Characterization and Multicentric Validation of a Common Standard for Toxoplasma gondii Detection Using Nucleic Acid Amplification Assays

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ABSTRACT

The molecular diagnosis of toxoplasmosis essentially relies upon laboratory-developed methods and suffers from lack of standardization, hence a large diversity of performances between laboratories. Moreover, the quantification of parasitic loads varies among centers, which in fact prevents the possible prediction of the severity of this disease as a function of parasitic loads. The objectives of this multicentric study performed in eight proficient laboratories of the 'Molecular Biology Pole' of the French National Reference Center for toxoplasmosis (NRC-T) were (i) to assess the suitability of a lyophilized preparation of *Toxoplasma gondii* as a common standard for use in this PCR-based molecular diagnosis, and (ii) to make this standard available to the community. High-quality written procedures were used for the production and qualification of this standard. Three independent batches of this standard, containing concentrations ranging from 10^4 to 0.01 *T. gondii* genome equivalents per PCR, were first assessed: the linear dynamic range was ≥6 log, the intra-assay coefficients of variation (CV) from a sample containing 10 *T. gondii* per PCR was 0.3%-0.42% and the inter-assay CV over a 2-week period was 0.76%-1.47%. A further assessment in eight diagnostic centers showed that it is stable, robust and reliable. These lyophilized standards can easily be produced at a larger scale when needed, and made widely available at the national level. To our knowledge, this is the first quality control assessment of a common standard which is usable both for self-evaluation in laboratories and for accurate quantification of parasitic loads in *T. gondii* prenatal infections.

Key-words: PCR, *Toxoplasma gondii*, congenital toxoplasmosis, assay standardization, standard, multicentric study.

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INTRODUCTION

Toxoplasmosis is a worldwide endemic protozoan disease, acquired mainly via eating or handling undercooked or raw infected meat or through contaminated soil, water or food, and caused by the parasitic protozoon *Toxoplasma gondii* (16). In healthy individuals, primary infection is most often asymptomatic and a symptom-free chronic infection established. Nevertheless, when the disease occurs in pregnant women or immunocompromised individuals such as AIDS or transplanted patients, a life-threatening disease may occur. The development of PCR in the 1990's has clearly improved the diagnosis of toxoplasmosis and molecular diagnosis is now essential for detecting the parasite in clinical specimens (2, 23). The development of quantitative real-time PCR (qrt-PCR) has then brought not only robustness to this diagnosis but also the possibility of quantifying parasitic loads (4), with the aim of establishing a correlation with the severity of the disease (10, 21). Yet, the molecular diagnosis of toxoplasmosis essentially relies upon laboratory-developed methods and still suffers from lack of standardization (19, 26). This in turn leads to variations in the performances (essentially sensitivity) of the PCR assays (14, 25), hence in the quality of patient management. In France, where congenital toxoplasmosis is considered as a public health problem and benefits from a national prevention program (28, 29), the Molecular Biology 'pole' of the French National Reference Center for Toxoplasmosis (NRC-T: http://cnrtoxoplasmose.chu-reims.fr) was created with the aim of standardizing this molecular diagnosis. However, the extreme diversity of methods used (26) and the continual development of novel qrt-PCR devices make a true standardization of the methods extremely difficult at the national level; rather, the NRC-T now aims at the harmonization of the performances of the different assays used in the proficient centers of the country. One of the tools developed for this strategy was to create standards freely available to the clinical microbiology community, *i.e.* reference biological materials which would have to be discriminating, reproducible, reliable, robust and producible in large amounts.

Another goal of the project is to standardize the quantification of the parasitic load in a biological sample. Practical recommendations were not provided in previous studies that correlated parasitic load and severity (10, 21). This is likely due to two main factors. First, these studies did not come exactly to the same conclusions and the community needs confirmation of this correlation. Second, the absolute quantification of parasitic loads poses numerous problems in routine practice and appears as a real challenge to standardization. Indeed, all centers in France allowed to perform prenatal diagnosis of congenital toxoplasmosis are now equipped with qrt-PCR apparatuses; and, in 2012, two thirds of these centers quantified the parasitic load in their results to the national external quality assessments (EQAs). Yet, these quantification results varied considerably among centers, and only 12 out of 30 of them were able to give measurements situated in the range of ±2-fold the expected concentration (Sterkers et al., unpublished data). Assay variability in parasitic load quantitation could lead to inaccurate estimation of disease severity. Within this frame, we feel that accuracy of *T. gondii* load measurements should be improved, in part by the use of a centrally validated and distributed standard to each participating laboratory. This study reports the creation and validation of such a standard for the molecular diagnosis of congenital toxoplasmosis, based on samples spiked with live pathogens rather than on DNA.

