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Comparative Assessment of a Commercial Kit and Two Laboratory-Developed PCR Assays for Molecular Diagnosis of Congenital Toxoplasmosis

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Toxoplasmosis is a worldwide infection that may cause severe disease and is regarded as a serious health problem in France. Detection of the parasite by molecular methods is crucial for diagnosing the disease. The extreme diversity of methods and performances of *Toxoplasma* PCR assays makes the use of commercial PCR kits an attractive alternative, as they offer a chance for standardization. We compared the performances of three molecular methods for the detection of *Toxoplasma gondii* DNA in amniotic fluid: a commercial method using nested PCR and two laboratory-developed methods, one using conventional PCR and the other one real-time PCR. This evaluation was based upon a *T. gondii* DNA serial dilution assay, three amniotic fluid samples spiked with *T. gondii* at different concentrations, and a clinical cohort of 33 amniotic fluid samples. The *T. gondii* DNA serial dilution assay showed a much lower sensitivity for the commercial kit than for the laboratory-developed methods. Moreover, out of 12 proven congenital toxoplasmosis cases, 91.7% were detected by the laboratory-developed assays, whereas only 50% were detected by the commercial kit. A lack of sensitivity of the method, partly due to the presence of PCR inhibitors, was the main drawback of the commercial method. This study emphasizes that commercial PCR diagnostic kits do not systematically perform better than carefully optimized laboratory-developed methods. There is a need for thorough evaluation of such kits by proficient groups, as well as for performance standards that commercial kits can be tested against to improve confidence in those selected by health care providers.

Toxoplasmosis is a worldwide endemic protozoan disease, acquired mainly through infected meat. Primary infection is most often asymptomatic in healthy individuals, and a symptom-free chronic infection is established. Nevertheless, essentially in two circumstances, a life-threatening disease may occur: (i) reactivation in immunocompromised patients (HIV-infected patients or transplant recipients) and (ii) primary infection during pregnancy, which is followed in about one-third of the cases by an infection of the fetus. In the latter, the consequences are borne by the fetus and not by the mother. Fetal infections may range from severe neurological abnormalities and chorioretinitis to infraclinical infection at birth, which, however, still poses a risk of late-onset ocular lesions (23). A rapid and accurate diagnosis is required in order to start the antiparasitic treatment. Prenatal diagnosis of congenital toxoplasmosis (CT) has been a national prevention policy in France since 1978 (22). It now essentially relies upon molecular methods, namely, PCR, and has considerably improved the prognosis and outcome for infected children wherever it has been implemented, in particular by eliminating the need for cordocentesis (reviewed in reference 1). Nevertheless, this molecular diagnosis remains unsatisfactory due to a complete lack of standardization and a considerable diversity among the nucleic acid extraction methods, amplification systems, and DNA primers used (21). This diversity is explained by the fact that most of these PCR assays are in-house or laboratory-developed methods, set up independently in each laboratory, which leads to important variations in protocols between laboratories (in particular in DNA extraction, choice of DNA target, design of primers, PCR conditions, and amplicon detection). The choice of the DNA target and primers is generally considered essential in this respect. Presently, the repeated "cryptic" DNA element described by Homan et al. (13), called rep529, should be preferred for the molecular diagnosis of toxoplasmosis, as several reports have found a better sensitivity when using this target than when using the more classical B1 gene target (7, 10, 11, 16, 17, 20). Although nucleic acid extraction methods have been commercialized for a long time, only a few turnkey systems for molecular detection of *Toxoplasma* spp. in humans have been marketed over the past few years; they respond to an increasing demand for quality management systems.

The aim of this study was to compare the technical performances of a commercially available PCR kit and two laboratory-developed PCR assays (conventional PCR [cnPCR] and real-time PCR [rtPCR]) routinely used in the laboratory for the molecular diagnosis of toxoplasmosis. Although several commercial kits were available at the beginning of the study, we tested only the one that targeted rep529. The study revealed that the performance of

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the commercial kit tested here was far lower than those of the laboratory-developed assays.

