Diagnostic Microbiology and Infectious Disease 82 (2015) 289–291

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Molecular diagnosis of toxoplasmosis: value of the buffy coat for the detection of circulating *Toxoplasma gondii*

Marie-Pierre Brenier-Pinchart [a,b,*], Elodie Capderou [a], Rose-Laurence Bertini [a], Sébastien Bailly [a], Hélène Fricker-Hidalgo [a], Emmanuelle Varlet-Marie [b,c], Jean-Benjamin Murat [a], Yvon Sterkers [b,c], Fériel Touafek [b,d], Patrick Bastien [b,c], Hervé Pelloux [a,b]

[a] *Laboratoire de Parasitologie et Mycologie, CHU de Grenoble et Université Grenoble Alpes, Grenoble 1 BP 217, 38043 Grenoble, France*
[b] *Pôle "Biologie Moléculaire" du Centre National de Référence de la Toxoplasmose, Montpellier, France*
[c] *Laboratoire de Parasitologie-Mycologie, CHU de Montpellier, CNRS UMR5290/IRD 224/UM1 ("MiVEGEC"), University of Montpellier 1, Montpellier, France*
[d] *Laboratoire de Parasitologie-Mycologie, Pôle de Biologie et Pathologie Médicale, CHU la Pité Salpêtrière, Charles Foix 47, bd de l'Hôpital, 75651 Paris Cedex 13, France*

ARTICLE INFO

Article history:
Received 19 January 2015
Received in revised form 23 March 2015
Accepted 20 April 2015
Available online 24 April 2015

Keywords:
Toxoplasmosis
Toxoplasma gondii
Diagnosis
PCR
Buffy coat
Whole blood

ABSTRACT

Early detection of *Toxoplasma* tachyzoites circulating in blood using PCR is recommended for immunosuppressed patients at high risk for disseminated toxoplasmosis. Using a toxoplasmosis mouse model, we show that the sensitivity of detection is higher using buffy coat isolated from a large blood volume than using whole blood for this molecular monitoring.

Disseminated toxoplasmosis is a life-threatening opportunistic infection that affects hematopoietic stem cell transplant (HSCT) and solid organ transplant (SOT) recipients (Martino et al., 2000; Schmidt et al., 2013). Primary infection in immunocompetent hosts leads to the formation of cysts in brain and muscles (Robert-Gangneux and Darde, 2012). In HSCT recipients, this infection occurs almost exclusively from reactivation of latent parasites in seropositive patients, while in SOT recipients, toxoplasmosis results mainly from transmission of the protozoan parasite with the transplanted organ from a *Toxoplasma*-seropositive donor to a *Toxoplasma*-seronegative recipient (Derouin et al., 2008). The diagnosis of toxoplasmosis in such patients at high risk for developing disease is difficult and often delayed (Schmidt et al., 2013). A prospective surveillance of immunocompromised patients who do not receive prophylaxis using PCR in peripheral blood samples has been recommended by several authors (Derouin et al., 2008; Edvinsson et al., 2009; Fricker-Hidalgo et al., 2009; Martino et al., 2005). However, today, modalities of PCR follow-up are not clearly defined, even with respect to biological testing. Whether this is for screening (when no signs/symptoms are present) or for diagnosis (in the presence of symptoms) of toxoplasmosis, serum/plasma, whole blood (WB), and buffy coat can be used to detect the parasitemia, without any consensus on the best sample to be used (Botterel et al., 2002; Caner et al., 2012; Khalifa et al., 1994; Kompalic-Cristo et al., 2007; Martino et al., 2005; Menotti et al., 2003). The molecular biology network of the French National Reference Center for Toxoplasmosis (http://cnrtoxoplasmose.chu-reims.fr/) wishes to propose some recommendations to the medical community on the best blood samples to detect parasitemia using PCR (Sterkers et al., 2010; Varlet-Marie et al., 2014). To achieve this, our objective was to study the usefulness of plasma, WB, and buffy coat to detect *Toxoplasma gondii* DNA by real-time PCR in murine circulating blood during experimental toxoplasmosis.