MATERIALS AND METHODS

Study scheme. The study was implemented in 2011 and 2012. The production of *T. gondii* stock suspensions and qualification process of the standard were performed in the coordinating center (Department of Parasitology-Mycology of the University Hospital Center of Montpellier, France). A multicentric assessment of the *T. gondii*

standard was then done by eight proficient laboratories from academic hospitals throughout France, including the coordinating center. These eight laboratories form the molecular biology 'pole' of the NRC-T. The study was performed in accordance with the regulations of the local medical ethics committee of the Hospital University Center (CHU) of Montpellier, in line with the revised Helsinki Declaration.

Production of *T. gondii* stock suspensions and standard vials. PCR negative amniotic fluid (AF) samples were spiked with live *T. gondii* tachyzoites to obtain stock suspensions and produce standard vials. Signed consents from patients with hydramnios were obtained and AF drawn for medical purposes in the academic hospital of Tours (France). The AF samples were confirmed as *T. gondii* PCR-negative and then pooled and used as a matrix. *T. gondii* tachyzoites were propagated *in vitro* under standard procedures by serial passage in HFF (human foreskin fibroblast) monolayers in DMEM (Dulbecco's modified Eagle's medium; Invitrogen) supplemented with 10% FBS (fetal bovine serum) (5). Tachyzoites were either the RH laboratory strain or a type II clinical strain (code name MTP040-COR/TgH25040A) (1). The RH strain is a well-known 'classical' type I laboratory strain, whereas MTP040-COR/TgH25040A is a clinical strain isolated from a placenta in a case of asymptomatic congenital toxoplasmosis diagnosed in Montpellier, France, in 2010. Harvested parasites were washed twice and then resuspended in sterile RPMI1640. Tachyzoites were counted in a haemocytometer, means and standard deviations (SDs) were calculated from 10 values, and the parasites were then diluted in the matrix to obtain 10^5 Tgg/mL. This suspension was aliquoted in 2-mL aliquots in glass vials closed with chlorobutyl vial lids (Fisher Scientific®). Aliquots were kept frozen at -20°C until freeze-drying by Lyofal® (Salon de Provence, France) and freeze-dried samples kept at +4°C until qualification. These 2-mL aliquots containing $2x10^5$ tachyzoites are herein termed 'stock suspensions'. Standards were obtained from the freeze-dried stock suspensions by adding 2 mL of sterile water (NAT grade) into the vial. For this study, three independent batches were produced: two using the RH strain and one with the more clinically relevant type II strain.

Qualification process of *T. gondii* standards. The methods described below have been used in routine practice in the coordinating center since 2009.

DNA extraction method used in the coordinating center. DNA was extracted using a method adapted from the Tween-Nonidet-NaOH (TNN; 0.5% Tween 20, 0.5% Nonidet P-40, 10 mM NaOH) method (12); briefly, it is based on a selective lysis of contaminating red blood cells and a heat-detergent extraction and thermolysis buffer method. The sample was first centrifuged at 16,000 × *g*, and the pellet was resuspended in a red blood cell lysis buffer (1% Tris 1M pH 7.2, 0.5% $MgCl_2$ 1M, 1% NaCl 1M). After a second centrifugation, the pellet was resuspended in 100 µL of TNN buffer and heated at 100°C for 10 min. An ultimate centrifugation was performed, and the supernatant was carefully collected. DNA extracts were stored at +4°C, and all the PCR tests performed within the next 15 days.

PCR method used in the coordinating center. The PCR assay targeted the non-coding repetitive DNA termed rep529 (13). *T. gondii* DNA amplification was performed by qrt-PCR using DNA primers, fluorescence resonance energy transfer (FRET) probes and conditions published by Reischl et al. (20) except the use of (i) a LightCycler 480 (LC480) (Roche Applied Science®) instead of a capillary LightCycler, (ii) the highest ramp of 2.2-4.4°C/sec in this PCR device instead of 20°C/sec in the Reischl study, (iii) the LightCycler® 480 Probes Master mix instead of the LightCycler® FastStart Master Hybridization Probes, and (iv) Sigma® instead of TibMolBiol® primers and probes. Good laboratory practices were followed; in particular a positive and negative control was added in each experiment. 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. (22), revealed in SybrGreen (LighCycler® 480 SYBR Green I Master, Roche Diagnostics) and running in the same experiment as the *T. gondii*-specific PCR. Two PCR inhibition internal controls were used for each sample by adding *T. gondii* DNA in two separate reaction tubes at a concentration of 1.5 Tgg/tube.

