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Multicentric comparative assessment of the commercial Bio-Evolution® *Toxoplasma gondii* detection kit with eight laboratory-developed PCR assays for the molecular diagnosis of congenital toxoplasmosis

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Abstract

Detection of *Toxoplasma gondii* in amniotic fluid is an essential tool for the prenatal diagnosis of congenital toxoplasmosis. The most efficient method for this is PCR. Although some consensus is emerging, this molecular diagnosis suffers from a lack of standardization and an extreme diversity of laboratory-developed methods. Commercial kits for the detection of *T. gondii* by PCR have been recently developed and offer certain advantages; however they must be assessed in comparison with optimized reference PCR assays. The present multicentric study aimed at comparing the commercial Bio-Evolution® *Toxoplasma gondii* detection kit with laboratory-developed PCR assays set up in eight proficient centers in France. The study compared 157 amniotic fluid samples and found a concordance of 99% and 100% using 76 *T. gondii*-infected samples and 81 uninfected samples, respectively. Moreover, taking into account the classification of the European Research Network on Congenital Toxoplasmosis, the overall diagnostic sensitivity of all assays was identical and calculated at 86% (54/63); specificity was 100% for all. Finally, the relative quantification results were in good agreement between the kit and the laboratory-developed assays. The good performances of this commercial kit are probably in part linked to the use of a number of good practices: detection in multiplicate, amplification of the repetitive DNA target ‘rep529’, use of an internal control for the detection of PCR inhibitors. The only drawbacks noted at the time were the absence of Uracil-N-glycosylase, as well as small defects in the reliability of the production of different reagents.

Introduction. Toxoplasmosis is a worldwide infectious disease, usually asymptomatic and not severe in humans except in certain circumstances. Thus, when primarily acquired during pregnancy, *Toxoplasma gondii* can lead to fetal infection, *i.e.* congenital toxoplasmosis. The diagnosis of congenital toxoplasmosis may prove a difficult task, combining clinical features and results from a battery of serologic and molecular tests in the prenatal, neonatal and postnatal periods (1). In France, the prenatal diagnosis of congenital toxoplasmosis has been based on PCR using amniotic fluid (AF) since the 1990s, when it superseded former methods based upon *Toxoplasma* isolation in fetal blood and AF by mouse inoculation, and the detection of specific antibodies in fetal blood (2-4). In France, amniocentesis is performed at least 4 weeks after *Toxoplasma* infection of the mother, but not before the 18th week of amenorrhea (<http://cnrtoxoplasmose.chu-reims.fr>); it is followed by PCR-based molecular diagnosis. A positive *Toxoplasma*-PCR result affirms congenital toxoplasmosis; a combinative treatment using pyrimethamine and sulfadiazine/sulfadoxine is then started to limit the presence of sequelae in the fetus, thus increasing the frequency of asymptomatic infection at birth. When *Toxoplasma*-PCR is negative, congenital toxoplasmosis cannot be ruled out, due to a rate of falsely negative results which, thanks to the constant progress of molecular methods (5) has been reduced to 10-20% (1, 6, 7). Using a high quality molecular diagnosis, and in spite of the persistence of false negatives, post-test risk curves using both negative and positive results can now prove

highly informative, allowing a good assessment of the actual risk of congenital toxoplasmosis (1). A national screening of acute *Toxoplasma* infection has been effective in pregnant women in France for decades (1, 6-8). Within this frame, only authorized proficient centers are able to realize this molecular diagnosis. In 2012, 186 cases of congenital toxoplasmosis were diagnosed in France, indicating a prevalence of 0.226 cases per 1000 births. In 72 cases of these, amniocenteses were performed, and the *Toxoplasma*-PCR was found positive in 60 cases and negative in 12. Thus, the overall sensitivity of the *Toxoplasma*-PCR in France was 83.3% and the rate of false negative results was 16.7% (<http://cnrtoxoplasmose.chu-reims.fr>). The molecular detection of *T. gondii* has therefore become an essential diagnostic tool in this clinical context; yet, its efficiency is hampered by a lack of standardization, due to the fact that almost all PCR assays used are 'laboratory-developed' assays, *i.e.* set up independently in each laboratory. This in turn leads to important variations in protocols between laboratories (in particular in DNA extraction, choice of DNA target, design of primers, PCR reaction conditions and amplicon detection), hence in performances (9, 10). The creation of the French National Reference Centre for Toxoplasmosis in 2006 was accompanied with the recommendation of improving and standardizing the molecular diagnosis of congenital toxoplasmosis at the national level. This included improving laboratory-developed PCR assays, establishing recommendations and eventually testing new methods. With this aim in mind, and also with the aim of fitting with

