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Evaluation of two commercial kits on the automated ELITE InGenius™ PCR platform for molecular diagnosis of toxoplasmosis

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Abstract

Molecular diagnosis of toxoplasmosis is essential for establishing the diagnosis of congenital contaminations and for primary infection or reactivation of immunocompromised patients. An integrated extraction and real-time PCR-based system is of particular interest in this context. Commercial kits for automated extraction and amplification steps are now available. Here we assessed two commercial PCR assays for this diagnosis, those of Bio-Evolution and Elitech, on the ELITE InGenius™ platform (ELITechGroup). The Bio-Evolution assay showed a specificity and a sensitivity of 100% on clinical samples, but a lower analytical detection threshold than the Elitech assay. The latter showed a specificity of 100% and a sensitivity of 96%. The SP1000 cartridges (Elitech), which allow DNA extraction from 1 mL of template, showed very interesting performances on amniotic fluids samples. Overall, the two kits had very good performances on the InGenius™ platform, which offers a turn-key solution suitable for the molecular diagnosis of toxoplasmosis.

Introduction

Toxoplasmosis is a zoonotic disease caused by the protozoan *Toxoplasma gondii* belonging to the phylum of *Apicomplexa*. It is an obligate intracellular parasite with a worldwide distribution, infecting mammals and birds. Nowadays, direct search of *T. gondii* for the diagnosis of congenital toxoplasmosis as well as acute disease in immunocompromised patients is based on molecular tools.

Although “in-house” quantitative PCR (qPCR) techniques are still widely used for these diagnoses (1), there is a growing interest of medical laboratories for commercial kits due to their ease and speed of use, their standardization fitting with the quality management system policy and their performances (2,3). DNA extraction is also a critical step in diagnoses as sensitive as toxoplasmosis. Automated DNA extraction methods were shown to be more reproducible than non-automated ones and perform well (4). Hence, a

combination of automated DNA extraction with qPCR not only takes advantages of both tools, but also reduces technical time, reporting delays and risk of errors. The ELITE InGenius™ platform (ELITechGroup, Inc., Bothell, WA, USA) is an open fully automated sample-to-result PCR system, allowing nucleic acid extraction and real-time PCR amplification of up to twelve samples. In this study, the performances of two commercial PCR assays, the Bio-Evolution *Toxoplasma gondii* detection kit (Bio-Evolution, Bussy-Saint-Martin, France) and the *Toxoplasma* ELITE MGB assay (ELITechGroup), were evaluated when performed on the ELITE InGenius™ platform.

Materials and methods

Samples.

A freeze-dried calibrated suspension (10^4 *Toxoplasma*/mL, *T. gondii* type II) was kindly provided by the Molecular Biology 'Pole' of the French National Reference Center for Toxoplasmosis (NRC-T) (<http://cnrttoxoplasme.chu-reims.fr/?lang=en>, last access on 03/17/2021) (5). A panel of 15 QCMD (Quality Control for Molecular Diagnostics, Glasgow, Scotland, UK) quality controls was included in this study. It consisted of eight vials of lyophilized amniotic fluid samples and seven vials of lyophilized plasma samples spiked or not with various concentrations of *T. gondii* (TGDNA18 and TGDNA20 panels). All these freeze-dried samples were reconstituted by adding 2.0 mL of sterile molecular-grade water as recommended. For the TGDNA18 panel, suspensions were centrifuged after 5 min, then 1.8 mL of supernatant were discarded and DNA from the pellet (200 μ L) was extracted (see below). For the TGDNA20 panel, which consisted of amniotic fluids only, three comparable vials of each three controls were tested with two extraction protocols: after 5 min, DNA from 1 mL or 200 μ L of homogenized suspension was extracted