Female Swiss-OF1 mice were purchased from Charles River, and the experiments were performed in accordance with a local animal ethics committee. For experimental infections, parasites of the ME49 strain (type II) were harvested from in vitro cultivated human foreskin fibroblasts counted in a Malassez cell, and 10^4 parasites in 200 μL of Hanks' medium were inoculated by intraperitoneal injection to 4–6 mice. At 6 days postinfection, mice were sacrificed by CO_2 inhalation, and then blood was sampled and pooled on 1 EDTA tube by cardiac puncture (BD Vacutainer® tube; Becton Dickinson and Company, Le Pont de Claix, France). The blood was separated into several Eppendorf tubes in order to obtain, for each experiment: 200 μL of plasma; 200 μL of WB; and buffy coat portions isolated from 200 μL (BC200), 600 μL (BC600), and 1200 μL of WB (BC1200). Four independent experiments

* Corresponding author. Tel.: +33-4-76-76-54-90; fax: +33-4-76-76-52-28.
E-mail address: MPBrenierPinchart@chu-grenoble.fr (M.-P. Brenier-Pinchart).

http://dx.doi.org/10.1016/j.diagmicrobio.2015.04.004
0732-8893/

were performed; for the last 2, buffy coats were also isolated from 750 μL (BC750) and 1600 μL of blood (BC1600). Buffy coats and plasma were collected after centrifugation of freshly isolated blood for 10 min at 600 g. DNA was extracted using the QIAamp DNA kit (Qiagen®, Courtaboeuf, France); elution was performed with 100 μL of elution buffer, and DNA was frozen at −20 °C until PCR. Real-time PCR (rt-PCR) was performed on a LightCycler 2.0 device (Roche Diagnostics®, Meylan, France) targeting the 529-bp repeat element of *T. gondii* (Reischl et al., 2003; Varlet-Marie et al., 2014). Each sample was analyzed in duplicate, and results were expressed in crossing points (Cps). To compare the different types of samples, the repeated-measures analysis of variance followed by the Tukey posttest was used. Linear regression and Pearson's correlation were calculated on data of Cp measured on buffy coats isolated from the 5 different blood volumes. Statistical analysis was performed with the software SAS version 9.3.

Four independent experiments were conducted, and a total of 56 samples were analyzed. The means of Cps (±SD) were 34.31 (±1.03), 29.08 (±1.63), 30.02 (±1.92), 28.31 (±1.19), and 27.10 (±1.26) on plasma, WB, BC200, BC600, and BC1200 samples, respectively (Table 1). The rt-PCR in plasma clearly provided insufficient sensitivity since *Toxoplasma* detection was very late compared to WB, with a difference of 5.23 Cps. On the other hand, 6 days after *Toxoplasma* inoculation, significant differences were also seen among the other sample preparations. Indeed, at that time of infection, *Toxoplasma* detection occurred significantly earlier in rt-PCR, corresponding to higher parasite loads, when testing buffy coats isolated from 1200 μL of blood compared to WB and to buffy coats obtained from 200 μL of blood ($P = 0.0006$ for WB versus BC1200; $P < 0.0001$ for BC200 versus BC1200). The delta of Cp measurements between WB and BC1200 was 1.98. This shows that, to detect circulating *Toxoplasma*, the buffy coat isolated from 1200 μL of blood is a better sample than 200 μL of WB. Moreover, parasite detection occurred significantly earlier when the buffy coats were isolated from larger than from smaller volumes of blood (Table 1). Linear regression analysis confirmed an inverse correlation between the parasitic load measured in Cps and the blood volumes used to prepare buffy coats ($P < 0.0001$, $\rho = -0.69$) (Fig. 1). The delta of Cp measurements observed between BC200 and BC1600 testing was 4.01, confirming that buffy coat isolated from a high blood volume is the most sensitive sample to detect *T. gondii* in peripheral blood.

