Letter to the Editor

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Molecular diagnosis of toxoplasmosis: evaluation of automated DNA extraction using eMAG® (bioMérieux) on buffy coat, cerebrospinal and bronchoalveolar lavage fluids

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To the Editor,

Toxoplasma gondii (Tg) is a protozoan parasite responsible for congenital toxoplasmosis when acquired during pregnancy and for life-threatening opportunistic disease in immunocompromised patients. After contamination, the parasite persists throughout its host's entire life within cysts in the brain, eyes and muscles [1]. Reactivation of latent cysts can occur in the case of immunosuppression, i.e. HIV infection or following a hematopoietic stem cell or solid organ transplant, and lead to cerebral or disseminated toxoplasmosis [2–4]. An early diagnosis of toxoplasmosis in immunocompromised patients is vital to improve their survival rate [1]. Biological diagnosis relies notably on molecular methods to detect parasite DNA, but because the circulating parasite load is often low, optimal sensitivity is required of these techniques. An important step in the molecular analysis is the nucleic acid extraction of the sample before DNA amplification. Three studies have already compared manual and automated DNA extraction methods, including the automated nucleic acid extraction platform easyMAG® (bioMérieux, Marcy l'Etoile, France), on amniotic fluid for the diagnosis of congenital toxoplasmosis [5–7]. However, no such studies have been performed on biological samples that can allow disseminated or cerebral toxoplasmosis to be diagnosed. The objective of this study was therefore to investigate the suitability of the automated method eMAG® (bioMérieux, Marcy l'Etoile, France), the newer version of easyMAG®, for the isolation of *T. gondii* DNA from artificially spiked buffy coats, cerebrospinal and bronchoalveolar lavage fluids, compared to a manual method using the QIAamp DNA Mini Kit (Qiagen, Courtaboeuf, France).

RH strain *T. gondii* tachyzoites were harvested from *in vitro* cultivated human foreskin fibroblasts. A stock suspension of 2.5×10^5 parasites/mL was prepared and diluted in phosphate-buffered saline (PBS) to obtain calibrated suspensions of 5, 10 and 50 Tg/20 µL immediately stored at –20 °C. Three different biological matrices were studied: buffy coat (BC) isolated from 4 mL of blood in EDTA tubes after centrifugation for 10 min at 1200 *g* [3], cerebrospinal fluids (CSF) and bronchoalveolar lavage fluids (BAF). To obtain two exactly identical samples, 400 µL samples of each matrix were pooled and artificially spiked with 40 µL of parasite suspension and 20 µL of internal control (IC 2, Argene, bioMérieux, Marcy l'Etoile, France) diluted 50-fold in PBS and were then separated in two aliquots of 200 µL. The two DNA extraction methods were performed on the same day: one aliquot was extracted manually with the QIAamp DNA Mini Kit (Qiagen, Courtaboeuf, France) and DNA was eluted with 200 µL of buffer as recommended by the manufacturer; the second was extracted with eMAG® ending with an eluate of 50 µL. Pre-analytical and analytical details are described in Table 1. The DNA extracts were frozen at –20 °C until PCR. Each spiked sample was then amplified at least twice by real-time PCR targeting the

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Table 1: Preanalytical and analytical steps for the DNA extraction methods.

Sample	Preanalytical step	Lyse	Extraction protocol	Silica, µL	Eluate, µL
QIAamp DNA Mini Kit (Qiagen)					
All	Add 10 µL of internal control, 20 µL proteinase K and 200 µL AL buffer, 56 °C for 10 min	Manual	Manual	/	200
eMAG®					
BC	Add 10 µL of internal control	Off board	B50_Off_Ves_SB	140	50
CSF	Add 100 µL of fetal bovine serum and 10 µL of internal control	On board	C40t_Off_Lys_SB	50	
BAF	Addition of 20 µL of proteinase K, 10 µL of internal control and four to five beads, vortex, 56 °C for 20 min	On board	B41t_Off_Lys_SB	50	

BC, buffy coat; CSF, cerebral-spinal fluid; BAF, bronchoalveolar lavage fluid.

repetitive DNA target rep529 using the LightCycler® 480 (Roche Diagnostics, Meylan, France) [8]. Three independent experiments were conducted for each matrix. The results are expressed as a PCR performance score corresponding to the number of positive amplifications over the total number of PCR reactions performed for the extraction method at a given parasite concentration. Performance scores and percentages were analyzed using Fisher's exact test while cycle thresholds were compared with Student test; a probability of 0.05 or less was considered to be significant.