quality management policies, we wished to compare the technical performances of a commercially available PCR assay to those of laboratory-developed PCR assays (i) routinely used in eight proficient laboratories from academic hospitals, and (ii) representative of the different methods used in France. Although nucleic acid extraction methods have been commercialized for several years, only a few turnkey systems for molecular detection of *T. gondii* in humans have been marketed over the past few years. Their use appears as an attractive alternative, as they offer a chance for standardization and they respond to an increasing demand of quality management systems. However (i) there is no report in the literature of a comparative study in which a *Toxoplasma*-PCR kit proved better than finely optimized laboratory-developed assays (18); and (ii) a few IVD-labeled *Toxoplasma*-PCR kit are currently available. The aim of this multicentric study was to compare a new commercial kit for the detection of *T. gondii* by PCR to optimized reference PCR assays using a panel of amniotic fluid samples from pregnant women acutely infected with *T. gondii*.

Materials and methods

Study scheme. The Departments of Parasitology-Mycology of eight French Academic Hospital Centers which constituted the 'Molecular Biology Pole' of the French National Reference Center for Toxoplasmosis (<http://cnrtoxoplasmose.chu-reims.fr>) participated to the study. Cryopreserved extracted DNA from AF samples drawn for prenatal diagnosis of congenital toxoplasmosis during routine practice in each participating

center was tested again with both the commercial kit and the local 'laboratory-developed' assay. These clinical samples could be negative or naturally infected with *T. gondii*. The study was performed in accordance with the regulations of the local medical ethics committee of each participating center, in line with the revised Helsinki Declaration. In that respect, written consent was obtained before any AF sampling.

Clinical cohort. AF samples were drawn by amniocentesis in pregnant women acutely infected with *T. gondii* during gestation. AF collection was done in frame with the routine practice of each participating laboratory (in particular with respect to the volume of fluid, see table 1 for details). Determination of the dates of maternal infections was done either after serologic conversion (*i.e.*, the shift from a negative to a positive *Toxoplasma* serology with specific IgMs and IgGs) or after studying the kinetics of specific IgG titer and taking into account the result of the test for avidity of *Toxoplasma*-specific immunoglobulin G. The delay between infection and amniocentesis was calculated in weeks of amenorrhea (WA). Of note, cases of early seroconversion in which amniocentesis were delayed until the 18th WA were excluded for this calculation. In each center, the criteria used in the present study to confirm or rule out the diagnosis of congenital toxoplasmosis (*i.e.*, reference diagnosis) were those defined by the European Research Network on Congenital Toxoplasmosis (11). These criteria allow classifying the likelihood of *T. gondii* infection into five mutually exclusive categories: definite, probable, possible, unlikely, and not infected.

