Evaluation of Toxoplasma ELITe MGB Real-Time PCR Assay for Diagnosis of Toxoplasmosis

Florence Robert-Gangneux, Marie-Pierre Brenier-Pinchart, Hélène Yera, Sorya Belaz, Emmanuelle Varlet-Marie, Patrick Bastien, Yvon Sterkers

Evaluation of the *Toxoplasma* ELITe MGB® real-time PCR assay for the diagnosis of toxoplasmosis

Florence Robert-Gangneux [1,5,7], Marie-Pierre Brenier-Pinchart [2,3,7], Hélène Yera[4,7], Sorya Belaz [1,5], Emmanuelle Varlet-Marie [6,7], Patrick Bastien [6,7], and the Molecular Biology study group of the French National Reference Center for Toxoplasmosis §

[1] Laboratoire de Parasitologie-Mycologie, Centre Hospitalier Universitaire de Rennes, Rennes, France

[2] Laboratoire de Parasitologie-Mycologie, Institut de Biologie et Pathologie, Centre Hospitalier Universitaire de Grenoble, France

[3] Institute for Advanced Biosciences, INSERM U1209 - CNRS UMR 5309, Université Grenoble Alpes, Grenoble, France

[4] Laboratoire de Parasitologie-Mycologie, Hôpital Cochin, Hôpitaux Universitaires Paris Centre, AP-HP, Faculté de Médecine, Université Paris Descartes, Paris, France;

[5] IRSET, Inserm U1085, Université Rennes 1, Rennes, France

[6] Département de Parasitologie-Mycologie, Centre Hospitalier Universitaire de Montpellier, Montpellier & UMR CNRS 5290 - IRD 224 - Université de Montpellier, France

[7] Centre National de Référence de la Toxoplasmose, Laboratoire Associé "Biologie moléculaire", France

Key-words: Toxoplasma gondii, PCR, Diagnosis, Congenital toxoplasmosis, rep529

Running title: Commercial PCR assay for toxoplasmosis diagnosis

ABSTRACT

Molecular diagnosis of congenital toxoplasmosis or disseminated toxoplasmosis is mainly based on PCR. The repeated DNA element rep529 has become the main DNA target used in most, whether laboratory-developed or commercial, PCR methods. In this multicenter study, we evaluated the *Toxoplasma* ELITe MGB® (Elitech®) commercial kit, by comparison with three reference quantitative PCR assays (RA) used in routine in three proficient laboratories of the French National Reference Center for Toxoplasmosis network, using *Toxoplasma* calibrated suspensions diluted to obtain a range of concentrations from 0.1 to 10,000 parasites/mL. These suspensions were extracted either with the DNA extraction kit (EXTRAblood®, Elitech®) recommended by the manufacturer or the QIAamp DNA-minikit (Qiagen). The *Toxoplasma* ELITe MGB® assay was also evaluated on a panel of 128 clinical samples, including 56 amniotic fluid, 55 placenta, and various other samples, of which 95 originated from patients with proven toxoplasmosis. The ELITe MGB assay amplified less frequently low-concentration replicates (<10 parasites/mL) of calibrated suspensions, than the RA of 2/3 laboratories. Additionally, the combination EXTRAblood® / *Toxoplasma* ELITe MGB® yielded poorer sensitivity than the combination QIAmp DNA-minikit/ ELITe MGB® for low parasite concentrations ($p<0.001$ for 1 parasite/mL). On clinical samples, the sensitivity and the specificity of the commercial assay were 89% and 100%, respectively. The sensitivity ranged from 79% to 100% with placenta and amniotic fluid samples, respectively. Overall, this study shows that the *Toxoplasma* ELITe MGB® assay is suitable for the diagnosis of toxoplasmosis from non-cell-rich or non-hemoglobin-rich samples, and that the EXTRAblood® kit is not optimal.