Serial dilution assays. A standard curve was generated by performing a logarithmic serial dilution of the DNA extract from the *T. gondii* standard. The seven concentrations tested ranged from 10^5 to 0.1 Tgg/mL corresponding to 10^4 to 0.01 *T. gondii* genome equivalent (Tgg) per PCR tube. Each batch of the standard was extracted three times in three independent experiments; and all dilutions were PCR-assayed in triplicate, yielding 9 PCR results per dilution.

Multicentric assessment of the *T. gondii* standards. The RH-based standard, batch TgRHJun11, was sent to eight centers, including the coordinating center. All participating laboratories used their own laboratory-developed, finely optimized, extraction and PCR methods (Table 1). Freeze-dried stock suspensions were shipped to the participating centers at room temperature. Participants stored the sample at +2-8°C until processing. All laboratories reconstituted and processed the lyophilized sample according to the instructions. For this purpose, the 2 mL-sample was concentrated as follows: after centrifugation at 16,000 × *g*, 1.8 mL of the supernatant was removed and the pellet resuspended in the 200 µL remaining volume. It should be stressed that in all cases, (i) the input volume of matrix for DNA extraction was 200 µL and (ii) the elution volume was 100 µL. The serial dilution assay was then performed by each participating center, following a strictly identical dilution protocol accompanying the sample. Seven concentrations ranging from 10^5 to 0.1 Tgg/mL corresponding to 10^4 to 0.01 Tgg/PCR tube were tested in multiplicate. At and around the sensitivity threshold of a given PCR method, only a proportion of the reaction tubes appear positive, which implies that for very low concentrations of the pathogen, several reactions have to be carried out for each experiment (thus increasing the probability of amplifying the pathogen DNA) (8, 9, 15, 30). As a consequence, in each participating center, the concentrations from 10^4 to 10 Tgg/reaction (PCR) tube were tested in duplicate, the concentration of 1 Tgg/reaction tube was tested in triplicate, the concentration of 0.1 Tgg per reaction tube in quadruplicate and that of 0.01 Tgg per reaction tube was tested in sextuplicate.

TABLE 1: Overview of the methods used for the molecular detection of *Toxoplasma gondii*

Center	DNA extraction	qrt-PCR device[a]	Amplicon detection[b]	Fluorophores[c]	DNA target and references for primers[d]	DNA extraction control	PCR inhibition control
A	Qiagen® QIAmp DNA mini kit	LC2 Roche®	FRET	FL/RED640	rep529, Reischl et al. BMC Infect. Dis. 2003	ß-globin gene	plasmidic competitive internal control
B	Qiagen® QIAmp DNA mini kit	LC2 Roche®	FRET	FL/RED640	rep529, Reischl et al. BMC Infect. Dis. 2003	optical density measure	ß-globin gene
C	Qiagen® QIAmp DNA mini kit	LC1 Roche®	FRET	FL/RED640	rep529, Reischl et al. BMC Infect. Dis. 2003	ß-globin gene	ß-globin gene
D	Tween-Noninet-NaOH method[d]	LC480 Roche®	FRET	FL/RED640	rep529, Reischl et al. BMC Infect. Dis. 2003	ß-globin gene	*T. gondii* DNA internal control
E	Qiagen® QIAmp DNA mini kit	Applied® 7000	TaqMan	FAM/BHQ1, LNA probe	rep529, Talabani et al. J Clin. Microbiol. 2009	optical density measure	plasmidic internal control
F	Qiagen® QIAmp DNA mini kit	Applied® 7500	TaqMan	FAM/TAMRA	rep529 et B1, Fekkar et al. J. Clin. Microbiol. 2008	albumin gene	*T. gondii* DNA internal control
G	Qiagen® QIAmp DNA mini kit	LC1 Roche®	FRET	FL/RED640	rep529, Reischl BMC Infect. Dis. 2003	two extractions with the same method	*T. gondii* DNA internal control
H	Roche® High pure PCR Template kit	LC2 Roche®	FRET	FL/RED640	rep529, Cassaing et al. J. Clin. Microbiol. 2006	ß-globin gene	ß-globin gene

