability of serial blood, serum, and CSF specimens gave us the opportunity to compare the performances of conventional PCR-ELISA and real-time quantitative PCR for diagnosis and treatment follow-up. Nineteen venous blood samples collected in EDTA from day 36 to day 225, two CSF samples collected on days 43 and 69, and 24 frozen sera collected between September 2001 (i.e., 4 months before stem cell transplant [SCT]) and day 200 were retrospectively tested in parallel by PCR-ELISA and real-time quantitative PCR.

Buffy coats were obtained from 7 ml of blood drawn in EDTA by using Histopaque (Sigma Aldrich, Saint Quentin Fallavier, France). DNA extraction was then performed on the leukocyte layer by using a QIAamp DNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Similarly, DNA extraction was performed on 200 μl of whole blood drawn in EDTA, serum, or CSF by using a

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QIAamp DNA minikit. Extracted DNA was resuspended in 100 μl of 10 mM Tris buffer (pH 9.0).

For PCR-ELISA, a 130-bp fragment of the *T. gondii* B1 gene was amplified by using primers B5 and B6, as described by Robert-Gangneux et al. (11). AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, Calif.) and reagents from the PCR-ELISA DIG Labeling Plus kit (Roche Applied Science, Meylan, France) were used for amplification. Ten microliters of extracted DNA sample (which represents DNA from 700 μl of whole blood when the leukocyte layer is used or from 20 μl of whole blood, serum, or CSF) was added to a final volume of 50 μl. Amplification was performed on a Perkin Elmer GeneAmp PCR system 2400 thermocycler (Applied Biosystems) with the following profile: 10 min at 25°C; 9 min at 95°C; 37 cycles of 30 s of denaturation at 95°C, 40 s of annealing at 60°C, and 40 s of extension at 72°C; and a 10-min terminal extension at 72°C. Digoxigenin-labeled PCR products were then detected with a PCR ELISA DIG detection kit (Roche Applied Science) and a biotin-labeled oligonucleotide probe (5′-GCAAGAGAAGTATTTGAGGTC-3′) specific to the amplified fragment of the *T. gondii* B1 gene. After immobilization on a streptavidin-coated microtiter plate, the probe-PCR product hybrids were visualized with a peroxidase-conjugated antidigoxigenin antibody and a colorimetric substrate. In each run, two dilutions of *T. gondii* DNA extracted from the peritoneal exudate of Swiss mice intraperitoneally infected with *T. gondii* strain RH were used as external standards. The diluted standards corresponded to DNA extracts of five and one tachyzoite, respectively.

For real-time PCR, we used the assay described by Costa et al. (3), which targets a 126-bp fragment of the *T. gondii* B1 gene with detection by fluorescence resonance energy transfer hybridization probes. For quantification, a 10-fold serial dilution of *T. gondii* strain RH DNA ranging from 0.1 to 10 parasites/μl was included in each amplification run. Real-time PCRs were performed in a final volume of 20 μl by using the DNA Master hybridization probe kit (Roche), with a 0.5 μM concentration of each primer, a 0.25 μM concentration of each probe, and 5 μl of extracted DNA sample. Carryover contamination was prevented by using heat-labile uracil-DNA-glycosylase (Roche Applied Science). The amplification was carried out in a LightCycler instrument (Roche) according to a previously described protocol (3). PCR results, expressed as parasite loads per milliliter, were calculated by interpolation from the standard parasite dilution curve.

In parallel to PCR determinations, immunoglobulin G (IgG) and IgM anti-*Toxoplasma* antibody titers were determined by ELISA (Platelia Toxo G and Platelia Toxo M; Bio-Rad, Marnes, France) on eight serum samples taken before and after SCT.

The results of PCR-ELISA and real-time PCR performed on the sequential blood specimens are presented in Table 1. Serum specimens were negative before transplant and until day 41. The first positivity was recorded for buffy coat on day 36, whereas whole blood was negative on the same date; no serum was available on that date. On day 43 (the day when treatment was initiated), parasite burden had markedly increased, as evidenced by real-time PCR. Parasite burden progressively decreased beginning on day 48 and became undetectable on day 61 in whole blood and day 74 in buffy coat by both real-time and conventional PCR. Thereafter, occasional positivity was recorded, by either conventional or real-time PCR (Table 1). Samples remained repeatedly negative after day 102, whatever the technique or sample used.

Parasite burden was constantly lower in serum than in whole blood and buffy coat. For example, on day 48, parasite burden was of 19 tachyzoites per ml of serum, versus 530 and 2,200 in whole blood and buffy coat, respectively. Parasite DNA was undetectable in some serum specimens from day 55 for real-time PCR and day 74 for PCR-ELISA and remained repeatedly negative after day 91 with both techniques.

In CSF, *T. gondii* DNA was detectable by both conventional and real-time PCR on day 43 and was undetectable on day 69, i.e., after 26 days of treatment.

The recipient was positive for anti-*Toxoplasma* antibodies before transplantation with high IgG titers (>2,400 IU) and without IgM antibodies. Retrospective analysis of a serum sample taken 3 months earlier showed similar results, suggesting a past infection with persistently high IgG titers. After SCT, IgG titers decreased to 160 IU on day 81 and then remained almost stable during follow-up.

Discussion. Toxoplasmosis may cause life-threatening infections in immunocompromised patients. SCT recipients are particularly susceptible to severe toxoplasmosis, mainly as a result of reactivation of a previously acquired latent infection (4, 5, 8, 9, 10, 12).