MATERIALS AND METHODS

Study scheme. The study was performed in accordance with the regulations of the local medical ethics committee of the Hospital University Center (CHU) of Montpellier, France, in line with the revised Helsinki Declaration. Written consent was obtained before any amniotic fluid sampling. The Departments of Parasitology-Mycology of the University Hospital Centers of Montpellier, Toulouse, and Grenoble participated in this study; they were all members of the Molecular Biology Pole of the French National Reference Center for Toxoplasmosis (https://www.chu-reims.fr/professionnels/cnr-toxoplasmose-1). The study used calibrated samples that are commercially available (Quality Control for Medical Diagnostics [QCMD], Glasgow, Scotland, United Kingdom), as well as amniotic fluid samples which were either (i) negative, (ii) naturally infected by *Toxoplasma gondii*, or (iii) spiked with low concentrations of *T. gondii*.

Freeze-dried calibrated samples and *Toxoplasma* serial dilution assay for analytical detection threshold. Calibrated samples were freeze-dried samples of *T. gondii* tachyzoites at a concentration of 50 tachyzoites (T)/ml from panel TG08 of the QCMD *T. gondii* External Quality Assessment (EQA) program. Freeze-dried samples were reconstituted by adding 1.0 ml of molecular-grade sterile water to each vial, according to the manufacturer's specifications. DNA extraction was performed according to the extraction method defined for each molecular assay (see below). The primary DNA extracted was then serially diluted and tested in multiplicate by PCR (20). The equivalents of 1, 0.4, 0.2, 0.1, 0.04, and 0.02 *Toxoplasma gondii* genome equivalent (Tgg) per PCR tube were tested. For each PCR assay, the concentration of 1 Tgg per reaction tube was tested in duplicate, and the range of concentrations of 0.4 to 0.02 Tgg per reaction tube was tested in quadruplicate (making up 22 reactions in total). This allowed defining criteria for assessing the performances of the assays as published previously (20) using (i) a PCR performance score, which was the total number of positive reactions out of the 22, and (ii) the analytical detection threshold, defined as the lowest concentration of a serial detection assay where at least half of the PCR tubes are positive (2 positive PCR tubes out of 4).

Clinical amniotic fluid samples and spiked amniotic fluid samples. Through the Molecular Biology Pole of the National Reference Center for Toxoplasmosis, we recruited amniotic fluids for which at least 6 ml was available. Information was collected and analyzed in order (i) to estimate the gestational age at which maternal infection occurred and (ii) to establish the diagnosis of CT. The diagnosis of CT was established in each center that sent amniotic fluid, based on the results of a series of parasitological (including PCR and mouse inoculation using amniotic fluid, placenta, and cord blood) and serological (at birth and during a long-term serological and clinical follow-up of the infants during the first year of life) tests (6). According to the classification system and case definitions of congenital toxoplasmosis developed by the European Research Network on Congenital Toxoplasmosis (15), all cases were defined as definite or unlikely congenital toxoplasmosis, not infected, or lost to follow-up.

To prepare *T. gondii*-spiked amniotic fluid samples, the Department of Parasitology-Mycology of the University Hospital Center of Grenoble added tachyzoites of type II *T. gondii* strains (BRC TgH 38034A and BRC TgH 20018A strains) (5) grown on human fibroblast cells to amniotic fluids collected from two women treated for hydramnios in which toxoplasmosis had been ruled out. Three different concentrations (500, 200, and 100 T/ml) were prepared in Grenoble; these corresponded after extraction to 100, 40, and 20 Tgg per PCR tube, respectively, taking into account a starting volume of amniotic fluid of 2 ml, a 50-μl volume of extracted DNA, and a 5-μl volume of DNA added to the PCR mixture (5 μl). All amniotic fluids were stored at −20°C until processing.

***Toxoplasma gondii* molecular detection methods.** All PCR tests were performed in the Department of Parasitology-Mycology of the University Hospital Center of Montpellier, which is proficient in the diagnosis of congenital toxoplasmosis at the regional and national levels (1, 2, 20).

The three molecular assays tested consisted of a DNA extraction method and a PCR method, all detailed below. All of them targeted rep529 (13). Method 1 is a ready-to-use commercial kit (AMS94/F; Clonit, Milan, Italy). Method 2 was a method used routinely in the department between January 2008 and June 2009. It is a finely optimized, laboratory-developed conventional method. Method 3 was a real-time PCR method and has become the method used in routine practice in the department since July 2009.