[a] LC: Light-Cycler, [b] FRET: FRET hybridization DNA probes, TaqMan: hydrolysis DNA probes (TaqMan technology; Applied Biosystems®); [c] FL: Fluorescein, FAM: Fluorescein, BHQ: Black-Hole-Quencher, TAMRA: Tetramethylrhodamin, [d] rep529: a 200- to 300-fold repetitive 529 bp DNA fragment in *Toxoplasma gondii*,[e] Hohlfeld et al., N. Engl. J. Med. 1994

Data and statistical analysis. Threshold cycle (Ct) values were determined by each center using the analysis software of the PCR device (Table 1). For example, in the coordinating center, Cts were calculated using the "absolute quantification/second derivative maxima" algorithm by the Light Cycler 480® Software release 1.5.0.39. Cts obtained by each center were used to elaborate the standard curve and to determine the efficiency of each PCR method. Indeed, the slope of standard curves allowed determining the PCR efficiency (EFF) in conformity with the formula $EFF = 10^{[-1/slope]}-1$. Spreadsheet (Microsoft Excel) was used for all statistical calculations including mean, SDs, coefficient of variation (CV) and correlations (R^2). The best-fitting line was determined using least-squares analysis.

RESULTS

With the aim of harmonizing the performances of the PCR assays used for the diagnosis of toxoplasmosis and standardizing the molecular quantification of *T. gondii* in AF, we produced a common standard consisting in freeze-dried AF spiked with *T. gondii* tachyzoites. Three standard batches were thoroughly evaluated and qualified in the coordinating center. One of these batches was also sent for evaluation in seven other proficient centers.

Qualification of the *T. gondii* standards. To qualify the *T. gondii* standards, we first determined the sensitivity, specificity, linear dynamic range, linearity, reproducibility and repeatability of the PCR assay used in routine in the coordinating center (Montpellier) using these samples. This assay adapted from Reischl *et al.* (20) has been published previously and is highly sensitive and specific (17). The analytical sensitivity threshold was 0.01 Tgg/PCR tube, equivalent to 0.1 Tgg/mL of AF (24). In addition to the specificity testing done by Reischl et al., we could not find any cross-signal when testing DNA of other Apicomplexa (*Plasmodium falciparum*, *P. ovale*, *Cryptosporidium* sp.) or other frequent blood parasites and fungi (*Leishmania infantum*, *Pneumocystis jirovecii*, *Aspergillus fumigatus* and *Candida albicans*). Moreover, to date, routine diagnostic use of the method for several years yielded no amplification of human DNA in >7500 negative clinical samples.

For qualification of this *T. gondii* standard, three independent serial dilution assays were performed from three different extracts of three *T. gondii* preparations. In all cases, the linear dynamic range of the assay was ≥6 log. Moreover, for all batches, at the highest dilution (0.01 Tgg/PCR tube), only a proportion of the reaction tubes was positive, thus allowing us (i) to assess the sensitivity threshold of the PCR assay (8, 9, 15, 30) and (ii) to confirm the correct range of concentrations tested.

The linearity of an analytical procedure corresponds to its capacity (over a defined range) to obtain results that are directly proportional to the concentration (quantity) of test material in the sample. Typical amplification curves generated and the standard curve derived from this dilution series are illustrated in Figure 1. The PCR assay showed a high R^2 value (>0.99) and a slope at -3.38, corresponding to a PCR efficiency > 97.5%. These values are satisfactory for all three regression lines; the PCR assay is therefore linear, allowing correct quantification of the DNA target.