Table 1. Main features of the PCR assays used in each center in this study

Centers	Extraction method	Extraction			PCR			
		Mean±SD volume of AF tested (mL)	Volume of elution	Volumes for PCR	PCR method	Revelation	Detection of PCR inhibitors ^a	Apparatus
A	QIAamp DNA mini kit (Qiagen®)	1.5±0	200µL kit buffer	5µL/20µL Triplicate	Reischl et al. 2003 Sterkers et al. JCM2010		Plasmidic internal control	LightCycler II (Roche®)
B		13.5±5	200µL kit buffer	7µL/20µL Triplicate	Reischl et al. 2003 Brenier-Pinchart et al. 2007 Sterkers et al. JCM2010		β-globin gene	LightCycler II (Roche®)
C		14±7	200µL NAT water	5µL/50µL Triplicate	Reischl et al. 2003 Yera et al. JCM2009 Sterkers et al. JCM2010		β-globin gene	LightCycler II (Roche®)
D	Tween-Nonidet-NaOH lysis method (Hohlfeld et al. 1994)	4±0	100µL TNN lysis buffer	5µL/20µL Triplicate	Reischl et al. 2003 Sterkers et al. JCM2012 Morelle et al. JCM2012	FRET	<i>T. gondii</i> DNA internal control	LightCycler 480 (Roche®)
E	QIAamp DNA blood mini kit (Qiagen®)	4.6±3	100µL kit buffer	5µL/20µL Triplicate	Reischl et al. 2003 Yera et al. JCM2009 Sterkers et al. JCM2010		<i>T. gondii</i> DNA internal control	LightCycler I (Roche®)
F	High Pure Template kit (Roche®)	7±0	200µL kit buffer	5µL/20µL Triplicate	Cassaing et al. 2006 Sterkers et al. JCM2010		β-globin gene	LightCycler II (Roche®)
G	QIAamp DNA mini kit (Qiagen®)	10±4	100µL NAT water	5µL/25µL Triplicate	Talabani et al. 2009 Sterkers et al. JCM2010		TaqMan	Plasmidic internal control
H		NA	NA	5µL/25µL Tetraplicate	Fekkar et al. 2008 Sterkers et al. JCM2010	<i>T. gondii</i> DNA internal control		ABI Prism 7500 (Applied Biosystems®)
All	Id.	Id.	Id.	#BE-A997-AB: 5µL/20µL, OR #BE-A997-CD: 5µL/25µL	Bio-Evolution® (#BE-A997)	Taqman	Internal control	ABI Prism 7000 and 7500 (Applied Biosystems®)(Bio-Evolution® #BE-A997-AB), OR LightCycler 480 (Roche®), LightCycler I and II (Roche®)(Bio-Evolution® #BE-A997-CD)

^aThe absence of reaction inhibition was verified by amplifying a positive internal control concurrently and in the same reaction tube as the test DNA after the addition of a control sequence of target DNA (internal control); this control DNA was either highly diluted *T. gondii* genomic DNA (equivalent to 1 or 0.5 tachyzoite genome), an artificial plasmidic DNA construct containing the primer sequences (amplified by the test primers), or a defined sequence of DNA amplified by a second primer pair, e.g., β-globin or albumin amplified under stringent conditions (to increase the PCR sensitivity to the presence of inhibitors in the sample). Dijon also systematically performed one PCR with the matrix DNA diluted.

DNA extraction and cryopreservation. DNA extraction of AF samples was done <48-72 hours after sampling and performed using the protocol used in routine practice in each participating laboratories. It consisted in either QIAamp DNA mini kit (QIAGEN®, Courtaboeuf, France) (five laboratories), or QIAamp DNA micro kit (QIAGEN®) (one laboratory), or Highpure template (ROCHE®, Meylan, France) (one laboratory) or “Tween-Nonidet-NaOH” method (12) (one laboratory). DNA was eluted in 200 µL (QIAamp DNA mini kit, QIAamp DNA micro kit, High pure template) or in 100 µL (QIAamp DNA mini kit, “Tween-Nonidet-NaOH” method). Then DNA-extracted AF samples were frozen at -80°C (7 laboratories) or -20°C (1 laboratory) during a period from five years to less than one year. When samples were tested prospectively, the extracted DNA was not frozen.

PCR assays. Each participating center was asked to test thawed DNA extracts from AF samples with (i) its own laboratory-developed PCR assay (used in routine practice) and (ii) the Bio-Evolution® kit for *T. gondii* detection by real-time PCR (BIO-EVOLUTION®, ref. # BE-A997, Bussy-Saint-Martin, France). All PCR assays targeted the repetitive non-coding “cryptic” DNA element (13) we termed ‘rep529’ (13). The primers and probes used were Tox-9/Tox-11 and HP1/HP2 (14) in five laboratories, the ones described by Talabani et al. (15) in one laboratory, those described by Cassaing et al. (16) in one laboratory, and those described by Fekkar et al. (17) in one laboratory (Table 1). The laboratory-developed PCR assays were performed using a LightCycler 1.0