(see below). Thirty-one clinical samples stored at -20°C following routine molecular diagnosis between 2018 and 2019 were also kindly provided by the French NRC-T. It included positive, weakly positive or negative (blind selection) peripheral bloods (n=18), cord bloods (n=3), placentas (n=6) and amniotic fluids (n=4). Before enrolment of a sample, diagnosis of toxoplasmosis was either excluded or asserted by molecular, serological and/or clinical follow-up of patients. All samples were tested in blind. For congenital toxoplasmosis, diagnosis of mothers and offsprings were classified as defined by the European Research Network on Congenital Toxoplasmosis group (6). Comparative immunoblot (IgG and IgM) at birth was performed to support diagnosis (7). Positive samples in immunocompromised were graded according to the European Group for Blood and Marrow Transplantation Infectious Diseases Working Party classification as "*Toxoplasma* infection" and "*Toxoplasma* disease" (8).

DNA extraction. Regarding pre-analytical steps on clinical samples, amniotic fluids (4 mL) were centrifuged (10 min at 16,000 g). Placentas (300 g) were ground and trypsinized with 0.6 g Trypsin at 37°C during 2 hours under constant magnetic stirring, then filtered. The pellet obtained by centrifugation 1,750 x g, 10 min was washed twice by PBS1X, and lysed with proteinase K (Euromedex, France). Buffy coat portions (300 μ L) isolated from whole blood (4 mL) after centrifugation for 10 min at 1,750 x g, were also lysed with proteinase K (Euromedex, France). Then, DNA extractions were carried on the ELITE InGenius™ platform (ELITechGroup). All samples were first processed using the extraction cartridges ELITE InGenius™ SP 200 (ELITechGroup) following the manufacturer's instructions, processing 200 μ L of pre-treated sample and eluting nucleic acids in a 100 μ L final volume. Four clinical samples of amniotic fluids

and the three control samples of amniotic fluids of the TGDNA20 panel, each in three different vials, were also extracted with the extraction cartridges ELITE InGenius™ SP 1000 (ELITechGroup) (nucleic acids from 1000 µL of pre-treated sample eluted in 100 µL). Manufacturer's internal control (CPE - Internal Control, ELITechGroup), compatible with the *Toxoplasma* ELITE MGB assay (ELITechGroup), was added during the extraction process.

PCR assays. Two commercial kits were evaluated, the *Toxoplasma* ELITE MGB assay (ELITechGroup) and the Bio-Evolution *Toxoplasma gondii* detection kit (Bio-Evolution) (Table 1). All DNA amplifications were performed on the ELITE InGenius™ apparatus (ELITechGroup), following the DNA extraction step on the same apparatus. The set parameters for DNA amplification complied with the protocols defined by each manufacturer. Clinical and control samples were tested in duplicate, and repeated in triplicate on the same initial DNA extract when discrepant qualitative results between both qPCR assays were observed. In all analyses the same detection threshold was used.

PCR performances. Limit of detection, range and linearity were approached using logarithmic serial dilutions of the DNA extract of the calibrated *Toxoplasma* suspension. The six concentrations tested ranged from 10^4 to 10^{-1} *T. gondii*/mL. All concentrations were tested in triplicate. A performance score was determined based on the number of positive samples divided by the total number of PCR wells (9).

Statistical analysis. Crossing threshold (Ct) values were used to elaborate the standard curve and to determine the efficiency of each PCR method. Delta Ct is the difference between Ct values obtained with each assay for a same sample. Means, standard deviations, coefficients of variation (CV) and correlations (R^2) were calculated using Microsoft® Excel® software (version 15.0.5031.1000). Slopes

of standard curves allowed determination of PCR efficiency in conformity with the formula $10^{(-1/\text{slope})} - 1$. Slopes between -3.1 and -3.6 giving reaction efficiencies between 90 and 110% are typically acceptable (Applied Biosystems application note “Amplification efficiency of TaqMan gene expression assays”, Thermo Fisher Scientific Inc., Waltham, MA, USA). Slopes were calculated when all triplicates were positive and $SD < 0.5$. Data obtained by both assays were compared using chi-square statistic with Yates correction when applicable. Agreement between both assays for the Ct values of positive clinical samples was evaluated by a Bland-Altman plot, which is a graphical representation of the difference of Ct in abscissa plotted against the mean of Ct values in ordinate, for each samples analysed by both assays (10).