DNA extraction was performed from 2 ml of each amniotic fluid and 1 ml of each calibrated sample. The DNA extraction method for method 1 (commercial kit) was performed strictly according to the manufacturer's specifications. The laboratory-developed methods 2 and 3 used the same extraction method, which is based on a selective lysis of contaminating red blood cells and a heat-detergent extraction and thermolysis buffer method: the Tween-Nonidet-NaOH (TNN; 0.5% Tween 20, 0.5% Nonidet P-40, 10 mM NaOH) method (12). Briefly, the sample was first centrifuged at 16,000 × *g*, and the pellet was resuspended in a washing buffer (method 1) or in a red blood cell lysis buffer (methods 2 and 3). After a second centrifugation, the pellet was resuspended in 50 μl of extraction solution (method 1) or of thermolysis buffer (methods 2 and 3) and heated at 100°C for 10 min. An ultimate centrifugation was performed for methods 2 and 3, and the supernatant was carefully collected. DNA extracts were stored at +4°C, and all the PCRs were performed within the next 15 days.

As for DNA amplification, for method 1, we punctiliously followed the manufacturer's specifications. Before amplification was started, the reagents were prepared on ice. The first amplification reaction mixtures were cycled in a Perkin Elmer 2400 thermal cycler by using the following conditions: 95°C for 3 min and 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 40 s. PCR tubes containing the reaction mixture were ready to use; 5 μl of patient DNA sample was added. Three microliters of the solution contained in the test tube used for the first amplification was added to a nested amplification master mixture. The nested amplification reactions were performed by using the following program: 95°C for 3 min and 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 40 s. Ten microliters of the reaction products was detected by electrophoresis on a (precast) PCR CheckIT gel with ethidium bromide (included in the kit). PCRs on one tube per patient, as well as one positive-control tube and one negative-control tube (both included in the kit) per run, were performed as recommended by the manufacturer.

Method 2 used unpublished primers H1 (5′-GAAGGGACAGAAGT CGAAGG-3′) and H2 (5′-CTAGATCGCATTCCGGTGTC-3′). The optimized conditions for a total reaction volume of 50 μl, including 5 μl of sample DNA, were the following: 5 μl of 10× buffer, 1.5 μl of bovine serum albumin (2 mg/ml), 1 μl deoxynucleoside triphosphates at a concentration of 10 mmol/liter each, 5 μl $MgCl_2$ (25 mmol/liter), 0.8 μl of each primer (50 pmol/μl), 1.0 μl of uracil-DNA glycosylase (UNG) (Roche Diagnostics, Meylan, France), and 0.45 μl of *Taq* DNA polymerase (Goldstar; Eurogentec). "Hot start," obtained by a wax barrier method, was used to increase specificity (Dynawax; Eurogentec). The reaction mixtures were cycled in an MJ Research thermal cycler by using the following conditions: 20°C for 10 min; 94°C for 3 min; 58°C for 30 s; 40 cycles of 72°C for 30 s, 94°C for 30 s, and 60°C for 30 s; and 72°C for 10 min. The reaction products were visualized under UV light after electrophoresis of 20 μl of the reaction solution in a 2% agarose gel.

In method 3, *Toxoplasma* DNA amplification was performed using the DNA primers and fluorescence resonance energy transfer (FRET) probes published by Reischl et al. (17) on a LightCycler 480 system (Roche Applied Science, Meylan, France) and under the conditions strictly described by Reischl et al. (17).