INTRODUCTION

Toxoplasmosis is a worldwide parasitic disease due to the intracellular coccidian parasite *Toxoplasma gondii*. Molecular diagnosis is an essential tool for the diagnosis of congenital toxoplasmosis, as well as of acute disease in immunocompromised patients with primary infection or reactivation of past infection (1). Prenatal diagnosis of congenital toxoplasmosis relies on *Toxoplasma* DNA detection in amniotic fluid (AF), and has been largely evaluated in field studies, particularly in French series, as a national prevention program implemented in 1992 requires a monthly serologic follow-up of seronegative pregnant women and recommends the realization of amniocentesis when a primary infection is documented. In France, 21 University Hospitals have a ministerial agreement for the prenatal diagnosis of congenital toxoplasmosis. Since several years now, all of them have moved for the use of real-time PCR (rtPCR) methods targeting the repeated DNA element *rep529* (GenBank accession number AF146527), as the sensitivity provided using this DNA target has proved higher than that of the formerly used B1 gene in most studies (2-5). About three quarters of these reference laboratories still use 'in-house' or laboratory-developed rtPCR techniques which have been evaluated in clinical studies; but there is an increasing trend to use commercial assays, which, in spite of being more expensive, are easier to use and allow a better quality management than the former. The manufacturers of these kits announce a sensitivity threshold, but the performance of these assays may be altered by the tested sample type or the DNA extraction method used. Indeed, *Toxoplasma* may be searched not only for the prenatal diagnosis of congenital toxoplasmosis, but also in immunocompromised patients in other sample types, such as blood, broncho-alveolar lavage fluid, cerebrospinal fluid (CSF), aqueous humor (AH) or various biopsies (6). In view of the globally excellent performances of 'in-house' methods in proficient diagnostic centers

Journal of Clinical Microbiology

(7), the evaluation of the analytical and clinical performances of commercial kits is absolutely needed to ensure the quality of results in routine use. Moreover, some commercial assays are validated for *Toxoplasma* detection in AF, but not in other sample types. In this multicenter study, we evaluated the performances of the *Toxoplasma* ELITe MGB® kit (Elitech®, Puteaux, France), a *rep529*-targeting assay, using serial dilutions of calibrated *Toxoplasma* suspensions in AF, and clinical samples including AF, placenta and various other types of samples.

MATERIALS & METHODS

Participating centers

The three participating centers (University Hospitals of Paris-Cochin, Rennes and Grenoble, France) are part of the Molecular Biology workgroup of the French National Reference Center for Toxoplasmosis (NRCT: http://cnrtoxoplasmose.chu-reims.fr/?lang=en), and have a ministerial agreement for the prenatal diagnosis of toxoplasmosis. They participate to national external quality controls on a regular basis with satisfactory results.

Samples

Calibrated Toxoplasma suspensions

The three participating centers used a calibrated *Toxoplasma* (type II) suspension produced by the Molecular Biology workgroup of the NRCT (University Hospital of Montpellier)(8). DNA was previously extracted from the suspension in each center using their routine method for molecular diagnosis (here, QIAamp DNA mini-kit®, Qiagen, Les Ulis, France). The three labs compared the sensitivity of detection of serial dilutions (from 10,000 tachyzoites/mL to 0.1/mL) after amplification using their own in-house PCR method (so-

called reference assay (RAs, Table 1) and the *Toxoplasma* ELITe MGB® kit, following the manufacturer's instructions. All dilution points were amplified in triplicate or quadruplicate. Another batch of calibrated suspension was extracted in parallel, using either the manual extraction device recommended by the manufacturer, i.e. EXTRAblood® (Elitech), or with the QIAamp DNA mini-kit®. In this experiment, the internal control was added at the time of extraction, following the manufacturer's instructions (see below).

Additionally, one center also evaluated a batch of 10 samples of external quality controls (EQC) from QCMD® (2014). This QC batch (TGDNA 14) consisted of 5 vials of lyophilized amniotic fluid samples spiked with various concentrations of *T. gondii* or unspiked (negative), and 5 vials of lyophilized plasma samples spiked with various concentrations of *T. gondii* or unspiked (negative), and was extracted using EXTRAblood® (Elitech).

Clinical samples

This part of the work used stored DNA from clinical samples obtained during routine molecular diagnosis (2005-2015). The three reference laboratories selected *Toxoplasma*-positive and -negative DNA preserved at -20°C or -80°C following routine molecular diagnosis (9). Thanks to the French prevention program for congenital toxoplasmosis, reference centers have a collection of samples (mainly AF and placenta samples) from patients with confirmed diagnosis. As already evaluated in previous studies, long-term storage of DNA at -20°C or below does not alter the result of *Toxoplasma* real-time PCR (3, 9), thus allowing the use of collections for diagnostic evaluations. Placenta and AF DNA samples from fetuses with suspected congenital toxoplasmosis were classified as true-positive or true-negative on the basis of the newborn serological follow-up (detection of specific IgM or IgA, and comparison of mother and newborn antibody profiles by western-blotting during a one-year follow-up),

so that the diagnosis could be definitely confirmed or ruled out, as previously described (10-13). The Elitech assay was certified CE-IVD in 2013 and validated for amniotic fluid samples and whole blood, thus we considered that placenta samples were comparable to blood samples. Other samples were collected from patients with retinochoroiditis or from immunocompromised patients, for whom the clinical diagnosis was recorded (disseminated, cerebral, or toxoplasmosis excluded). Overall, 128 DNA samples were included: 55 isolated from placentas, 56 from AF, 4 from AH, 6 leukocyte pellets isolated from buffy-coats, 2 from biopsies, and 5 from CSF. Of these, 33 samples were classified as PCR-negative and 95 as PCR-positive, according to serological and clinical follow-up of patients.