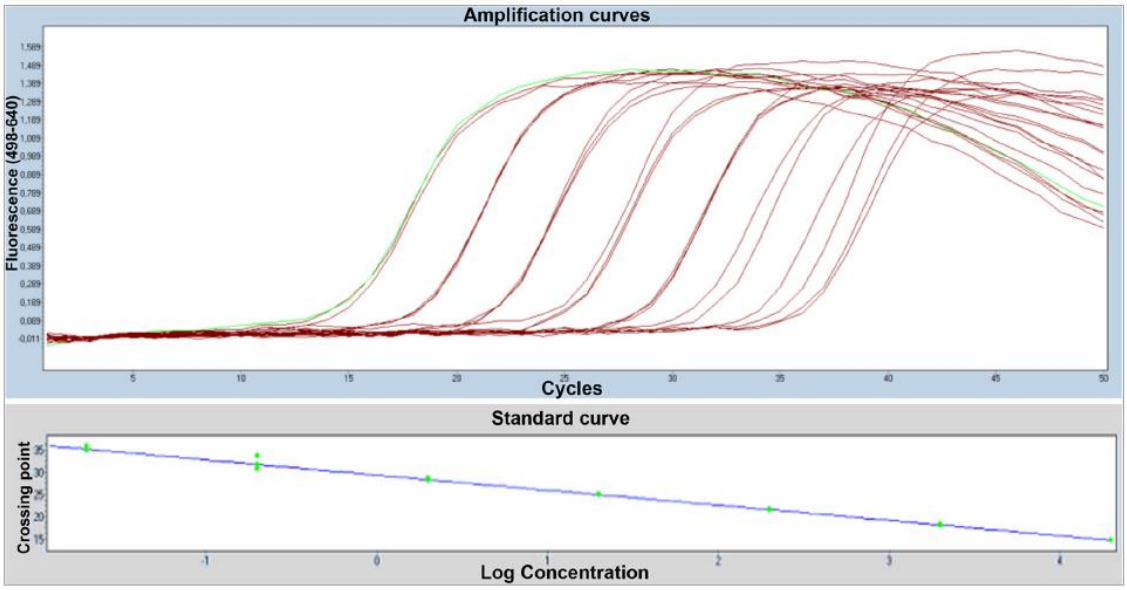

FIG. 1. LightCycler® real-time PCR amplification of *Toxoplasma gondii* DNA performed during qualification of the *T. gondii* standard in one serial dilution assay (Batch TgRHJun11, vial 1). (Top) Amplification curves based on real-time fluorescence measurements during PCR. All tests were performed in triplicate on seven samples representing a 10-fold serial dilution ranging from 10^4 to 0.01 Tgg/PCR tube. (Bottom) Standard curve demonstrating a linear relationship between logarithm of copy number and Ct.

Highly similar results were obtained for RH strain- and type II-based standards (Table 2). All replicates at each concentration down to 0.1 Tgg/PCR tube were found positive; at the lowest concentration (0.01 Tgg/PCR tube), 6/9 and 8/9 reactions were found positive using the RH- and Type II-based standard, respectively. Individual Ct values for *T. gondii* detection were in the range of 16-38.2 cycles, and the mean values ranged from 16.5 to 37.7. At DNA concentrations between 10^4 and 10 Tgg/PCR tube, intra- and inter-assay comparisons yielded SDs varying from 0.01 to 0.16, and 0.12 to 0.39 Cts, respectively, and logically increasing at the lowest concentrations (1 and 0.1 Tgg/PCR tube). The intra-assay coefficients of variation (CV), calculated from triplicate testing of a sample containing 10 Tgg/PCR tube, varied from 0.3% to 0.42%. The inter-assay CV, calculated by testing a sample containing 10 Tgg/PCR tube in five separate assays over a 2-weeks period, varied from 0.76% to 1.47%. Thus, reproducibility and repeatability were high for both intra- and inter-assay experiments.

TABLE 2. Results of the PCR tests performed for qualification of the three *T. gondii* standard batches in the coordinating center.

	Concentrations (Tgg/PCR tube)[a,b]	**10^4**	**10^3**	**10^2**	**10**	**1**	**0.1**	**0.01**
RH strain Batch TgRHJun11	Number of positive tubes/number of test tubes	9/9	9/9	9/9	9/9	9/9	9/9	7/9
	Mean CP values	16.97	20.96	24.61	28.1	31.7	35.66	37.7[c]
	Intra-assay SDs	0.02-0.08	0.01-0.08	0.02-0.1	0.07-0.16	0.14-0.51	0.18-0.78	0.52-0.97[c]
	Inter-assay SDs	0.26	0.16	0.17	0.23	0.52	0.41	0.54
RH strain Batch TgRHJul11	Number of positive tubes/number of test tubes	9/9	9/9	9/9	9/9	9/9	9/9	6/9
	Mean CP values	16.45	19.73	23.21	26.72	30.34	33. 32	36.53[c]
	Intra-assay SDs	0.01-0.08	0.01-0.1	0.03-0.05	0.07-0.12	0.17-0.62	0.19-0.87	0.48-0.74[c]
	Inter-assay SDs	0.18	0.14	0.12	0.13	0.44	0.69	0.53
Type II clinical strain TgIIJul11	Number of positive tubes/number of test tubes	9/9	9/9	9/9	9/9	9/9	9/9	8/9
	Mean CP values	16.05	19.41	22.90	26.45	29.40	32.57	35.65[c]
	Intra-assay SDs for Type II strain	0.02-0.06	0.02-0.09	0.02-0.06	0.06-0.13	0.05-0.47	0.21-0.75	0.50-1.60[b]
	Inter-assay SDs[a]	0.37	0.28	0.22	0.39	0.61	0.96	1.49