For methods 2 and 3, the presence of PCR inhibitors was checked in each DNA extract by amplifying an internal positive control consisting of *T. gondii*-extracted DNA equivalent to 0.8 Tgg, which was added to one

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TABLE 1 Comparison of the performance scores and analytical detection thresholds of the three PCR assays using a *Toxoplasma gondii* DNA serial dilution assay[a]

Method[b]	Concn (Tgg/reaction tube)						PCR performance score
	1	0.4	0.2	0.1	0.04	0.02	
1 (commercial nested PCR)	2/2	1/4	2/4	2/4	1/4	0/4	8/22
2 (laboratory-developed cnPCR)	2/2	4/4	4/4	4/4	4/4	4/4	22/22
3 (laboratory-developed rtPCR)	2/2	4/4	4/4	4/4	4/4	3/4	21/22

[a] The equivalents of 1, 0.4, 0.2, 0.1, 0.04, and 0.02 *Toxoplasma gondii* genome equivalent (Tgg) per PCR tube were tested. The PCR performance score was calculated by determining the number of positive reaction tubes for each concentration out of the 22 reactions performed. The analytical sensitivity threshold of each method was defined as the lowest concentration that was detected in at least half of the PCR tubes (2 positive reactions out of 4) (20). For example, the numbers of positive reactions/total number of reactions using method 1 (commercial kit) were 2/2, 1/4, 2/4, 2/4, 1/4, and 0/4 for 1, 0.4, 0.2, 0.1, 0.04 and 0.02 Tgg, respectively, per reaction tube. Therefore, the performance score for method 1 was 8/22. The analytical sensitivity threshold was estimated at 0.1 Tgg per reaction tube for method 1 and was not reached for methods 2 and 3, i.e., it was below 0.02 Tgg per reaction tube.

[b] cnPCR, conventional PCR; rtPCR, real-time PCR.

supplemental PCR tube for each patient. A DNA extraction control tube was also included for each DNA extract, consisting of the amplification of a fragment of the human beta-globin gene with the primers described by Saiki et al. (19). Finally, three negative-control tubes, which each received 5 μl of H_2O instead of DNA, were included in each PCR run to detect contamination.

RESULTS AND DISCUSSION

Design and rationale of the study. A positive PCR using amniotic fluid or cord blood affirms the diagnosis of congenital toxoplasmosis (15); moreover, it is the most informative biological test for the diagnosis of patent toxoplasmosis in the prenatal period (reviewed in reference 1). In order to fit with a quality management system policy, we wished to compare the technical performance of a commercially available PCR assay to those of two laboratory-developed (conventional and real time) PCR assays routinely used in the laboratory. Although several commercial kits were available at the beginning of the study, we tested the only one which targeted rep529 (13) at the time (AMS94/F; Clonit Milan, Italy). Since it has been shown that the parasitic load in congenital toxoplasmosis is often very low, with a median concentration in amniotic fluid estimated around 10 tachyzoites per ml (9, 18), the PCR assays used for this diagnosis must be highly sensitive. Therefore, in addition to clinical samples, we deliberately chose to test relatively low parasite concentrations: 1, 0.4, 0.2, 0.1, 0.04, and 0.02 genome equivalent per reaction; these values correspond to 50, 20, 10, 5, and 2 tachyzoites and 1 tachyzoite per ml of amniotic fluid, respectively, considering a starting volume of amniotic fluid of 1 ml, a 50-μl volume of extracted DNA, and a 1-μl volume of DNA added to the PCR mixture.

In the study, a total of 110 DNA preparations and 670 PCRs were carried out: 91 reactions, including 16 controls, for method 1, 326 reactions, including 148 controls, for method 2, 174 reactions, including 74 controls, for method 3, and 72 reactions targeting the beta-globin gene to test the DNA preparation in method 2 and method 3.

Analytical sensitivity threshold and detection of low concentrations of *T. gondii* in simulated specimens. The first step of the study was to determine the analytical sensitivity threshold for the three methods using serial dilutions of commercially available calibrated samples (QCMD). The principle of the test is based on the fact that, when the detection limit of the method is reached, only a proportion of PCRs are positive (8, 20). This allowed us to determine a PCR performance score as well as the analytical sensitivity threshold for each method (20) (see Materials and Methods). The PCR performance scores for the *T. gondii* DNA serial dilution assay were 8/22, 22/22, and 21/22 for method 1 (commercial kit), method 2 (laboratory-developed cnPCR), and method 3 (laboratory-developed rtPCR), respectively (Table 1). Concerning the analytical sensitivity threshold, it was estimated at 0.1 Tgg per reaction tube for method 1. The analytical sensitivity threshold was not reached for methods 2 and 3, i.e., it was below 0.02 Tgg per reaction tube and therefore at least 5-fold lower for laboratory-developed methods than for the commercial one (Table 1).