Molecular techniques

DNA extraction of clinical specimens

Clinical specimens were processed in the setting of routine diagnosis. After appropriate pre-analytical steps (centrifugation of fluids, buffy-coat, pre-digestion of placenta or biopsies with proteinase K), 200 µL of clinical samples were extracted using the QIAamp DNA mini-kit® according to the manufacturer's instructions, and eluted in 100 µL (all three centers used the same technique for routine diagnosis).

DNA extraction of* Toxoplasma *calibrated suspensions

In each center, the same batch of *Toxoplasma* calibrated suspension was extracted using QIAamp DNA mini-kit® and amplified in parallel with the RA and the ELITe MGB assay. In one center, one batch of calibrated suspension was also extracted using EXTRAblood®, with or without adding 5 µL of internal control (IC)(CPE-DNA Internal Control, Elitech®), following the manufacturer's instructions. Briefly, 200 µL of samples were mixed with 25 µL of proteinase K, 200 µL of lysis buffer, 10 µL of carrier RNA and 5 µL of IC, incubated at 70°C for

10 min, then centrifuged for 5 sec at 11,000 rpm. After addition of 210 µL of absolute ethanol and a brief centrifugation, the lysate was loaded into a column. After several washing steps, DNA was eluted in 60 µL of buffer. Quality control samples (QCMD® 2014) were also extracted using this technique.

DNA amplification

Reference methods

The in-house methods used by the three participants targeted the *rep529* sequence and has been previously evaluated and published (5, 8, 10, 14). Technical details can be found in Table 1. All three RA satisfy annual external quality control program, managed by the National Reference Center for Toxoplasmosis (Centre Hospitalier Universitaire de Montpellier). All DNA from clinical samples were re-amplified in parallel with the RA method and the ELITe MGB® assay. Newly-extracted *Toxoplasma* suspensions were amplified with both techniques.

Toxoplasma ELITe MGB® method

The *Toxoplasma* ELITe MGB® assay was performed according to the manufacturer's instructions, using 10 µL of the DNA template and 20 µL of mix. When the Elitech IC was not added during extraction (calibrated suspensions or clinical samples previously extracted using the QIAamp DNA mini-kit®), 2 µL of Elitech IC was added in 10 µL of template DNA, and 10 µL of this solution were used for amplification, as suggested by the manufacturer.

The assay has been validated only on Applied Biosystems devices. In this study, ELITe MGB® amplification was performed using a StepOnePlus® device or ABI® Prism 7000 (ThermoFisher), and the following program: 2 min at 50°C, 2 min at 94°C, and 45 cycles of 10 sec at 94°C, 30 sec at 60°C and 20 sec at 72°C. Each clinical sample was analyzed in a single

Journal of Clinical Microbiology

reaction, as well as the QCMD® EQC DNA. If the result was not concordant with that of the previous routine diagnosis, a second PCR was performed with the Elitech® method and the in-house PCR. The LOD (95% sensitivity) announced by the manufacturer in whole blood samples is 34.29 or 88.72 *T. gondii* /mL, and 5.47 or 1.91 *T. gondii*/extraction in AF samples, using manual extraction (EXTRAblood®, Elitech) or automated extraction (NucliSENS EasyMAG®, BioMérieux), respectively.

Performance score

For each technique, a performance score was calculated from the results obtained with calibrated suspensions, as follows: number of positive replicates/total number of amplifications.

Statistical analysis

The Mann-Whitney test was used to compare the mean Ct obtained with qPCR assays for the calibrated *Toxoplasma* suspensions. When the number of positive replicates was below three, a two-way ANOVA analysis was used instead.

For clinical samples, the analysis of qualitative results obtained with both techniques was analyzed using a Chi-square test or a Fisher's exact test.

Statistical analysis was made using GraphPad® Prism V5 (GraphPad software, USA). $P < 0.05$ was considered significant.