Ethical approval and informed consent.

This study corresponds to a non-interventional retrospective study and followed the relevant guidelines and regulations according to the French Health Public Law (CSP Art L1121-1.1). In particular, it does not include potentially identifying patient/participant informations. Informed consent was obtained for sampling and analyzing amniotic fluids; the rest of the study is exempted from informed consent requirement and does not require approval by an ethics committee.

Results

PCR performance score and efficiency using *Toxoplasma* calibrated suspensions.

Serial dilutions of a calibrated *Toxoplasma* suspension were performed from 10^4 to 10^{-1} parasites/mL. Qualitative and quantitative results are reported in Table 2. The lowest parasite concentrations, *i.e.* 1 and 0.1 T/mL. were inconstantly amplified (Table S1). The PCR performance score were 15/18 (83.3%) with Bio-Evolution assay, and reached 17/18 (94.4%) with Elitech.

	<i>Toxoplasma gondii</i> detection kit (Bio-Evolution)	<i>Toxoplasma</i> ELITE MGB assay (Elitech)
DNA target	rep529	rep529
Type of probe	TaqMan (FAM-BHQ1)	TaqMan (FAM-MGB)
Internal control	Not specified (for DNA amplification only)	Artificial DNA sequence (for DNA extraction and amplification)
DNA input volume (µL)	5	10
PCR mix volume (µL)	20	20
No. of cycles	40	45
Uracil-DNA glycosylase	No	Yes

Table 1. Main technical characteristics of the two PCR assays.

Concentration (<i>T. gondii</i> parasites/mL)	Qualitative results (No. of positive reactions/no. of reactions performed)		Quantitative results (mean Ct values ± SD*)	
	Bio-Evolution	Elitech	Bio-Evolution	Elitech
10,000	3/3	3/3	19.2 ± 0.1	21.3 ± 0.1
1,000	3/3	3/3	23.4 ± 0.1	25.9 ± 0.2
100	3/3	3/3	27.0 ± 0.3	29.3 ± 0.3
10	3/3	3/3	29.5 ± 0.4	32.1 ± 0.2
1	2/3	3/3	34.6 ± 2.6	36.1 ± 1.0
0.1	1/3	2/3	33.4	37.6 ± 2.2
PCR performance score	15/18 (83.3%)	17/18 (94.4%)	/	/
Efficiency (-1 + 10 ^(-1/slope))	/	/	94.9%	90.3%

Table 2. Performance of the two PCR assays using DNA serial dilutions of a calibrated *Toxoplasma* suspension.

*SD: standard deviation. SDs were calculated on the basis of the positive values only.

The chi-square statistic with Yates correction is 0.281. The p -value is 0.596; the difference was not found significant at $p < 0.050$. The PCR efficiencies were 94.9% and 90.3% for Bio-Evolution and Elitech, respectively. Linearity and range were evaluated by constructing a calibration curve when all triplicates were positive with $SD < 0.5$. Linearity ($R^2 > 0.987$) and range extended from 10^4 parasites/mL for both assays (Figure 1). The analytical detection threshold, defined as the lowest concentration of a serial detection assay where at least half of the PCR tubes are positive, was 10-fold lower for the Elitech assay compared to the Bio-Evolution one (0.1 parasite/mL *versus* 1 parasite/mL, respectively). When testing QCMD samples, all qualitative results were concordant (26 positive, 2 negative) for both qPCR assays except for 2 discrepant results (Table S1). The discrepant results were about TGDNA20C1-01 and TGDNA20C1-05 samples when extracted with SP200

cartridges: the first was positive and the second was negative with Bio-Evolution assay, and vice versa with Elitech. These two samples are expected positive at 6 ± 4 *Toxoplasma*/mL by the manufacturer. They were finally found positive when re-tested (in 2/3 reactions wells with the Elitech assay for the first sample, and in 1/3 reactions wells with the Bio-Evolution assay for the second). Mean Ct values were systematically higher with Elitech than with Bio-Evolution (mean delta Ct = 2.2 ± 1.1 ; p -value < 0.001).