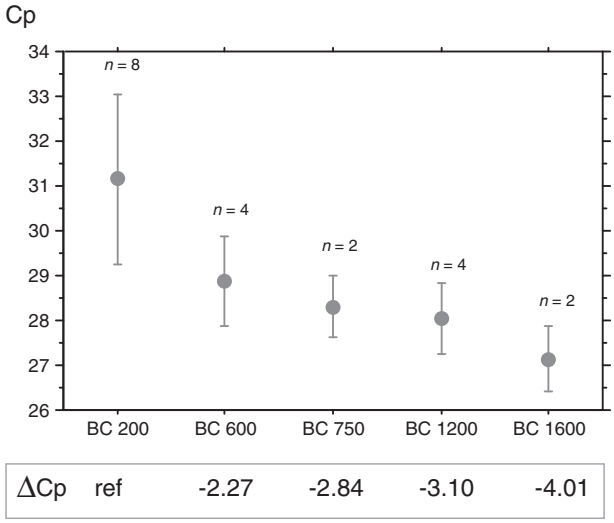

Fig. 1. Means of crossing points (±SDs) of 5 different blood volumes used to isolate the buffy coat.

In this model of experimental toxoplasmosis in mice using a type II *Toxoplasma* strain, the most common strain in human toxoplasmosis (Ajzenberg et al., 2009), the buffy coat isolated from a high volume of blood, as compared to plasma, WB, and buffy coats isolated from smaller volumes of blood, therefore appears as the best sample to detect *Toxoplasma* in peripheral blood. The sensitivity of DNA detection increased with the increase in the blood volume used to prepare the buffy coat. This result is consistent with the biology of *T. gondii* since this protozoon is an obligate intracellular parasite (Robert-Gangneux and Darde, 2012). We hypothesize that the number of nucleated cells used to perform the DNA extraction and detection is a major parameter for the quality of this molecular diagnosis. Thus, in biological diagnosis, an increase in the concentration of blood nucleated cells, following an increase in the volume of sampled blood, would also raise the number of infected cells, thereby leading to an earlier PCR detection especially because some patients at high risk for developing toxoplasmosis disease are neutropenic patients. Another parameter is the concentration of

Table 1
Detection of *T. gondii* in different samples from peripheral blood in mouse toxoplasmosis.

Experiments	No. of mice	Plasma	WB	BC200	BC600	BC750	BC1200	BC1600
1	4	>35 (n = 2)	30.33 ± 1.83 (n = 4)	29.56 ± 1.00 (n = 4)	28.54 ± 0.39 (n = 2)		27.33 ± 0.27 (n = 2)	
2	6	34.03 ± 1.14 (n = 2)	27.64 ± 0.84 (n = 4)	28.19 ± 0.76 (n = 4)	27.17 ± 1.06 (n = 2)		25.77 ± 0.57 (n = 2)	
3	6	34.06 ± 1.53 (n = 2)	29.07 ± 1.65 (n = 4)	31.93 ± 1.97 (n = 4)	29.15 ± 1.08 (n = 2)	28.05 ± 0.37 (n = 1)	28.17 ± 1.18 (n = 2)	26.75 ± 0.96 (n = 1)
4	6	34.61 ± 0.78 (n = 2)	29.26 ± 0.85 (n = 4)	30.37 ± 1.55 (n = 4)	28.60 ± 0.99 (n = 2)	28.57 ± 0.99 (n = 1)	27.90 ± 0.22 (n = 2)	27.49 ± 0.24 (n = 1)
Experiments 1 to 4	Means ± SD	34.3 ± 1.03 (n = 8)	29.08 ± 1.63* (n = 16)	30.01 ± 1.91**,*** (n = 16)	28.31 ± 1.19† (n = 8)	/	27.10 ± 1.25 (n = 8)	
	ΔCp[a]	+5.23	Reference	+0.94	−0.77		−1.98	
Experiments 3 and 4	Means ± SD			31.15 ± 1.90 (n = 8)	28.88 ± 1.00 (n = 4)	28.31 ± 0.67 (n = 2)	28.04 ± 0.80 (n = 4)	27.13 ± 0.74 (n = 2)
	ΔCp[b]			Reference	−2.27	−2.84	−3.10	−4.01

Means and SDs of the Cp measurements are shown for the different types of samples: plasma; WB; and buffy coat isolated from 200 μL (BC200), 600 μL (BC600), 750 μL (BC750), 1200 μL (BC1200), and 1600 μL (BC1600) of blood.
n = number of samples analyzed.
ΔCp: Cp of each type of sample − Cp found when testing WB[a] or Cp found when testing BC200[b] (considered as reference).
* WB versus BC1200: $P = 0.0006$.
** BC200 versus BC1200: $P < 0.0001$.
*** BC200 versus BC600: $P < 0.0001$.
† BC600 versus BC1200: $P = 0.0578$.

potential PCR inhibitors, of which heme is known as the major one (Akane et al., 1994), which is likely to be higher in WB than in buffy coat. Finally, the differences observed in *T. gondii* DNA detection among the different samples studied here were high: they could reach 4.01 Cps (BC200 versus BC1600) and 7.21 Cps (plasma versus BC1200), which corresponds to a 10- to 20-fold theoretical increase in terms of DNA load.