RESULTS

Comparative testing using* Toxoplasma *calibrated suspensions

The first step of the study was to determine the PCR performance scores using serial dilutions of calibrated *Toxoplasma* DNA suspension. PCR performance scores were

calculated as described elsewhere (7, 15), using the *T. gondii* (Tg) DNA serial dilution assay, and are reported in Table 2. In all three centers, the scores obtained using the in-house method and the Elitech® assay were close, but the RA method had a higher score than the commercial assay in two out of three centers ($p<0.01$, Table 2). Taken together, the lowest parasite concentrations, i.e. "1" and "0.1" *Toxoplasma*/mL, were inconstantly amplified. For "1 T.g./mL, the difference was statistically significant (5 positive out of 10 replicates and 8 out of 10 replicates with ELITe MGB and RAs, respectively)($p<0.01$).

The mean cycle threshold (Ct) of amplification obtained with *Toxoplasma* ELITe MGB® was calculated for concentrations of calibrated suspensions between 1 and 10,000 Tg/mL, and the results were compared for the two DNA extraction methods. Surprisingly, the mean Cts obtained with the combination EXTRAblood®/Elite MGB® (recommended by the manufacturer) were significantly higher than those obtained with the combination QIAamp ®/Elite MGB® (29.2±0.17 versus 27.8±0.18, $p<0.05$ for 100 Tg/mL and 39.3±0.07 versus 34.6±0.4, $p<0.001$ for 1 Tg/mL) (Figure 1).

Comparative testing using clinical samples

When using clinical samples, the concordance between the in-house method and the Elite MGB® assay was 92% (118/128). All negative DNAs were tested negative (33/33); but false negative results were obtained using the Elite MGB® kit as compared with the RAs for 10 out of 95 positive samples, yielding a specificity of 100% and a relative sensitivity of 89.5% for the kit. This sensitivity was 100% for AF (38/38) and other fluid samples (AH, CSF), but only 79% (34/43) for placenta and 80% (4/5) for buffy coat samples (Table 3). In 9/10 cases, the false negative results were obtained for placenta samples with high Ct values (>37). To rule out PCR inhibition, samples were diluted to $1/10^{th}$ and re-tested. Additionally, as the nature

of the Elitech® IC was not known and could be suspected to interfere with small amounts of parasite DNA, these false negative samples were also tested without IC. Amplification was restored from plain DNA in the absence of IC (2 cases, with Ct of 39.7 and 40.7) and after dilution to 1/10th and absence of IC in one case (Ct = 36.7). No amplification was observed after dilution to 1/10th when IC was not removed.

Overall, the sensitivity for fluid samples was higher than for cellular samples (100% versus 80%, p<0.01)(Table 3).

Finally, the qualitative results obtained using the QCMD® 2014 quality control samples were concordant for the RA1 and Elite MGB® assays (7/7 positive samples and 3/3 negative samples; Table 3). Mean Cts obtained with both techniques were not statistically different (p=0.8, data not shown).

Influence of IC on amplification performances

As the observation above suggested a competing effect of the IC, another series of DNA extractions of a calibrated *Toxoplasma* suspension using different conditions was launched to confirm this hypothesis. It appeared that DNA extraction with EXTRAblood® (with 5 µL of IC added in the sample as recommended) followed by amplification with Elite MGB® performed as well as extraction with EXTRAblood® followed by addition of IC at the time of amplification with ELITe MGB® (Figure 2A). Surprisingly, at the "1 Tg/mL" concentration, the mean Ct was significantly lower when the IC was withdrawn from the extraction and amplification steps than that obtained after extraction with EXTRAblood® including IC (33.57 ±0.93 *versus* 38.75 ±1.18, p<0.001) (Figure 2A). Similarly, the addition of the IC to the mix of amplification also led to an increase of the mean Ct at the "1 Tg/mL" concentration (33.57 ±0.93 *versus* 37.97 ±0.007, p<0.001). In another experiment, both extraction methods

(QIAamp DNA mini-kit® and EXTRAblood®) were compared using the same amplification method (RA1). Whatever the parasite concentration, Ct values were much lower when DNA was extracted with the QIAamp DNA mini-kit® than with EXTRAblood® (36.62 ± 1.38 *versus* 45, and 21.52 ±0.08 *versus* 25.35 ±0.2, for the lowest and highest parasite concentrations, respectively; p<0. 001) (Figure 2B).