In total, 70 samples were studied corresponding to 142 PCRs. The 18 control samples without parasites (nine samples extracted by each of the two extraction methods) were found to be PCR negative (36 PCRs). None of the buffy coat samples with 10 or less Tg per sample were detected when extracted with Qiagen, whereas 7/12 PCRs were positive with eMAG® extracts (see Table 2). There was no statistically significant difference in PCR scores between the two extraction methods with higher parasite concentrations (50 Tg/sample). Qiagen extracts with 10 or less Tg were detected in 7/24 PCRs (29.2%) vs. 21/24 PCR (87.5%) with eMAG® extracts (p-value $<10^{-4}$) in CSF and BAF. Regardless of the matrix, the eMAG® extracts were detected with a significantly higher yield than the manual method (45/54 [83.3%] vs. 20/54 [37%]; $p<0.001$) especially in samples with low concentrations of *Toxoplasma* (≤10 Tg). Moreover, for all three types of spiked samples with 50 Tg, the mean cycle threshold (Ct) of amplification was significantly higher when DNA was extracted with the QIAamp DNA minikit than with eMAG® (35.1 ± 2.33 vs. 33.6 ± 1.92; $p<0.05$). The mean Cts of the internal control for the 70 samples were also statistically different when extracted with Qiagen compared to eMAG® (35.5 ± 1.6 vs. 33.2 ± 2.1 respectively, $p<10^{-10}$).

Table 2: Performance scores of PCR assays performed on three independent series of artificially-spiked samples after DNA extraction with QIAamp DNA Mini Kit (Qiagen) and eMAG® (bioMérieux).

Sample	0 Tg		5 Tg		10 Tg		50 Tg	
	Qiagen	eMAG®	Qiagen	eMAG®	Qiagen	eMAG®	Qiagen	eMAG®
BC								
PCR+[a]	0/6	0/6	0/4[b]	3/4[b]	0/8	4/8	5/6	5/6
Ct ± SD	/	/	/	36.2 ± 0.9	/	36.6 ± 0.8	35.6 ± 1.5	35.1 ± 1.3
CSF								
PCR+	0/6	0/6	4/6	6/6	0/6	5/6	4/6	6/6
Ct ± SD	/	/	35.9 ± 0.8	35.0 ± 1.6	/	36.0 ± 2.4	33.7 ± 2.6	32.8 ± 2.2
BAF								
PCR+	0/6	0/6	3/6	6/6	0/6	4/6	4/6	6/6
Ct ± SD	/	/	36.3 ± 0.2	35.3 ± 0.6	/	36.0 ± 1.4	35.9 ± 2.8	33.3 ± 1.7
Performance score								
PCR+	0/18	0/18	7/16	15/16[c]	0/20	13/20[d]	13/18	17/18
Ct ± SD	/	/	36.1 ± 0.6	35.4 ± 1.2	/	36.2 ± 1.6	35.1 ± 2.3	33.6 ± 1.9

[a]Number of positive PCR/number of performed PCR. [b]Two samples extracted by Qiagen and eMag® were deleted due to a technical problem during manual extraction; [c]p-value <0.01; [d]p-value <0.0001. Tg, *Toxoplasma gondii*; Ct, cycle threshold; SD, standard deviation; BC, buffy coat; CSF, cerebral-spinal fluid; BAF, bronchoalveolar lavage fluid.

Multiplex molecular panels are currently being developed to diagnose respiratory tract, bloodstream and meningitis/encephalitis infections [9]. Even though these tests are performed on the same samples used to diagnose toxoplasmosis, none of these panels include the detection of *Toxoplasma* DNA. In the absence of a syndrome-based approach, the molecular diagnosis of severe toxoplasmosis remains the result of a targeted diagnostic approach performed in specialized laboratories. In this context, it is of paramount importance to optimize the specific molecular diagnosis of disseminated and cerebral toxoplasmosis in immunocompromised patients. DNA extraction methods are gradually developing towards automation [10]. This allows more standardization, repeatability, traceability required for biological diagnosis and also for Laboratory Quality Assurance. In this study, we evaluated how suitable eMAG® is for isolating *Toxoplasma* DNA from BC, CSF and BAF compared to a manual extraction method. For each *Toxoplasma* concentration, the two artificial samples were strictly identical, and the two extraction methods were performed on the same day and by the same operator to avoid methodological bias. We showed that the automated DNA extraction method with eMAG® had a higher extraction yield. According to the manufacturer's instructions, the volume of elution buffer was not the same in these two extraction methods (Table 1). The smaller elution volume obtained with eMAG® extraction (50 µL) compared to Qiagen (200 µL) could in part, explain why the performance scores of this automated method were significantly higher. It should be noted that with eMAG® extracts, the 10 Tg group yielded fewer positive results than the 5 Tg group. This could be due to the fact that two different series of dilution were used to obtain the 5 Tg and 10 Tg aliquots. Thus, there may be variability between the two final concentrations due to the dilution protocol used. The automated DNA extraction method with eMAG® allowed a lower limit of detection compared to the manual extraction Qiagen method with the preanalytical steps and extraction protocols used in our study. This may allow for earlier diagnosis of life-threatening toxoplasmosis in immunocompromised patients, which would in turn enable treatment to be started sooner and improve the survival of these patients. These results are consistent with previous studies comparing Qiagen with easyMAG® extraction on amniotic fluids [5, 6].

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