(ROCHE®, Meylan, France) in three laboratories, a LightCycler 2.0 (ROCHE®) in two laboratories, and a LightCycler 480 (ROCHE®, Meylan, France), an ABI Prism 7000 and an ABI 7500 (APPLIED BIOSYSTEMS®, Villebon-sur-Yvette, France) in one laboratory each. Real-time PCR amplification with the Bio-Evolution® kit was performed as recommended by the manufacturer and was done using the same real-time PCR apparatus as the one used for the laboratory-developed PCR assay in each center. The volume of DNA extract added to the PCR mix was 5µL (seven laboratories) or 7µL (one laboratory) in the laboratory-developed methods, and 5µL using the kit (as recommended by the manufacturer). The presence/absence of PCR inhibitors was tested using positive controls for each DNA-extracted sample with the laboratory-developed PCR assays and with the commercial kit (see Table 1 for details). Negative controls were included in each PCR run.

Data analysis. All DNA extracts were tested in triplicate with the commercial kit and also in triplicate when possible with the laboratory-developed PCR assays. Detection of *T. gondii* was considered positive when at least one reaction tube was positive. Detection was considered negative when all three reactions were negative, in the confirmed absence of PCR inhibitors. When the two types of detection (laboratory-developed PCR assays or commercial kit) were performed at the same time in the same center and found positive in triplicate (all centers but center E who

performed only one replicate), PCR crossing points (Cp) were compared in a Bland-Altman plot. For this, the mean Cp value obtained for each sample by each method (laboratory-developed method and commercial kit) was calculated and the mean of these two values, as well as the difference between these two values, were plotted with the mean of both methods in abscissa and the difference between both methods in ordinate.

Results and Discussion

Proficiency of the participating laboratories.

The eight participating laboratories are proficient in the molecular detection of *T. gondii* at a regional and national level in France and also at an international level. Indeed, all of them are authorized by the French national authority “Agence de la Biomédecine” to perform prenatal diagnosis of congenital toxoplasmosis. They are also all members of the 'Molecular Biology Pole' of the French National Reference Center for Toxoplasmosis (<http://cnrttoxoplasmose.chu-reims.fr>), and each of them has developed a highly performing laboratory-developed PCR assay (1, 6, 9, 10, 15, 17-22), all targeting the repetitive DNA element rep529, shown as the most efficient target to date for this diagnosis (10, 14, 21, 23-25)(for details, see Table 1 and S1). This same working group earlier recommended that laboratories should work toward a sensitivity threshold of 0.75 to 2.5 tachyzoites/ml of AF (10) and a 100% specificity. All participants to this study are able to detect at least 5 tachyzoites (T) per mL of AF, as checked by, *e.g.* annual, external quality (26). The Bio-Evolution® kit does not

include an extraction step, hence the use of a proper DNA extraction method in each center.

Description of the cohort.

One hundred and fifty-seven DNA-extracted AF samples were included in the study; 140 were retrospective samples kept in biobanks, and 17 were prospectively enrolled (Table S1). Storage conditions and the absence of effect of long-term conservation have been described elsewhere (27). The dates of maternal infection, according to gestational age, were established using serologic tests for 137/157 (87%) patients. According to the classification system proposed by the European research Network on Congenital toxoplasmosis (11), primary maternal infection during pregnancy was definite for 112/157 (71%) patients: 26%, 60% and 14% of them were infected during the first, second and third trimester of pregnancy, respectively. The mean date of amniocentesis was 29 WA [19 WA – 42 WA], and the mean time interval between infection and amniocentesis was 8 weeks [0-28 weeks]. Eleven and five samples from amniocentesis performed at 18 WA and during the delivery, respectively, were excluded from these calculations (see Material and Methods). Of note, the characteristics of the cohort should not be compared to previously published cohorts because the AF samples were retrospectively selected in the biobanks (see Material and Methods) and therefore, these figures do not depict the natural situation. A definite final diagnosis of congenital toxoplasmosis in the fetus/infant was asserted in 84/157 (54%) cases. Details about the cohort are visible in Table S1.