All three *T. gondii*-spiked amniotic fluid samples (testing 50, 20, and 10 Tgg per PCR tube) were found positive with the two laboratory-developed methods, but only 2 of them were so with the commercial method. For the lowest concentration, which was not detected with the commercial method, we repeated the test for a total of 3 PCR tubes and found it inconsistently positive (2 out of 3) (Table 2). These data are in agreement with the sensitivity of the system, which was estimated by the manufacturer as ≥30 Tgg.

Therefore, using two different analytical tests (dilution assay and spiked samples), both laboratory-developed assays showed a higher sensitivity than the commercial kit.

Of note, here, as stated elsewhere (3), real-time PCR did not appear to be more sensitive than conventional PCR.

Diagnostic performances in a clinical cohort. We then wanted to test the three methods in a retrospective clinical cohort. We tested 33 amniotic fluids that were preserved at −20°C in three laboratories of the Molecular Biology Pole of the French National Reference Center of Toxoplasmosis from children for whom a definite diagnosis of congenital toxoplasmosis was documented (see Materials and Methods).

Of the 33 children investigated, 12 were congenitally infected with *Toxoplasma gondii* and 21 proved to be uninfected. Out of the 12 congenitally infected children, 11 (91.7%) were found positive with both methods 2 and 3 but only 6 (50%) were so with method 1 (Table 2). Here again, among the two laboratory-developed methods, real-time PCR did not appear to be more sensitive than conventional PCR. In methods 2 and 3, PCR analysis was performed in multiplicate. All six PCR-negative samples by method 1 that were PCR positive by methods 2 and 3 were tested again using method 1, for a total of 3 PCR tubes. Two of these were then found inconsistently positive, one corresponding to a clinical sample and the other one to spiked amniotic fluid (Table 2). This confirms that the use of two (or three) reaction tubes per patient in routine practice is an important measure to avoid falsely negative results in *Toxoplasma* PCR (8).

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TABLE 2 Comparison of the performances of the three PCR assays using amniotic fluid clinical samples