Diagnostic performances in a clinical cohort. Among the 31 specimens included in the retrospective clinical cohort and tested in duplicate, qualitative results were concordant for 30 specimens (97%): 18 positive results (both PCR wells of all duplicates were positive) and 12 negative results (Table S1). The only discordant result concerned a whole blood specimen, which was positive with Bio-Evolution in one reaction tube out of two (Ct=35.89), but negative for the duplicate with Elitech.

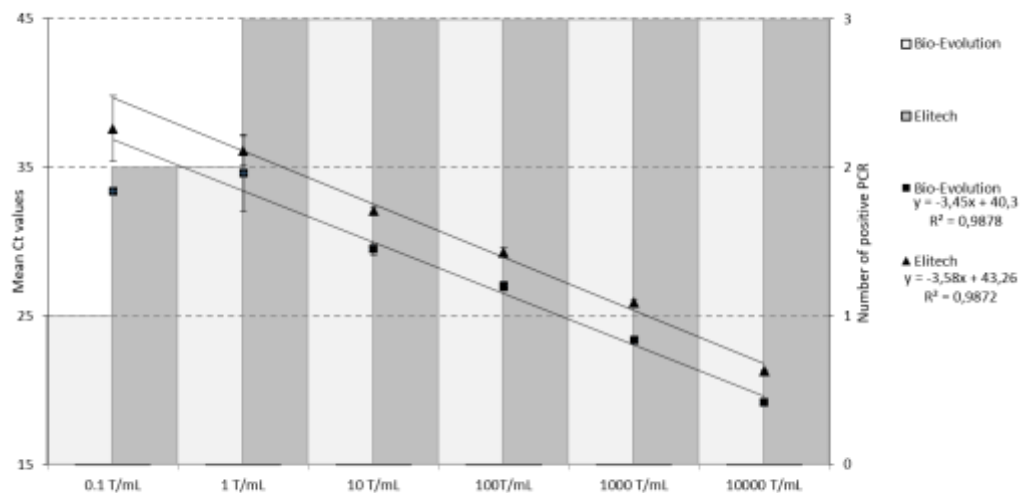


Figure 1. Serial dilution assays. Ten-fold serial dilution assays of a freeze-dried calibrated suspension (10^4 *Toxoplasma*/mL, *T. gondii* type II) were performed in triplicate with Bio-Evolution (black square) and Elitech (black triangle) PCR assays. Histograms represent the number of positive PCR wells with Bio-Evolution (light grey) and Elitech (dark grey) (ranging from 1/3 to 3/3). Calibration curves were drawn based on positive samples with all triplicate wells positive and $SD < 0.5$; they allowed determining slopes and R^2 .

This specimen is a low positive sample. It had been found positive (in one reaction tube out of three) by the laboratory of the

Molecular Biology “Pole” (University Hospital of Montpellier) of the French NRC-T on their own extract analysed with

their “in-house” qPCR using the PCR method published by Reischl et al. (11). This positivity was also supported by the clinical context, as this was a blood specimen collected seven days before a disseminated toxoplasmosis case. This discrepant specimen was finally also found positive with the Elitech assay (in 2/3 reaction wells). In addition, all DNA extracts were found to be free of PCR inhibitors using the internal control (IC) of both assays. Considering QCMD and clinical samples, mean Ct values for IC were 28.97 ± 0.60 and 31.41 ± 0.94 for Bio-Evolution and Elitech assays, respectively. Considering the whole clinical cohort, as observed with spiked samples, mean Ct values were systematically higher for the Elitech assay (mean delta Ct = 1.8 ± 0.7 ; p -value < 0.001).

Good agreement of both PCR assays.