The superiority of the buffy coat to diagnose human toxoplasmosis has been reported in the literature only for 1 case : Menotti et al. (2003) showed that the sensitivity of detection was higher when the PCR tests were performed using a buffy coat isolated from 7 mL of blood than using 200 μL of WB or serum analyzed in parallel. This superiority of the buffy coat to detect circulating *T. gondii* might explain why Martino et al. (2005) reported that, in some cases of probable or documented toxoplasmosis, the PCR tests using peripheral blood were negative and why they underlined that negative results of peripheral blood PCR testing should not rule out the presence of the disease in a seropositive HSCT recipient with a compatible clinical presentation. Unfortunately, this multicentric study report did not specify the types of blood samples used (Martino et al., 2005). Furthermore, the PCR used before 2005 amplified target in *T. gondii* genome less repeated (e.g., B1) than the 529-bp repeat element used at present (Reischl et al., 2003).

Disseminated toxoplasmosis is a rapidly progressing infection. Systematic screening by repeated PCR testing of peripheral blood has been suggested during the early weeks or months following transplantation (Derouin et al., 2008; Fricker-Hidalgo et al., 2009; Martino et al., 2005). Even if the potential drawback of such systematic screening strategy is the possible detection of parasite circulating DNA in patients with no clinical signs (Edvinsson et al., 2008; Martino et al., 2005), a recent retrospective study suggests also that a biological follow-up using PCR on blood could guide pre-emptive treatment and improve the outcome of allo-HSCT patients (Robert-Gangneux et al., 2015). Consequently, a sensitive biological diagnosis to detect the circulating parasites in patients at high risk for toxoplasmosis early is probably essential. In this respect, the choice of the best suited sample for molecular detection is highly relevant. In conclusion, our study in mouse model strongly suggests that molecular diagnosis using buffy coat isolated from a large blood volume may be the most helpful to diagnose disseminated toxoplasmosis. However, the interest of buffy coat isolated from 5 to 7 mL of blood in screening immunocompromised patients with a high risk of toxoplasmosis reactivation and in diagnosing toxoplasmic infection often presenting with nonspecific symptoms would require a clinical trial.

Acknowledgments

We thank Mohamed-Ali Hakimi for the helpful discussion and Sabine Durville for reading the manuscript.

Potential conflicts of interest: All authors, no conflicts.

References

Ajzenberg D, Yera H, Marty P, Paris L, Dalle F, Menotti J, et al. Genotype of 88 *Toxoplasma gondii* isolates associated with toxoplasmosis in immunocompromised patients and correlation with clinical findings. J Infect Dis 2009;199(8):1155–67. http://dx.doi.org/10.1086/597477.

Akane A, Matsubara K, Nakamura H, Takahashi S, Kimura K. Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from bloodstains, a major inhibitor of polymerase chain reaction (PCR) amplification. J Forensic Sci 1994;39(2):362–72.

Botterel F, Ichai P, Feray C, Bouree P, Saliba F, Tur Raspa R, et al. Disseminated toxoplasmosis, resulting from infection of allograft, after orthotopic liver transplantation: usefulness of quantitative PCR. J Clin Microbiol 2002;40(5):1648–50.

Caner A, Dönmez A, Döşkaya M, Değirmenci A, Tombuloğlu M, Cağirgan S, et al. Determining *Toxoplasma gondii* high-risk autologous and allogeneic hematopoietic stem cell transplantation patients by systemic pre-transplantation PCR screening of stem cell originated buffy coat. Parasitol Int 2012;61(4):565–71. http://dx.doi.org/10.1016/j.parint.2012.05.005.