Sample type	Contamination or concn[a]	Criteria for clinical diagnosis[b]	Definitive classification of clinical cases[c]	Result for method: 1	2	3[d]
Clinical amniotic fluid	5–26	Positive amniotic fluid; positive IgM and IgA; increased IC at mo 1; positive serology at 1 yr	Definite congenital toxoplasmosis	Positive	Positive	Positive (27.72 ± 0.1)
	11	Positive amniotic fluid; evocative sonography (cerebral lesion)	Definite congenital toxoplasmosis (termination of pregnancy at 34 WA)	Positive	Positive	Positive (27.67 ± 0.06)
	22	Positive amniotic fluid; evocative sonography (cerebral lesion, ascitis)	Definite congenital toxoplasmosis (termination of pregnancy at 30 WA)	Positive	Positive	Positive (26.80 ± 0.11)
	22	Positive amniotic fluid; evocative sonography (cerebral lesion, hepatosplenomegaly)	Definite congenital toxoplasmosis (termination of pregnancy at 30 WA)	Inhibited	Positive	Positive (28.92 ± 0.03)
	21–28	Positive amniotic fluid and cord blood; positive IgM and IgA; positive serology at 1 yr; retinochoroiditis	Definite congenital toxoplasmosis	Positive	Positive	Positive (27.28 ± 0.23)
	22–24	Positive amniotic fluid; evocative sonography (cerebral calcification); positive IgM and IgA; increased IC at mo 3; positive serology at 1 yr	Definite congenital toxoplasmosis	Inhibited	Positive	Positive (33.58 ± 0.02)
	18–29	Positive amniotic fluid; positive serology at 1 yr	Definite congenital toxoplasmosis	Inconsistently positive (2/3[e])	Positive	Positive (28.72 ± 0.03)
	24	Positive amniotic fluid; evocative sonography (cerebral lesion)	Definite congenital toxoplasmosis (termination of pregnancy at 28 WA)	Positive	Positive	Positive (22.51 ± 0.12)
	25–26	Positive amniotic fluid; positive IgM and IgA; increased IC at mo 1 and mo 2; positive serology at 1 yr; retinochoroiditis	Congenital toxoplasmosis	Inhibited	Positive	Positive (31.57 ± 0.06)
	31	Positive amniotic fluid; positive IgM and IgA; positive serology at 1 yr	Congenital toxoplasmosis	Inhibited	Positive	Positive (33.81 ± 0.04)
	33	Positive amniotic fluid	Congenital toxoplasmosis	Positive	Positive	Positive (26 ± 0.05)
	27	Increased IC at mo 3 and mo 4; positive comparative WB at mo 3; positive serology at 1 yr	Congenital toxoplasmosis	False negative	False negative	False negative
	≤7	Comparable mother-child ICs; negative IgM and IgA; serology in regression at mo 4	Unlikely congenital toxoplasmosis	Negative	Negative	Negative
	17–23	Comparable mother-child ICs and WBs at birth; negative IgM and IgA; serology in regression at mo 3	Unlikely congenital toxoplasmosis	Negative	Negative	Negative
	28–29	Negative IgM and IgA; serology in regression at mo 2	Unlikely congenital toxoplasmosis	Negative	Negative	Negative
	≤2	Comparable mother-child ICs; negative IgM	Lost to follow-up	Negative	Negative	Negative
	6–12	Comparable mother-child ICs; negative IgM and IgA	Lost to follow-up	Negative	Negative	Negative
	11–19	Comparable mother-child ICs; negative IgM and IgA	Lost to follow-up	Negative	Negative	Negative
	21	Comparable mother-child WBs	Lost to follow-up	Negative	Negative	Negative
	29	Comparable mother-child ICs; negative IgM and IgA	Lost to follow-up	Negative	Negative	Negative
	≤2	Comparable mother-child ICs; negative IgM and IgA; negative serology at mo 10	Not infected	Negative	Negative	Negative
	10	Negative IgM and IgA; negative serology at mo 6	Not infected	Negative	Negative	Negative
	12	Comparable mother-child ICs; negative IgM and IgA; negative serology at mo 14	Not infected	Negative	Negative	Negative
	12–13	Comparable mother-child ICs; negative IgM and IgA; negative serology at mo 4	Not infected	Negative	Negative	Negative
	14–15	Comparable mother-child ICs and WBs; negative IgM and IgA; negative serology at mo 4	Not infected	Negative	Negative	Negative
	17	Comparable mother-child ICs and WBs; negative IgM and IgA; negative serology at mo 6	Not infected	Negative	Negative	Negative
	19–20	Negative serology at mo 6	Not infected	Negative	Negative	Negative
	21–22	Comparable mother-child WBs; negative IgM and IgA	Not infected	Negative	Negative	Negative
	26	Comparable mother-child ICs; negative IgM; negative serology at mo 11	Not infected	Negative	Negative	Negative
	Seropositive mother	Seropositive mother treated for hydramnios	Not infected	Negative	Negative	Negative
	Seropositive mother	Seropositive mother treated for hydramnios	Not infected	Negative	Negative	Negative
	Seronegative mother	Seronegative mother	Not infected	Negative	Negative	Negative
T. gondii-spiked amniotic fluid	500	NR[f]	NR	Positive	Positive	Positive (23.92 ± 0.03)
	200	NR	NR	Positive	Positive	Positive (25.75 ± 0.03)
	100	NR	NR	Inconsistently positive	Positive	Positive (26.03 ± 0.03)

[a] Contamination for clinical amniotic fluids is indicated as weeks of amenorrhea or seropositivity or seronegativity of the mother, and concentration for *T. gondii*-spiked amniotic fluids is indicated as tachyzoites/ml.
[b] The diagnosis of congenital toxoplasmosis was based on the results of a combination of both parasitological and serological tests and was established in each center that sent amniotic fluid (4, 6). IC, immune charge; WB, Western blot.
[c] All cases were defined as definite or unlikely congenital toxoplasmosis, not infected, or lost to follow-up according to the classification developed by the European Research Network on Congenital Toxoplasmosis (15). WA, weeks of amenorrhea.
[d] The numbers in parentheses indicate the values of the cycle thresholds (which give a relative form of quantification of the parasitic load in the sample, the highest cycle thresholds corresponding to the lowest parasitic loads).
[e] Two of three samples positive.
[f] NR, not relevant.