The Ct values obtained using both methods were compared for each of the 54 concordant positive samples and plotted in a Bland-Altman graph (Figure 2A). There was a very good agreement between both methods, with only four samples out of the range of $\pm 1.96SD$. When Ct of Bio-Evolution were plotted against those obtained by Elitech (Figure 2B), correlation was high with $R^2=0,949$.

In addition, the DNA extraction step was also tested on the four amniotic fluids of the cohort and on nine QCMD samples. They were extracted with each of the two DNA extraction cartridges (ELITE InGenius™ SP200 and ELITE InGenius™ SP1000). Whatever the PCR assay, mean Ct values were decreased systematically with SP1000 cartridges, at least 0.22 Ct to 5.93 Ct, and for 2 samples a positive result was obtained only when extraction was performed with SP1000 cartridges (**Table 3**).

Discussion

Design and rationale of the study.

Diagnosis of congenital and disseminated toxoplasmosis mainly relies on molecular diagnosis. The repeated DNA element *rep529* (GenBank accession number AF146527) (11,12) was shown to provide high sensitivity to qPCR assays (9,11) and has become the main DNA target used in most PCR methods, whether laboratory-developed or commercial (1). In this study, both assays target the *rep529* region. The study was conducted to determine if the performances of the Bio-Evolution and Elitech PCR assays are maintained on the easy-to-use sample-to-result ELITE InGenius™ PCR platform. Indeed, the Bio-Evolution PCR assay is not validated on this apparatus by the manufacturer and the previous evaluation of the Elitech PCR assay was performed using manual extraction methods and other thermocyclers (3). The possibility to combine extraction and amplification steps on the same apparatus ensures technical time saving for the lab staff with a reduced hands-on time (13) (this platform has a turnaround time of approximately 2.5 hours, from setup to result) and limits human errors. Yet, it is relevant to check the correct match between the DNA extraction and the PCR method (1). Many clinical specimens (peripheral blood, cord blood, placentas and amniotic fluids) and artificial spiked amniotic fluid samples of external quality controls from QCMD were tested in the study. Cerebrospinal fluid and broncho-alveolar lavage samples were not included. The retrospective nature of the design of the study may induce some limitations as clinical samples included have been frozen since their sampling. Yet it has been previously published that freezing and storage at $-20\text{ }^{\circ}\text{C}$ provides adequate preservation of *Toxoplasma gondii* DNA for retrospective molecular

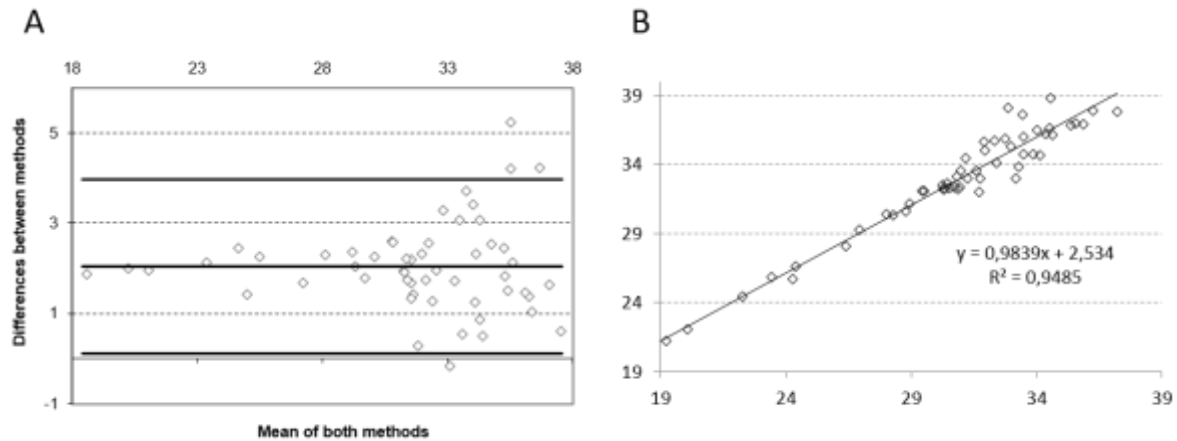


Figure 2. Bland-Altman and correlation plots representing the concordance between both Bio-Evolution and Elitech PCR assays performed on the Elitech platform. A. Bland-Altman plot. For each positive samples by both methods (losange), Ct values were plotted, with the mean of both methods in abscissa and the difference between both methods in ordinate. The thick lines represent the means and the ± 1.96 standard deviations. B. Correlation plot. Ct values obtained by Bio-Evolution PCR assay were plotted against those obtained by Elitech. Linear regression with its equation and R2 values is presented.