Comparison of the *Toxoplasma gondii* detection with the commercial kit versus the laboratory-developed PCR assays. Among the 157 AF DNA extracts analyzed, 76 (48%) were found *T. gondii*-positive with the laboratory-developed PCR assay of the corresponding laboratory at the time of the initial diagnosis, and all, but nine that could not be tested again, were confirmed in the second test performed in this study. Among these 76 samples, 75 were also found positive with the commercial kit (Table 2). The concordance between the laboratory-developed PCR assays and the commercial kit was 99%. Only one discrepant result was found: it was positive in all triplicates ($C_p = 38.4 \pm 0.6$) at the time of initial diagnosis, and positive in one sample out of two ($C_p > 45$) in the confirming test performed here and negative when tested by the commercial kit. It should be stressed (i) that only 40 cycles were performed with the commercial kit; and (ii) that no PCR inhibitors were detected by the kit. Although some DNA

degradation during preservation cannot be ruled out, *T. gondii* DNA was still detected using the laboratory-developed method at the time of the study. The 81 remaining samples were all classified *T. gondii*-negative using the laboratory-developed PCR assay and the commercial kit. All DNA extracts were found free of PCR inhibitors, using the positive controls of both the laboratory-developed PCR assays and the commercial kit. Taking into account the patients' follow-up, we used the "classification system and case definitions of *Toxoplasma gondii* infection in immunocompetent pregnant women and their congenitally infected offspring" developed by the European Research Network on Congenital Toxoplasmosis (11) to determine the diagnostic performances of the assays used: the overall sensitivity of the eight laboratory-developed PCR assays and of the commercial kit with this cohort can be estimated at 54/63 (86%) (Table 2). Specificity was 100% for all assays: no false positive results were detected by any method.

Table 2. Comparison of the detection of *Toxoplasma gondii* using laboratory-developed PCR assays and commercial kit: overall performances of the methods.

		Detection of <i>T. gondii</i>		Performances ^a	
		Positive	Negative	Sensitivity	Specificity
Laboratory-developed assays	PCR	76	81	54/63 (86%)	29/29 (100%)
	Commercial kit	75	82	54/63 (86%)	29/29 (100%)

^aTo calculate sensitivity and specificity cases with loss of follow-up are excluded

Comparison of qualitative results and relative quantification between the commercial kit and the laboratory-developed PCR assays. At and around the sensitivity threshold of a given PCR method,

only a proportion of the reaction tubes appears positive; which implies that, for very low concentrations of the pathogen, several PCRs have to be carried out for each experiment (thus increasing the probability of amplifying the

pathogen DNA) (10, 20, 28-30). In the current study, inconsistently positive results were found in 7 occurrences, both by the reference methods and by the commercial kit.

To evaluate further the concordance between the laboratory-developed PCR assays and the commercial kit, the Cp values obtained using both methods were compared for each of 63/76

positive samples, and plotted in a Bland-Altman graph (Fig. 1). In all cases but two, the values were within the ± 1.96 standard deviation interval, allowing us to conclude that the relative quantification using the commercial kit was in good agreement with all the laboratory-developed methods.