DISCUSSION

In the diagnosis of congenital toxoplasmosis, a high sensitivity of the PCR assay is needed, as parasite loads in amniotic fluids from congenitally infected infants are frequently as low as 10 tachyzoites/mL or less (16). Early treatment of pregnant women who benefit from serological screening in France, could account for such low parasite loads (17). High performance of molecular assays is also a key issue in immunocompromised patients, for whom rapid and accurate diagnosis is essential. Furthermore, toxoplasmosis is of increasing importance in HIV-negative immunocompromised patients, due to the growing number of transplantations and to the use of immunosuppressive drugs for the treatment of chronic inflammatory diseases (6). Here, when applied to calibrated DNA samples, the *Toxoplasma* ELITe MGB® assay showed similar performance as the three laboratory-developed reference methods down to 10 T.g/mL, but the lowest concentrations were inconstantly detected (Table 2), despite a higher DNA input into the amplification reaction (10 µL) than in the reference methods (Table 1).

When using clinical samples, the *Toxoplasma* ELITe MGB® assay showed a 100% sensitivity for amniotic fluids, and yielded congruent results on a batch of external quality controls (QCMD® 2014). Although the kit is commercialized, i.e. validated, only for amniotic fluids

and whole blood samples, we included other sample types which are frequently sent to laboratories for diagnosis, such as aqueous humor or CSF. We also included placenta samples, which offer the advantage to mimic blood samples and to be linked to a clinical history and follow-up of neonates, thus can be definitely classified as true-positive or not. The sensitivity for other fluid samples was as good as for AF, although the number of samples included was small. However, the sensitivity was much lower for other sample types, in particular placenta samples. These results show that this commercial assay performs better on samples with low cellularity or reduced contamination with red blood cells, which confirms the lower sensitivity using whole blood, reported by the manufacturer himself. As routinely done in the three reference laboratories, when a PCR inhibition is suspected from the observation of high Ct values with their IC, samples were re-tested after sample dilution. This strategy was thus applied to the false-negative samples obtained with the ELITe MGB assay, but it didn't fit for amplification restoration. Additionally, as the nature of the Elitech® IC was not known and could be suspected to interfere with small amounts of parasite DNA, these false-negative samples were also tested without IC. This allowed to restore *Toxoplasma* amplification in 30% of these samples, thus demonstrating that competition of the Elitech IC could partially explain the false negative results. The inhibitory effect of the Elitech® IC was definitely confirmed using low concentrations of *Toxoplasma* calibrated suspensions (Figure 2A). However, the technical instructions from the manufacturer do not mention that the reactions should be performed in duplicate, plain and diluted, or with and without IC, as this significantly augments the cost of the test. With respect to the interpretation of IC amplification, it is stated in the manufacturer's instructions that a Ct ≤ 35 is suitable for a correct interpretation of results. During our

Journal of Clinical Microbiology

assays, the Ct obtained for IC was always < 30, thus it could be reasonably considered that the test was valid; yet false negative results have been found.

As the DNA extraction method is also known to influence the performance of PCR amplification (18), we decided to compare the Ct results of calibrated parasite suspensions, extracted using either the extraction kit (EXTRAblood®) as recommended by the manufacturer, or the QIAamp DNA blood mini-kit®, as routinely done in the three labs. Subsequent amplification with the RA1 showed that the combination QIAamp® extraction/RA1 performed much better than the combination EXTRAblood®/RA1 (Figure 2B), stressing the need to evaluate the extraction and PCR methods together, as emphasized earlier (19). Moreover, the combination EXTRAblood®/Elite MGB (recommended by the manufacturer) performed significantly worse than the combination QiaAmp®/Elite MGB to amplify low parasite concentrations (Figure 1), thus should be avoided. The evaluation of the combination extraction / amplification methods is of peculiar importance for off-label use of commercial PCR assays, particularly with cell-rich samples types, as its efficiency is determining to detect very low parasite amounts. Furthermore, the validation of the entire process is necessary to meet the requirements of quality assurance system.

Regarding the handiness and good laboratory practices, the mix provided in the kit contains uracil-*N*-glycosylase (UNG) to limit carryover contaminations from previously amplified PCR products. The kit does not include a standard to perform a curve to quantify parasite loads. The PCR assay, the internal control and the positive control are purchased in three separate vials, thus the management of batch traceability is not easy.

Several qPCR commercial assays are available to date to detect *Toxoplasma* DNA, but very few have been evaluated using clinical samples or in routine use. A previous multicenter

Journal of Clinical Microbiology

study conducted by the Molecular Biology Group of the French NRCT evaluated another commercial kit by Bio-Evolution® using 157 amniotic fluid samples, and found a 99% concordance for *T. gondii*-infected samples (13). In the present study, we enrolled a smaller amount of amniotic fluid samples (56), but the *Toxoplasma* ELITe MGB® assay showed also a good concordance using these samples.