Samples	Mean Ct value					
	Bio-Evolution assay			Elitech assay		
	SP200	SP1000	ΔCt^\dagger	SP200	SP1000	ΔCt^\dagger
AF*1	26.40	24.27	2.13	28.08	25.69	2.39
AF*2	34.02	32.40	1.62	36.48	34.13	2.35
AF*3	20.09	17.65	2.44	22.05	19.52	2.53
AF*4	33.85	30.52	3.33	34.71	32.27	2.44
TGDNA20C1-01	34.76 ^s	31.69	3.07	Neg	31.97	/
TGDNA20C1-01	34.54	31.94	2.60	38.77	35.02	3.75
TGDNA20C1-01	31.89	31.26	0.64	35.61	33.00	2.62
TGDNA20C1-04	34.38	33.47	0.91	36.20	34.72	1.48
TGDNA20C1-04	35.57	30.24	5.33	36.94	32.46	4.49
TGDNA20C1-04	34.51 ^s	32.75	1.76	36.64	35.82	0.82
TGDNA20C1-05	Neg	32.31	/	35.94	35.73	0.22
TGDNA20C1-05	33.30 ^s	31.74	1.57	33.85	33.01	0.84
TGDNA20C1-05	32.88 ^s	30.87	2.01	38.12 ^s	32.20	5.93

Table 3. Comparison of two DNA extraction cartridges: SP200 and SP1000.

*AF: amniotic fluid. $^\dagger \Delta Ct$: difference between Ct_{SP200} and Ct_{SP1000} . ^sOnly one positive in the duplicate. Neg: negative result at the first run.

analysis (14). Another potential bias of this study concerns the potential competing effect of the IC in case of low parasitic concentrations. Such effect was not tested

in this study although it was previously described with this IC after manual extraction with EXTRAblood (ELITechGroup) (3). However, as the

expected effect of this IC is deleterious in terms of performance, this does not call into question the conclusions of this study. The amplifications were performed in duplicate, except for the standards which were tested in triplicate. Indeed, it has been shown that the parasitic loads in congenital toxoplasmosis are often very low, with a median concentration in amniotic fluid estimated around 10 tachyzoites per ml (15), and low parasitic loads are also encountered during disseminated toxoplasmosis (16). Hence, (i) the PCR assays used for this diagnosis must be highly sensitive; and (ii) the French NRC-T recommends the analysis of clinical samples in duplicate or triplicate to be able to detect these low parasitic loads (1).

A secondary aim of the study was to test the ELITE InGenius™ SP1000 cartridge on amniotic fluids (allowing the extraction of 1 mL of template), as it is not validated on this matrix contrary to the SP200 cartridge (allowing the extraction of 0.2 mL of template).

DNA extraction step. A main difference between Bio-Evolution and Elitech PCR assays is the absence of an IC for the extraction step in the first one. Controlling DNA extraction is important as a failed extraction may lead to false negative results and cannot be detected in the absence of a specific control. In order to verify this step with Bio-Evolution, a second PCR targeting a commercial or a “laboratory-made” DNA extraction control is required. The Elitech PCR assay uses a stabilized solution containing two plasmid DNAs and the genomic RNA of MS2 phage as IC which checks extraction and amplification steps. When choosing a commercial PCR assay, the presence of an IC should be taken into account, especially in a context of quality management. It is necessary to remain critical concerning the IC chosen by the manufacturer. Indeed, as demonstrated by a recent study, the use of heterologous targets may not be considered as the best choice (17).