[a] Tgg: *T. gondii* genome equivalent, [b] Mean Crossing point (CP) values and Standard deviations (SDs) were calculated from three independent serial dilution assays performed using three different vials of each batch; [c] based on the positive values only

Multicentric assessment of the *T. gondi*i standard.

In order to perform a multicentric assessment of our standards, we sent the RH-based standard, batch TgRHJun11, to eight proficient centers (including the coordinating center), which also correspond to a representative panel of the methods used in France for the diagnosis of congenital toxoplasmosis. Indeed, each of the eight participating laboratories used its own laboratory-developed molecular diagnosis method(s), all targeting the repetitive DNA element 'rep529' (13) but differing in the DNA extraction method, primers, PCR technology, and amplicon detection (Table 1). Data for all participating laboratories are shown in Table 3. All methods reliably detected the parasite down to 1 Tgg/PCR tube. At and below 0.1 Tgg/PCR tube, only a portion of the reactions were positive with certain methods: two laboratories found all quadruplicates positive, five laboratories found two or three, and one laboratory found only one. At 0.01 Tgg/PCR tube, one and two laboratories found 4/6 and 1/6, respectively, positive reactions, whereas the five other assays yielded negative results.

TABLE 3. Results of the multicentric assessment of the RH *T. gondii* standard

Center and PCR efficiency	Concentrations (Tgg[a]/PCR tube)[b]	10^4	10^3	10^2	10	1	0.1[c]	0.01[c]
A	Nb positives [d]	2/2	2/2	2/2	2/2	3/3	1/4	1/6
EFF[e] = 98%	Mean CP values[f] ± SD	17.55 ±0.56	22.2 ±0.15	24.90 ±0.04	28.22 ±0.42	32.37 ±1.54	33.27	33.04
B	Nb positives [c]	2/2	2/2	2/2	2/2	3/3	3/4	1/6
EFF = 110%	Mean CP values ± SD	17.04 ±0.04	20.62 ±0.05	23.88 ±0.05	26.67 ±0.78	31.05 ±0.9	31.53 ±0 .96	33.27
C	Nb positives [c]	2/2	2/2	2/2	2/2	3/3	4/4	0/6
EFF = 90%	Mean CP values ± SD	17.05 ±0.15	20.72 ±0.07	24.11 ±0.05	27.5 ±0.38	31.32 ±0.97	34.98 ±1.81	/
D	Nb positives [c]	2/2	2/2	2/2	2/2	3/3	4/4	4/6
EFF = 93%	Mean CP values ± SD	17.22 ±0.08	21.16 ±0.04	24.7 ±0.1	28.35 ±0.12	32.09 ±0.43	35.9 ±0.60	37.7 ±1.15
E	Nb positives [c]	2/2	2/2	2/2	2/2	3/3	2/4	0/6
EFF = 100%	Mean CP values ± SD	17.57 ±0.35	20.22 ±0.03	23.58 ±0.30	27.26 ±0.02	30.13 ±1.31	34.14 ±1.09	/
F	Nb positives [c]	2/2	2/2	2/2	2/2	3/3	2/4	0/6
EFF = 92%	Mean CP values ± SD	19.41 ±0.07	22.72 ±0.03	26.28 ±0.07	29.62 ±0.28	33.78 ±1.66	36.61 ±2.04	/
G	Nb positives [c]	2/2	2/2	2/2	2/2	3/3	2/4	0/6
EFF = 93%	Mean CP values ± SD	17.46 ±0.3	21.89 ±0.04	24.62 ±0.08	27.9 ±0.11	35.55 ±3.4	32.15 ±1.27	/
H	Nb positives [c]	2/2	2/2	2/2	2/2	3/3	3/4	0/6
EFF = 94%	Mean CP values ± SD	18.15 ±0.22	21.80 ±0.06	24.94 ±0.32	28.20 ±0.07	33.33 ±1.15	34.82 ±2.54	/