Taken together, this study showed that the *Toxoplasma* ELITe MGB® assay appears suitable for prenatal diagnosis. However, a note of caution is in order when using cell-rich or hemoglobin-rich samples, as this carries the risk of false negative results. In our hands, the use of the EXTRAblood® DNA extraction lowered the performance of the *Toxoplasma* ELITe MGB® assay for low parasite concentrations, and dramatically lowered the sensitivity of the in-house PCR method tested. Like for all molecular diagnostic methods, clinical microbiologists who would aim at implementing this technique in their laboratory should evaluate the combination of extraction and amplification methods before changing one or the other.

ACKNOWLEDGMENTS

This study was in part funded by the Institut de Veille Sanitaire (InVS) through financial support of the Molecular Biology Group of the French National Reference Centre for Toxoplasmosis (Centre National de Référence de la Toxoplasmose).

§ Molecular Biology study group of the French National Reference Center for Toxoplasmosis: Patrick Bastien (University Hospital of Montpellier), Marie-Pierre Brenier-Pinchart (University Hospital of Grenoble), Sophie Cassaing (University Hospital of Toulouse), Frédéric Dalle

(University Hospital of Dijon), Laurence Delhaes (University Hospital of Bordeaux), Denis Filisetti (University Hospital of Strasbourg), Jean Ménotti (University Hospital of Saint-Louis, Paris), Hervé Pelloux (University Hospital of Grenoble), Florence Robert-Gangneux (University Hospital of Rennes), Yvon Sterkers (University Hospital of Montpellier), Feriel Touafek (University Hospital of Pitié-Salpêtrière, Paris), Emmanuelle Varlet-Marie (University Hospital of Montpellier), Hélène Yera (University Hospital of Cochin, Paris).

REFERENCES

1. **Robert-Gangneux F, Darde ML.** 2012. Epidemiology of and diagnostic strategies for toxoplasmosis. Clin Microbiol Rev **25:**264-296.
2. **Cassaing S, Bessieres MH, Berry A, Berrebi A, Fabre R, Magnaval JF.** 2006. Comparison between two amplification sets for molecular diagnosis of toxoplasmosis by real-time PCR. J Clin Microbiol **44:**720-724.
3. **Belaz S, Gangneux JP, Dupretz P, Guiguen C, Robert-Gangneux F.** 2015. A 10-year retrospective comparison of two target sequences, REP-529 and B1, for Toxoplasma gondii detection by quantitative PCR. J Clin Microbiol **53:**1294-1300.
4. **Kasper DC, Sadeghi K, Prusa AR, Reischer GH, Kratochwill K, Forster-Waldl E, Gerstl N, Hayde M, Pollak A, Herkner KR.** 2009. Quantitative real-time polymerase chain reaction for the accurate detection of Toxoplasma gondii in amniotic fluid. Diagn Microbiol Infect Dis **63:**10-15.
5. **Reischl U, Bretagne S, Kruger D, Ernault P, Costa JM.** 2003. Comparison of two DNA targets for the diagnosis of Toxoplasmosis by real-time PCR using fluorescence resonance energy transfer hybridization probes. BMC Infect Dis **3:**7.
6. **Robert-Gangneux F, Sterkers Y, Yera H, Accoceberry I, Menotti J, Cassaing S, Brenier-Pinchart MP, Hennequin C, Delhaes L, Bonhomme J, Villena I, Scherer E, Dalle F, Touafek F, Filisetti D, Varlet-Marie E, Pelloux H, Bastien P.** 2015. Molecular diagnosis of toxoplasmosis in immunocompromised patients: a 3-year multicenter retrospective study. J Clin Microbiol **53:**1677-1684.
7. **Sterkers Y, Varlet-Marie E, Cassaing S, Brenier-Pinchart MP, Brun S, Dalle F, Delhaes L, Filisetti D, Pelloux H, Yera H, Bastien P.** 2010. Multicentric comparative analytical

Journal of Clinical Microbiology

performance study for molecular detection of low amounts of Toxoplasma gondii from simulated specimens. J Clin Microbiol **48:**3216-3222.

8. **Varlet-Marie E, Sterkers Y, Brenier-Pinchart MP, Cassaing S, Dalle F, Delhaes L, Filisetti D, Pelloux H, Touafek F, Yera H, Bastien P.** 2014. Characterization and multicentric validation of a common standard for Toxoplasma gondii detection using nucleic acid amplification assays. J Clin Microbiol **52:**3952-3959.