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No false-positive result was found with any of three methods tested. Carryover contaminations with amplicons are a general concern for diagnostic PCR assays. This is particularly important with nested PCR and with highly sensitive methods. Here, carryover contaminations by amplicons were prevented by (i) drastic physical separation measures (rooms, equipment, and personnel) and (ii) decontamination procedures (e.g., UV exposure of rooms, consumables, and materials; bleaching of all materials and surfaces; and the use of uracil-DNA glycosylase in method 2).

Good laboratory practices and controls. Commercial kits often offer convenient turnkey protocols that save time and are easier to accredit than laboratory-developed methods. But the prime aim for any molecular assay is the diagnostic performance. In that respect, a number of rules should be respected when a molecular assay is designed, particularly for microbiological diagnosis: (i) the analysis should be performed in multiplicate; (ii) the method should include a "true" positive control that tests for the presence of reaction inhibitors (at best an internal control) and not only for the quality of reagents and thermocycling; and (iii) it should include a control for DNA extraction (Table 2). The commercial kit (method 1) did not follow any of these three good laboratory practices. Neither inhibitors of the PCR nor extraction defects using beta-globin PCR (not shown) could be detected in the samples tested by methods 2 and 3. In method 1, the quality of DNA extraction was not evaluated and all "positive controls" included were found positive. However, these were not "true" positive controls since they do not test for the presence of inhibitors within the sample, but only for the capacity of amplifying the DNA target in a given run. Although this was not recommended by the supplier, we tested for the presence of inhibitors by using an internal positive control at a concentration of 2 Tgg. This allowed us to detect the presence of inhibitors in four amniotic fluid clinical samples found falsely negative by method 1 (Table 2). This abnormally high inhibition rate was unexpected using amniotic fluid. Both DNA preparation methods were similar (see Materials and Methods), and a likely difference that might impact the quality of the DNA extract may be the last centrifugation step, which is absent in method 1 and which we believe is a critical step to eliminate PCR inhibitors.

Conclusion. The extreme lack of standardization of *Toxoplasma* PCR due to the abundance of laboratory-developed methods is definitely a hindrance to optimal care of patients affected with toxoplasmosis (21). In such a context, the use of commercial PCR kits appears to be a suitable and attractive alternative, as they tend to be generally user-friendly and standardized; hence, they can be accredited. Until 2010, no commercial kits were used in France for the molecular diagnosis of toxoplasmosis (21; our unpublished data), but in Europe, commercial kits were used by six centers and the Clonit kit specifically was used by 2 out of 38 surveyed laboratories (14). However, our study shows that, with a clinical sensitivity of <50%, the commercial kit tested here exhibited much lower performance than two laboratory-developed assays. This clearly shows that commercial molecular assays for the diagnosis of toxoplasmosis should be thoroughly evaluated before being used in routine diagnosis. In addition, further studies are needed to define international recommendations for minimum performance. In that sense, we earlier recommended that laboratories should work toward a sensitivity threshold of 0.75 to 2.5 tachyzoites/ml of amniotic fluid (20) and a 100% specificity. Finally, performance standards that commercial kits can be tested against should be worked out to improve confidence in those selected by health care providers. In our view, however, these standards should not be based on preextracted DNA but should aim at evaluating the performance of both the extraction method and the PCR itself.