[a]Tgg: *T. gondii* genome equivalent; [b] RH standard batch TgRHJun11 was used for this assessment; [c]calculation of standard deviations(SDs) was based on positive values only; [d]Nb positives: number of positive reactions / number of reactions performed; [e]EFF: PCR efficiency; [f]CP: crossing point,

DISCUSSION

Molecular diagnosis of toxoplasmosis suffers from a lack of standardization, leading to a large diversity of performances between laboratories, even in countries where congenital toxoplasmosis is considered a public health problem and benefits from a national prevention program. To date, this is the first report of a quality control assessment of a common standard for the molecular diagnosis of congenital toxoplasmosis, and in particular for the quantification of parasitic loads in *T. gondii* prenatal infections. Its assessment, both at the production stage and then at a multicenter level, showed that it is stable, robust and reliable. Its use in the medical parasitology community has proven successful.

A variety of methods can be used to produce standards. Ideally, due to varying PCR-inhibiting properties depending on the biological fluid examined, the standard should use the same matrix as the one examined in diagnosis (AF, blood…); also, the amount of target present prior to DNA extraction and PCR should be as accurate as possible. Yet, the nature of the reference material to be quantified may vary widely, from heterologous plasmids to plasmids containing inserts of *T. gondii* DNA sequence, or to whole pathogens. Here, we used calibrated samples of human AF because (i) it perfectly mimics diagnostic samples for congenital

toxoplasmosis, (ii) it constitutes an easily obtainable and abundant source of matrix and (iii) the development of standard material from this type of matrix is more advanced than that from blood samples (3, 14). A minor drawback is that AF drawn from hydramnios is often naturally diluted, hence paucicellular, and may not precisely reproduce the composition of AF samples tested in routine. We used whole pathogens rather than DNA for the following reason: we assume this helps better reproducing the conditions of the assay in routine diagnosis because we consider that DNA extraction and amplification are intimately linked for the success of the method (see also below). Moreover, here, as opposed to the previously described methods using *Toxoplasma* tachyzoites drawn from mouse ascites, we used cultivated parasites, which have the advantage of being easier to obtain and purify, more standardized, easier to count in a counting chamber, and less subject to degradation than ascites-drawn tachyzoites (Bastien P. *et al.*, unpublished data). One of our preparations also has the advantage of being based on a naturally occurring *T. gondii* strain (type II), which therefore mimics the routine situation. The testing of this standard yielded near identical results to the one based on the RH laboratory strain. Hence the type II strain-based preparation was selected for further distribution at the national level. Finally, the use of freeze-drying further enhances the stability and robustness of the standard, and offers other advantages such as transport at ambient temperature, long-term conservation at +4°C, non-infectiousness, and flexibility in use. Freeze-dried samples have proved stable for at least three years under storage at +4°C (not shown). As assessed from PCR testing of the panels, freeze-drying only resulted in a slight reduction of Ct values ranging between 0.2 and 1 Ct, regardless of the concentrations of tachyzoites tested (from 10000 to 0.1 Tgg/PCR tube) (not shown). Another advantage is that freeze-dried preparations can be produced in bulk, allowing large-scale distribution when needed. Moreover, the results of the national internal and external quality assessments show that a short stay at ambient temperatures during shipment of the freeze-dried standards does not result in loss of detection signal (not shown). It should be stressed that this standardized material is fundamentally different than the use of a simple DNA extract, which is often distributed in EQAs (18). The use of DNA extracted in the coordinating center has the advantage of simplicity, as it can be used as such in its own buffer. It may also appear as more 'homogeneous', hence more 'standardized', than whole pathogens. Yet, we decided not to send previously extracted DNA, since a previous study, using the same *T. gondii* DNA extracted by a Roche® system revealed variations in the results among different centers, that did not reflect the true performances of the PCR assays used by each participant: indeed, when compared with DNA extraction performed independently in each center, using this DNA extract resulted in a reduction in sensitivity of all PCR assays except the one which used the Roche® extraction system (25; and Molecular Biology 'pole' of the NRC-T, unpublished data). This could only be explained by a lack of conjoint optimization between the DNA extraction method of the sender and the PCR methods used by the participants (unpublished data). Thus, the use of a DNA extract does not assess the DNA extraction method, yielding the risk of inaccurate assessments of the methods used by the participants. In contrast, our protocol allowed the testing of the whole process (DNA extraction, amplification, and detection) of each molecular assay set up independently by each participant to reliably compare the performances of all assays. Using the whole pathogen thus helps mimicking the conditions of the assay in routine diagnosis.