Concerning DNA extraction performed with SP1000 cartridge, interestingly, Ct values for *T. gondii* target were significantly decreased as compared to those obtained with the SP200 cartridge. This result is relevant as DNA is theoretically five-time more concentrated with the SP1000 cartridge comparatively with the SP200. For the QCMD samples extracted with the two cartridges, whereas all were positive when extracted with the SP1000 cartridges, several were inconstantly positive or negative when extracted with the SP200 cartridges. Despite the low number of samples tested, the particular interest of this reagent for the amniotic fluid samples is confirmed due to the often low parasitic load. A larger study based on amniotic fluids not artificially spiked with *T. gondii* should be performed to confirm these preliminary results. Centrifugation of large volume of amniotic fluid samples or all pauci cellular samples followed by elimination of the supernatant and testing either 200 µL or 1000 µL could also be tested. Furthermore, it might be interesting to assess the performances of the SP1000 cartridges on whole blood, which has not been validated by the manufacturer for this matrix.

DNA amplification step. Among the 31 clinical specimens tested, one whole blood sample was found inconsistently positive between both PCR assays. The Elitech assay finally found a positive result for this discrepant specimen after re-testing in triplicate (2 out of 3 positives). Among all the QCMD specimens, each PCR assay obtained one false negative result after the first PCR sets, and the results were finally concordant after re-testing in triplicate (1 or 2 out of 3 positives). These cases illustrate the interest of increasing the number of reactions when getting close to the sensitivity threshold of the methods. It would be interesting that PCR assay manufacturers include this recommendation in their manuals despite the potential extra cost induced.

The specificity was excellent for both assays, without any false-positive result. However, contrary to the Elitech assay, the Bio-Evolution assay does not include the use of UDG, which is recommended to prevent carry-over contamination (1).

In total, PCR assays for the molecular diagnosis of toxoplasmosis commercialised by Bio-Evolution and Elitech presented good performances when used with the ELITE InGenius™ PCR platform, in accordance with the published performances with other PCR platforms (2,3). Interestingly, during the evaluation of the Elitech PCR assay performed by Robert-Gangneux et al., a similar

calibrated *Toxoplasma* suspension prepared also by the Molecular Biology “Pole” of the French NRC-T was tested. Compared to those in this study, the results were poorer for the lowest concentrations, i.e. 1 and 0.1 parasite/mL, resulting in lower PCR performance scores (3). Although it is not possible to affirm that the calibration of the initial suspension and the serial dilutions were perfectly comparable between both studies, these discrepancies may highlight the impact of the extraction methods and the PCR platform on PCR performances, particularly for the lowest parasitic loads.

Conclusion

Overall, both PCR assays produced very good and comparable performances on the automated and integrated ELITE InGenius™ PCR platform. They are rapid, accurate, and easy-to-use methods for detection of *T. gondii* in clinical specimens with low parasitic loads, from whole blood to amniotic fluid. The DNA extraction cartridges proposed by the manufacturer of the PCR platform have a real interest for these key samples. The study reminds also the need for replicates.

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C. Nourrisson is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Author contributor roles:

CN: formal analysis, investigation, visualization, writing-original draft; MM: investigation; PP: resources, writing – review and editing; YS: conceptualization, methodology, supervision, writing – review and editing.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Table S1. Raw data. (Available online only)

For clinical samples, final diagnosis of congenital toxoplasmosis is adapted on case definitions according to Lebech *et al.* (6), with the only adjustment that molecular biology diagnosis is assimilated to cultivation approach. For disseminated samples in immunocompromised patients, clinical classification was either 'toxoplasma disease' or 'toxoplasma infection' according to the European Group for Blood and Marrow Transplantation Infectious Diseases Working Party classification (8).

Pos: positive; Neg: negative; Inc. Pos: inconstantly positive; AF: amniotic fluid; NN: neonatal; EQA: external quality assessment; SDA: serial dilution assay; *: related sample