9. **Delhaes L, Filisetti D, Brenier-Pinchart MP, Pelloux H, Yera H, Dalle F, Sterkers Y, Varlet-Marie E, Touafek F, Cassaing S, Bastien P.** 2014. Freezing and storage at -20 degrees C provides adequate preservation of Toxoplasma gondii DNA for retrospective molecular analysis. Diagn Microbiol Infect Dis **80:**197-199.

10. **Robert-Gangneux F, Dupretz P, Yvenou C, Quinio D, Poulain P, Guiguen C, Gangneux JP.** 2010. Clinical relevance of placenta examination for the diagnosis of congenital toxoplasmosis. Pediatr Infect Dis J **29:**33-38.

11. **Delhaes L, Yera H, Ache S, Tsatsaris V, Houfflin-Debarge V.** 2013. Contribution of molecular diagnosis to congenital toxoplasmosis. Diagn Microbiol Infect Dis **76:**244-247.

12. **Filisetti D, Yera H, Villard O, Escande B, Wafo E, Houfflin-Debarge V, Delhaes L, Bastien P.** 2015. Contribution of neonatal amniotic fluid testing to diagnosis of congenital toxoplasmosis. J Clin Microbiol **53:**1719-1721.

13. **Filisetti D, Sterkers Y, Brenier-Pinchart MP, Cassaing S, Dalle F, Delhaes L, Pelloux H, Touafek F, Varlet-Marie E, Yera H, Candolfi E, Bastien P.** 2015. Multicentric comparative assessment of the bio-evolution Toxoplasma gondii detection kit with eight laboratory-developed PCR assays for molecular diagnosis of congenital toxoplasmosis. J Clin Microbiol **53:**29-34.

14. **Talabani H, Asseraf M, Yera H, Delair E, Ancelle T, Thulliez P, Brezin AP, Dupouy-Camet J.** 2009. Contributions of immunoblotting, real-time PCR, and the Goldmann-Witmer coefficient to diagnosis of atypical toxoplasmic retinochoroiditis. J Clin Microbiol **47:**2131-2135.

15. **Morelle C, Varlet-Marie E, Brenier-Pinchart MP, Cassaing S, Pelloux H, Bastien P, Sterkers Y.** 2012. Comparative assessment of a commercial kit and two laboratory-developed PCR assays for molecular diagnosis of congenital toxoplasmosis. J Clin Microbiol **50:**3977-3982.

16. **Romand S, Chosson M, Franck J, Wallon M, Kieffer F, Kaiser K, Dumon H, Peyron F, Thulliez P, Picot S.** 2004. Usefulness of quantitative polymerase chain reaction in amniotic fluid as early prognostic marker of fetal infection with Toxoplasma gondii. Am J Obstet Gynecol **190:**797-802.

17. **Wallon M, Peyron F, Cornu C, Vinault S, Abrahamowicz M, Kopp CB, Binquet C.** 2013. Congenital toxoplasma infection: monthly prenatal screening decreases transmission rate and improves clinical outcome at age 3 years. Clin Infect Dis **56:**1223-1231.

18. **Yera H, Filisetti D, Bastien P, Ancelle T, Thulliez P, Delhaes L.** 2009. Multicenter comparative evaluation of five commercial methods for toxoplasma DNA extraction from amniotic fluid. J Clin Microbiol **47:**3881-3886.

19. **Bastien P, Procop GW, Reischl U.** 2008. Quantitative real-time PCR is not more sensitive than "conventional" PCR. J Clin Microbiol **46:**1897-1900.

410 **LEGENDS TO FIGURES**

411 **Figure 1:** Evaluation of the *Toxoplasma* Elite MGB® kit using serial dilutions of *Toxoplasma*
412 calibrated suspensions. Calibrated suspensions were extracted using either QIAamp DNA
413 mini-kit® or Extrablood®, before being amplified using *Toxoplasma* Elite MGB®.
414 Amplifications were performed in triplicate. Data are expressed as means ± SEM; *, $p<0.05$,
415 ***, $p<0.001$.

416

417 **Figure 2:** Evaluation of the impact of the internal control (IC) and of the extraction method
418 on amplification efficacy. (A) Comparison of the Ct of amplification obtained with Elite MGB®
419 PCR on DNA extracted with EXTRAblood®, with addition of internal control (+IC) in the
420 sample before extraction, or without internal control (-IC), or after addition of IC in DNA at
421 the time of amplification; (B) Comparison of the Ct obtained with the reference assay 1
422 (RA1) after DNA extraction with EXTRAblood® or with QIAamp DNA mini-kit®. Amplifications
423 were performed in triplicate; data are expressed as means ± SEM; **, $p<0.01$, ***, $p<0.001$.