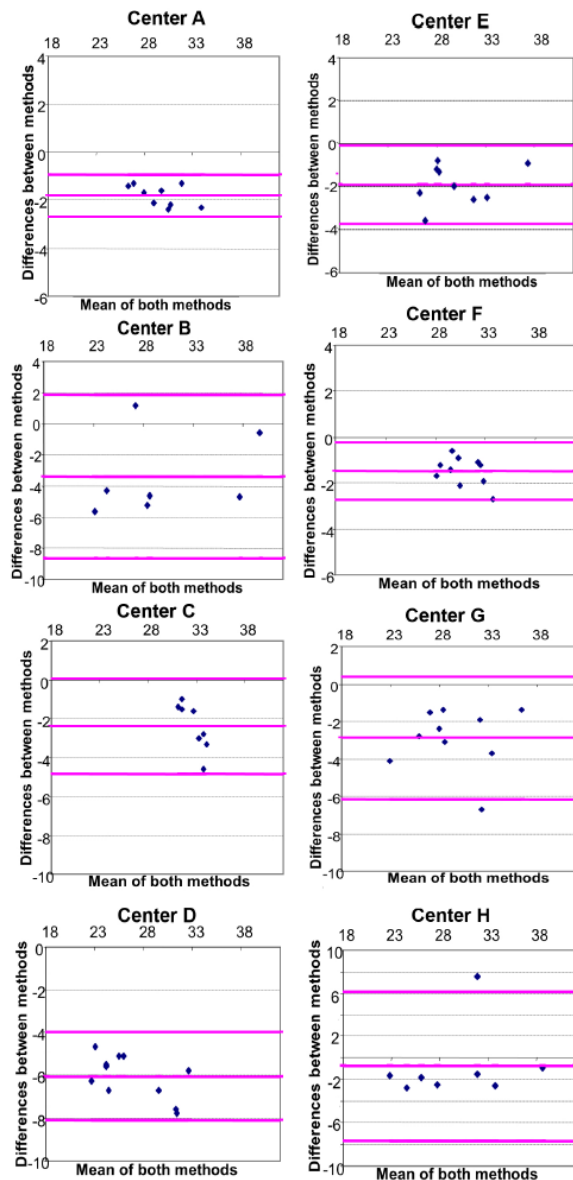


Figure 1. Concordance between Cp values obtained using laboratory-developed methods and the Bio-Evolution® commercial kit for the molecular detection of *Toxoplasma gondii*. The Cp values obtained for each sample by each method (laboratory-developed method and commercial kit) were calculated for each

center independently (A-H). For each sample, the means of the Cps obtained with both methods, and the differences between the Cps obtained with both methods were calculated. To obtain the Bland-Altman plots, these values were plotted, with the mean of both methods in abscissa and the difference between both methods in ordinate for each center. Pink lines represent the mean and ± 1.96 standard deviations.

Evaluation of handiness and respect of good laboratory practices. The Bio-Evolution® kit was felt as an easy to handle turnkey kit. It included detailed and well-written instructions to users. More importantly, with respect to good laboratory practices, the manufacturer (i) pointed out that the analysis should be performed in triplicate, and (ii) included positive and negative controls corresponding to a 200 eqT/ μ L and dH₂O, respectively. However, the commercial kit did not include the use of uracil-N-glycosylase (UNG) to limit carry-over contaminations from previously amplified PCR products. In addition, all the components of the kit, *i.e.* positive, negative controls and mix vials, are stored in the same box. Besides, six of the eight laboratories reported problems with the volumes of the reagents of the commercial kit since the volume found was below the volume stated on the vials. Finally, the manufacturer’s recommendation

was to set the threshold manually in the real-time PCR analysis software (so-called “fit point” method): however, the LightCycler software (Roche®) offers the possibility of using an automated analysis (so-called “second

derivative” method) which is unbiased, hence more reproducible than the fit-point method. This point has been amended in the newest version of the kit's instruction manual.

Conclusion. In total, the commercial kit tested here for *T. gondii* detection by real-time PCR (Bio-Evolution®, ref.# BE-A997) is a well-designed and useful kit, which leaves room for some improvement, but in our view, represents an excellent tool for the molecular detection of *T. gondii* (18). The use of the recommended DNA target rep529 is likely an important factor of efficiency for this kit, although this, in itself, is not sufficient to guarantee good results (18). Although it is not yet labeled 'IVD' (In Vitro Diagnosis) and, at the time of testing, suffered from some defects in the reliability of the production of different reagents, this commercial method showed equivalent performances to eight finely optimized laboratory-developed PCR assays used in proficient French laboratories.

Conflict of interest. DF and EC work in a laboratory which received research funds from the manufacturer to test the pilot version of the commercial kit. Commercial kits were provided for free by the manufacturer to the eight participating laboratories.

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Available online: Supplemental Dataset 1. Raw data per center for the 157 cases analyzed in this study, including dates of maternal infection and of prenatal diagnosis, technical data, detailed results, and final CT diagnosis.