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REFERENCES

1. **Bastien P.** 2002. Molecular diagnosis of toxoplasmosis. Trans. R. Soc. Trop. Med. Hyg. **96**(Suppl 1):S205–S215.
2. **Bastien P, Jumas-Bilak E, Varlet-Marie E, Marty P.** 2007. Three years of multi-laboratory external quality control for the molecular detection of *Toxoplasma gondii* in amniotic fluid in France. Clin. Microbiol. Infect. **13**:430–433.
3. **Bastien P, Procop GW, Reischl U.** 2008. Quantitative real-time PCR is not more sensitive than "conventional" PCR. J. Clin. Microbiol. **46**:1897–1900.
4. **Bessieres MH, et al.** 2009. Diagnosis of congenital toxoplasmosis: prenatal and neonatal evaluation of methods used in Toulouse University Hospital and incidence of congenital toxoplasmosis. Mem. Inst. Oswaldo Cruz **104**:389–392.
5. **Brenier-Pinchart MP, Bertini RL, Maubon D, Pelloux H.** 2010. In vitro differential phenotypic characteristics among type-II *Toxoplasma gondii* strains from congenital toxoplasmosis in humans. J. Parasitol. **96**:798–799.
6. **Brenier-Pinchart MP, et al.** 2007. Adapting a conventional PCR assay for *Toxoplasma gondii* detection to real-time quantitative PCR including a competitive internal control. Parasite **14**:149–154.
7. **Cassaing S, et al.** 2006. Comparison between two amplification sets for molecular diagnosis of toxoplasmosis by real-time PCR. J. Clin. Microbiol. **44**:720–724.
8. **Chabbert E, Lachaud L, Crobu L, Bastien P.** 2004. Comparison of two widely used PCR primer systems for detection of toxoplasma in amniotic fluid, blood, and tissues. J. Clin. Microbiol. **42**:1719–1722.
9. **Costa JM, et al.** 2000. Real-time PCR for diagnosis and follow-up of *Toxoplasma* reactivation after allogeneic stem cell transplantation using fluorescence resonance energy transfer hybridization probes. J. Clin. Microbiol. **38**:2929–2932.
10. **Edvinsson B, Lappalainen M, Evengård B.** 2006. Real-time PCR targeting a 529-bp repeat element for diagnosis of toxoplasmosis. Clin. Microbiol. Infect. **12**:131–136.
11. **Hierl T, et al.** 2004. Preliminary evaluation of one conventional nested and two real-time PCR assays for the detection of *Toxoplasma gondii* in immunocompromised patients. J. Med. Microbiol. **53**:629–632.
12. **Hohlfeld P, et al.** 1994. Prenatal diagnosis of congenital toxoplasmosis with a polymerase-chain-reaction test on amniotic fluid. N. Engl. J. Med. **331**:695–699.
13. **Homan WL, Vercammen M, De Braekeleer J, Verschueren H.** 2000. Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in *Toxoplasma gondii*, and its use for diagnostic and quantitative PCR. Int. J. Parasitol. **30**:69–75.
14. **Kaiser K, et al.** 2007. Multicenter proficiency study for detection of *Toxoplasma gondii* in amniotic fluid by nucleic acid amplification methods. Clin. Chim. Acta **375**:99–103.
15. **Lebech M, et al.** 1996. Classification system and case definitions of

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Toxoplasma gondii infection in immunocompetent pregnant women and their congenitally infected offspring. European Research Network on Congenital Toxoplasmosis. Eur. J. Clin. Microbiol. Infect. Dis. **15**: 799–805.
16. **Menotti J, et al.** 2010. Evaluation of a new 5′-nuclease real-time PCR assay targeting the *Toxoplasma gondii* AF146527 genomic repeat. Clin. Microbiol. Infect. **16**:363–368.
17. **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. doi:10.1186/1471-2334-3-7.
18. **Romand S, et al.** 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.
19. **Saiki RK, et al.** 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230**:1350–1354.
20. **Sterkers Y, et al.** 2010. Multicentric comparative analytical performance study for molecular detection of low amounts of *Toxoplasma gondii* from simulated specimens. J. Clin. Microbiol. **48**:3216–3222.
21. **Sterkers Y, Varlet-Marie E, Marty P, Bastien P.** 2010. Diversity and evolution of methods and practices for the molecular diagnosis of congenital toxoplasmosis in France: a 4-year survey. Clin. Microbiol. Infect. **16**: 1594–1602.
22. **Thulliez P.** 1992. Screening programme for congenital toxoplasmosis in France. Scand. J. Infect. Dis. Suppl. **84**:43–45.
23. **Wallon M, et al.** 2004. Long-term ocular prognosis in 327 children with congenital toxoplasmosis. Pediatrics **113**:1567–1572.

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