424
425
426
427
428

429 **Table 1**: Technical description of the four PCR methods used

430

Technique	DNA target	Type of probe	DNA input (µL)	Internal control	No of cycles	PCR device
RA1	rep529	Taqman (FAM-TAMRA)	5	Universal extraction & inhibition DNA control (Diagenode®)	40	StepOnePlus® (ThermoFisher)
RA2	rep529	Taqman	5	non-competitive exo-genous DNA inserted in PCR 2.1 vector	50	AB7000® (ThermoFisher)
RA3	rep529	FRET	7	PhiX (DNA Bacteriophage)	40	LC2.0® (ThermoFisher)
Toxoplasma Elite MGB®	rep529	Taqman (FAM-MGB)	10	Artificial DNA sequence	45	StepOnePlus® (ThermoFisher)

431 RA: reference assay

432

433

434 **Table 2:** Performance scores (No of positive reactions/No of reactions performed) for the

435 four PCR assays using DNA serial dilutions of a calibrated *Toxoplasma* suspension

436

Concentration	Lab 1			Lab 2		Lab 3	
	RA1 (Qiagen)	Elite MGB (Qiagen)	Elite MGB (Extrablood+IC)	RA2 (Qiagen)	Elite MGB (Qiagen)	RA3 (Qiagen)	Elite MGB (Qiagen)
10,000 Tg/mL	3/3	3/3	3/3	3/3	3/3	4/4	4/4
1,000 Tg/mL	3/3	3/3	3/3	3/3	3/3	4/4	4/4
100 Tg/mL	3/3	3/3	3/3	3/3	3/3	4/4	4/4
10 Tg/mL	3/3	3/3	3/3	3/3	3/3	4/4	4/4
1 Tg/mL	3/3	2/3	2/3	1/3	0/3	4/4	3/4
0.1 Tg/mL	0/3	0/3	0/3	0/3	1/3	1/4	1/4
PCR performance score (%)	15/18* (83.3)	14/18 (77.8)	14/18 (77.8)	13/18 (72.2)	13/18 (72.2)	21/24* (87.5)	20/24 (83.3)

437 Tg, *Toxoplasma gondii*; RA, reference assay

438 *p<0.01, compared to Elite MGB/Qiagen

439

440 **Table 3**: Performances of the *Toxoplasma* ELITe MGB® assay using clinical samples,

441 according to the sample type

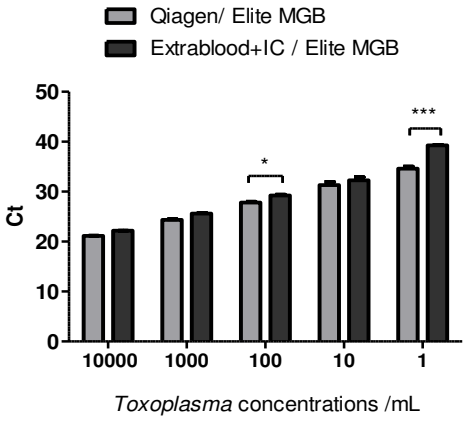
442

443

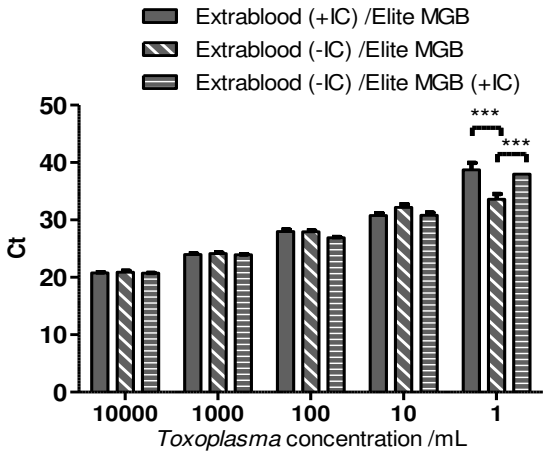
Sample type	Sensitivity % (n/N)	Specificity % (n/N)
Clinical samples (n = 128)	89.5 (85/95)	100 (33/33)
Amniotic fluid (n = 56)	100 (38/38)	100 (18/18)
Other fluids[a] (n = 9)	100 (7/7)	100 (2/2)
Placenta (n = 55)	79 (34/43)	100 (12/12)
Blood (buffy coat) (n = 6)	80 (4/5)	100 (1/1)
Biopsies (n = 2)	100 (2/2)	na
QCMD® samples (n = 10)	100 (7/7)	100% (3/3)

444 [a] consisting in 5 cerebrospinal fluid and 4 aqueous humor samples

445 na, not applicable



A



B