A multicentric qualification process. The production and qualification of the standard presented here followed strict rules according to international standards (6) and the observed quality criteria were highly satisfactory. The multicentric assessment allowed to definitely validate its quality and utility. Data obtained by the eight proficient laboratories cannot be statistically compared since the calculation of Cts depends on the assays used, in particular

the PCR device, the software, and the detection method. This being said, at the highest concentrations, the *T. gondii* standard test results were found similar among centers: at 10 Tgg/mL, intra-center ΔCts were below 1.0 in all centers, and the slopes ranged from -1.554 for laboratory C to -1.310 for laboratory B. By contrast, below 10 Tgg/PCR tube, the participants showed more divergent results: at 1 Tgg/PCR tube, intra-center ΔCts ranged from 1.81 (31.32 +/-0.97) for laboratory C to 6.19 (35.55 +/-3.4) for laboratory G; and below 0.1 Tgg/PCR tube, detection became unpredictable, showing that some methods were close to their detection limits (8, 9, 15, 30).

Use of a serial dilution assay. Using a serial dilution assay had several advantages. First, when serially diluted, the standards can be used for quantification. In the serial dilution assay, the linear dynamics spanned over 6 orders of magnitude, showing that quantification should be accurate in this range of concentrations. From our experience, the 23 last positive clinical samples recorded in our routine activity exhibited a mean Ct of 30.5 +/-3.7 (25-36 Cts) indicating that the range of the standard curve is appropriate for quantification in routine practice. More importantly, the use of dilutions of extracted DNA, combined with the repetition of the PCR DNA target, allowed avoiding the pitfall of the Poisson's law which is applicable at very low pathogen concentrations. We have previously taken advantage of this strategy to discriminate between molecular methods (25).

Multi-purpose standards. There are several advantages in using such a standard. (i) It allows a self-assessment of the technical performances (in particular analytical sensitivity) of the *Toxoplasma*-PCR assay in each center, hence PCR assay validation. (ii) It allows each center to follow-up the performances of its molecular diagnosis, and particularly after equipment or reagent changes. (iii) It provides a source of material for assay validation and the production of secondary standards to be used as run controls or working reagents. (iv) It facilitates external quality assessments and comparisons among different centers. (v) It should allow the standardization of quantification of parasitic loads in clinical samples. As noted above, notification of parasitic loads to physicians remains rare in France, both because of the difficulties encountered in absolute quantification and because there is a general assumption in the community that the two studies about this matter (10, 21) must be confirmed. The need for standardization here is critical. One of the concerns is the strict correlation between the results obtained using serial dilutions of our standard and those obtained after the DNA extraction of samples containing low amounts of parasites, including clinical samples. In our hands, highly similar results were obtained in several centers when directly extracting samples at 10 and 50 Tgg/mL or using DNA diluted to the same concentrations from the highly concentrated standard (not shown). This standard therefore appears as an essential step towards that goal.

In total, we showed the feasibility of using a stable, standardized, validated, discriminant and centrally distributed, freeze-dried whole-pathogen standard, for the evaluation of commercial or laboratory-developed PCR assays for the diagnosis of congenital toxoplasmosis. We showed that the serial dilution assay was in a suitable range for the wide variety of assays used by the participants. Our standard helped assessing and improving the PCR assays in use in different centers, and proved a powerful tool for the homogenization of their performances. Finally, the quantification of parasitic loads in toxoplasmic infections can be significantly improved by using such a common standard.

At the moment, a *T. gondii* standard containing $1x10^5$ parasites (RH strain) per mL is freely available to the members of the NRC-T network upon request, as long as they answer a standardized questionnaire reporting their molecular detection method and results. The standard vial comes with detailed instructions about the resuspension and processing of the sample and it is crucial that participants follow these instructions, in order

to get reliable quantification results, which can also be used for further improvements. A confirmation of the practical usefulness of this standard is that most French centers which perform the molecular diagnosis of toxoplasmosis are now asking for this standard on a regular basis. Apart from its usefulness for quality control, the extensive use of such a standard should allow for multicentric studies correlating parasitic loads in AF and fetal prognosis. One limitation of our standard is that it can be used only for the diagnosis of congenital toxoplasmosis. The future developments of this standard will need to address the implementation of quantification in whole blood, which represents a new challenge, undoubtedly useful for the follow-up of immunosuppressed patients.

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