The case reported here illustrates several major aspects of prevention, diagnosis, and follow-up of toxoplasmosis in SCT recipients.

Because of a positive *Toxoplasma* serology before transplantation, our patient belonged to a group of SCT recipients that is known to be at risk for severe disseminated toxoplasmosis. Therefore, he was a candidate for specific prophylaxis using sulfadoxine plus pyrimethamine (7), as usually prescribed in our institution for patients with consistent engraftment (neutrophils $> 10^9$/liter). However, due to a delayed hematological recovery, no specific prophylaxis was administered.

The second point is the unusual radiological presentation of cerebral toxoplasmosis in this case, although non-ring-enhanced lesions have rarely been observed in proven cerebral toxoplasmosis (6).

Finally, the diagnosis of toxoplasmic encephalitis was strongly suspected on the basis of positive PCR results for blood and CSF and ascertained by the resolution of clinical and radiological symptoms upon specific therapy. Serology did not contribute to diagnosis, as IgG titers decreased early after SCT and then remained stable during follow-up. Retrospective analysis of sequential blood samples gave us the opportunity to compare the respective performances of conventional and real-time PCR for early diagnosis of toxoplasmic encephalitis. Conventional PCR-ELISA and real-time PCR gave concordant results in terms of detection of *T. gondii* DNA for 54 of 62 samples. Discrepant results (confirmed on repeated tests) were noted for eight samples. Positive PCR-ELISA and negative real-time PCR were noted for six samples, whereas the reverse was observed for two samples with very low parasitic burdens, close to the detection limit of real-time PCR.

TABLE 1. Anti-*Toxoplasma* antibody titers and results of conventional and real-time *T. gondii* PCR on sequential samples of blood and CSF

Sample collection day[a]	Anti-*Toxoplasma* serology		No. of tachyzoites/ml in[c]:							
			Whole blood		Buffy coat		Serum		CSF	
	IgG titer (IU/ml)	IgM (index)[b]	PCR-ELISA	Real-time PCR	PCR-ELISA	Real-time PCR	PCR-ELISA	Real-time PCR	PCR-ELISA	Real-time PCR
−127	2,400	0					0	0		
−15	2,400	0					0	0		
−8							0	0		
−1							0	0		
+7							0	0		
+14							0	0		
+20							0	0		
+27							0	0		
+34							0	0		
+36			0	0	+	86				
+41							+	26		
+43			+	2,200	+	26,000			+	37
+48			+	530	+	2,200	+	19		
+50			+	130	+	1,400				
+53			+	240	+	1,400				
+55							+	0		
+61			0	0	+	11				
+62							+	18		
+67	300	0	+	0	+	8	+	0		
+69							+	16	0	0
+71			+	0	+	6				
+74			0	19	0	0	0	0		
+81	160	0	0	0	+	0.5	+	0		
+84			0	0	0	0				
+91			0	37	+	0	0	0		
+99							0	0		
+102	250	0	0	0	0	0	0	0		
+106			0	0	0	0				
+126							0	0		
+165	240	0	0	0	0	0	0	0		
+179	198	0	0	0	0	0	0	0		
+197			0	0	0	0				
+200	187	0					0	0		
+214			0	0	0	0				
+225			0	0	0	0				

[a] − and + indicate days before and after SCT, respectively.
[b] Values were calculated according to the manufacturer's instructions.
[c] +, PCR-ELISA positive.

T. gondii DNA could be detected in whole blood, buffy coat, and serum. However, it was detected first in buffy coat at day 36, whereas serum and whole blood were negative on the same date. Moreover, clinical symptoms appeared 1 week after PCR positivity in buffy coat. After treatment, *T. gondii* DNA was detectable in buffy coat longer and more consistently than in whole blood or serum. This suggests that PCR should be preferentially performed on buffy coat for optimum sensitivity. However, we confirm that PCR on serum can be used for retrospective diagnosis of toxoplasmosis from stored samples (1, 3).

The real-time PCR offered the possibility of assessing disease progression and treatment efficacy. In our patient, the parasitic burden initially increased over a 7-day period and then decreased 5 days after the initiation of therapy. Transient positivity with a low parasitic burden was observed between days 74 and 91; from day 102, i.e., after 8 weeks of treatment, PCRs were constantly negative. In a previous study, Bretagne et al. obtained negative results 3 days posttreatment in a case of late toxoplasmosis in an SCT patient (2); this patient had mild clinical symptoms and no evidence of visceral localization upon CT scan and chest X ray. In our patient, the longer delay to negative results could be related to the severity of the disease and the profound immunodeficiency related to cord blood cell transplantation. Our results also indicate that *T. gondii* may not be rapidly eradicated from tissues and fully justifies the 4- to 6-week full-dose treatment with pyrimethamine plus sulfadiazine. The achievement of negative PCR results, together with clinical and radiological improvement, constituted a good basis for switching to maintenance treatment.

Both conventional PCR-ELISA and real-time PCR proved sensitive for detection of *T. gondii* DNA in blood and serum samples. When performed on buffy coat, both PCR techniques can be highly contributive for early diagnosis of toxoplasmic encephalitis and can be proposed for close follow-up of patients at high risk for toxoplasmosis, especially when prophylaxis cannot be given or has to be delayed. Real-time PCR has a lower risk of false-positive reactions but remains costly; however, this technique allows good quantification of parasite burden and thus appears to be better suited for monitoring treatment efficacy.

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