Derouin F, Pelloux H, on behalf of the ESCMID Study Group on Clinical Parasitology. Prevention of toxoplasmosis in transplant patients. Clin Microbiol Infect 2008;14(12):1089–101. http://dx.doi.org/10.1111/j.1469-0691.2008.02091.x.

Edvinsson B, Lundquist J, Ljungman P, Ringden O, Evengard B. A prospective study of diagnosis of *Toxoplasma gondii* infection after bone marrow transplantation. APMIS 2008;116(5):345–51. http://dx.doi.org/10.1111/j.1600-0463.2008.00871.x.

Edvinsson B, Lappalainen M, Antilla VJ, Paetau A, Evengard B. Toxoplasmosis in immunocompromised patients. Scand J Infect Dis 2009;41(5):368–71. http://dx.doi.org/10.1080/00365540902783319.

Fricker-Hidalgo H, Bulabois CE, Brenier-Pinchart MP, Hamidfar R, Garban F, Brion JP, et al. Diagnosis of toxoplasmosis after allogeneic stem cell transplantation: results of DNA detection and serological techniques. Clin Infect Dis 2009;48(2):e9-15. http://dx.doi.org/10.1086/595709.

Khalifa KES, Roth A, Roth B, Arasteh KN, Janitschke K. Value of PCR for evaluating occurrence of parasitemia in immunocompromised patients with cerebral and extracerebral toxoplasmosis. J Clin Microbiol 1994;32(11):2813–9.

Kompalic-Cristo A, Frotta C, Suárez-Mutis M, Fernandes O, Britto C. Evaluation of a real-time PCR assay based on the repetitive B1 gene for the detection of *Toxoplasma gondii* in human peripheral blood. Parasitol Res 2007;101(3):619–25.

Martino R, Maertens J, Bretagne S, Rovira M, Deconinck E, Ullmann AJ, et al. Toxoplasmosis after hematopoietic stem cell transplantation. Clin Infect Dis 2000;31(5):1188–95.

Martino R, Bretagne S, Einsele H, Maertens J, Ullmann AJ, Parody R, et al. Early detection of *Toxoplasma* infection by molecular monitoring of *Toxoplasma gondii* in peripheral blood samples after allogeneic stem cell transplantation. Clin Infect Dis 2005;40(1):67–78.

Menotti J, Vilela G, Romand S, Garin YJ, Ades L, Gluckman E, et al. 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. J Clin Microbiol 2003;41(11):5313–6.

Reischl U, Bretagne S, Kruger D, Ernault P, Costa JM. Comparison of two DNA targets for the diagnosis of toxoplasmosis by real-time PCR using fluorescence resonance energy transfer hybridization probes. BMC Infect Dis 2003;3:7.

Robert-Gangneux F, Darde ML. Epidemiology of and diagnostic strategies for toxoplasmosis. Clin Microbiol Rev 2012;25(2):294–6. http://dx.doi.org/10.1128/CMR.05013-11.

Robert-Gangneux F, Sterkers Y, Yera H, Accoceberry I, Menotti J, Cassaing S, et al. Molecular diagnosis of toxoplasmosis in immunocompromised patients: a three-year multicenter retrospective study. J Clin Microbiol 2015;53(5):1677–84. http://dx.doi.org/10.1128/JCM.03282-14.

Schmidt M, Sonneville R, Schnell D, Bigé N, Hamidfar R, Mongardon N, et al. Clinical features and outcomes in patients with disseminated toxoplasmosis admitted to intensive care: a multicenter study. Clin Infect Dis 2013;57(11):1535–41. http://dx.doi.org/10.1093/cid/cit557.

Sterkers Y, Varlet-Marie E, Cassaing S, Brenier-Pinchart MP, Brun S, Dalle S, et al. Multicentric comparative analytical performance study for molecular detection of low amounts of *Toxoplasma gondii* from simulated specimens. J Clin Microbiol 2010;48(9):3216–22. http://dx.doi.org/10.1128/JCM.02500-09.

Varlet-Marie E, Sterkers Y, Brenier-Pinchart MP, Cassaing S, Dalle F, Delhaes L, et al. Characterization and multicentric validation of a common standard for *Toxoplasma gondii* detection using nucleic acid amplification assays. J Clin Microbiol 2014;52(11):3952–9. http://dx.doi.org/10.1128/JCM.01906-14.