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

Emmanuelle Varlet-Marie,[a,b,c] Yvon Sterkers,[a,b] Marie-Pierre Brenier-Pinchart,[b,d] Sophie Cassaing,[b,e] Frédéric Dalle,[b,f] Laurence Delhaes,[b,g] Denis Filisetti,[b,h] Hervé Pelloux,[b,d] Fériel Touafek,[b,i] Hélène Yera,[b,j] Patrick Bastien[a,b]

CHU (University Hospital Centre) of Montpellier/University Montpellier I, Laboratoire de Parasitologie-Mycologie, Montpellier, France[a]; Pôle Biologie Moléculaire du Centre National de Référence de la Toxoplasmose, Montpellier, France[b]; University Montpellier I, Faculty of Pharmacy, Laboratoire de Biophysique & Bioanalyse, Montpellier, France[c]; CHU A. Michallon/University Joseph Fourier Grenoble I, Laboratoire de Parasitologie-Mycologie, Département des Agents Infectieux, Grenoble, France[d]; CHU of Toulouse, Service de Parasitologie-Mycologie, Toulouse, France[e]; CHU of Dijon, Laboratoire de Parasitologie-Mycologie, Faculty of Medicine, Université de Bourgogne, UMR 1347 Aggroécologie, Dijon, France[f]; CHU of Lille/Université Nord de France (UDSL), Service de Parasitologie-Mycologie, Faculty of Medicine, Institut Pasteur de Lille, Lille, France[g]; Hôpitaux Universitaires de Strasbourg/University of Strasbourg, Laboratoire de Parasitologie et Mycologie Médicales, Strasbourg, France[h]; AP-HP, Groupe Hospitalier Pitié-Salpêtrière, Laboratoire de Parasitologie-Mycologie, Paris, France[i]; University Paris Descartes, AP-HP, Groupe Hospitalier Cochin, Laboratoire de Parasitologie-Mycologie, Paris, France[j]

The molecular diagnosis of toxoplasmosis essentially relies upon laboratory-developed methods and suffers from lack of standardization, hence the large diversity of performances between laboratories. Moreover, quantifications of parasitic loads differ among centers, a fact which 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* organisms per PCR were 0.3% to 0.42%, and the interassay CV over a 2-week period was 0.76% to 1.47%. A further assessment in eight diagnostic centers showed that the standard is stable, robust, and reliable. These lyophilized standards can easily be produced at a larger scale when needed and can be 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.

Toxoplasmosis is a protozoan disease of worldwide endemicity that is acquired mainly via eating or handling undercooked or raw infected meat or through contact with contaminated soil, water, or food and is caused by the parasitic protozoon *Toxoplasma gondii* (1). In healthy individuals, primary infection is most often asymptomatic, with a symptom-free chronic infection established. Nevertheless, when the disease occurs in pregnant women or immunocompromised individuals such as AIDS patients or patients with organ transplants, a life-threatening disease may occur. The development of PCR in the 1990s has clearly improved the diagnosis of toxoplasmosis, and molecular diagnosis is now essential for detecting the parasite in clinical specimens (2, 3). The development of quantitative real-time PCR (qrt-PCR) has therefore brought to this diagnosis not only robustness but also the possibility of quantifying parasitic loads (4), with the aim of establishing a correlation with the severity of the disease (5, 6). Yet the molecular diagnosis of toxoplasmosis essentially relies upon laboratory-developed methods and still suffers from lack of standardization (7, 8). This in turn leads to variations in the performances (essentially in sensitivity) of the PCR assays (9, 10) and hence in the quality of patient management. In France, where congenital toxoplasmosis is considered a public health problem and benefits from a national prevention program (11, 12), 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 (8) 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 the creation of standards, i.e., reference biological materials which would have to be discriminating, reproducible, reliable, robust, and producible in large amounts, freely available to the clinical microbiology community.

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 (5, 6). This is likely due to two main factors. First, these studies did not come exactly to the same conclusions,

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Editor: P. H. Gilligan
Address correspondence to Patrick Bastien, p-bastien@chu-montpellier.fr.

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and the community needs confirmation of this correlation. Second, the absolute quantification of parasitic loads poses numerous problems in routine practice and is 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 with respect to the national external quality assessments (EQAs). Yet these quantification results differed considerably among centers, and only 12 of 30 of them were able to give measurements situated in a ±2-fold range of the expected concentration (Y. Sterkers, E. Varlet-Marie, and P. Bastien, 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 standard distributed to each participating laboratory. This report describes the creation and validation of such a standard for the molecular diagnosis of congenital toxoplasmosis determined on the basis of samples spiked with live pathogens rather than on the basis of DNA.

MATERIALS AND METHODS

Study scheme. The study was implemented in 2011 and 2012. The production of *T. gondii* stock suspensions (see below) and the process of qualification of the standard were performed in the coordinating center (Department of Parasitology-Mycology of the University Hospital Center of Montpellier, 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 to produce standard vials. Signed consent forms from patients with hydramnios were obtained and AF samples drawn for medical purposes in the academic hospital of Tours (France). The AF samples were confirmed to be *T. gondii* PCR negative and then pooled and used as a matrix. *T. gondii* tachyzoites were propagated *in vitro* by serial passage in human foreskin fibroblast (HFF) monolayers in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS) (13) using standard procedures. Tachyzoites were either the RH laboratory strain or a type II clinical strain (code name MTP040-COR/TgH25040A) (14). 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 RPMI 1640. Tachyzoites were counted in a hemocytometer, means and standard deviations (SDs) were calculated from 10 values, and the parasites were then diluted in the matrix to obtain 10^5 *T. gondii* genome equivalents (Tgg)/ml. This suspension was divided into 2-ml aliquots in glass vials closed with chlorobutyl vial lids (Fisher Scientific). Aliquots were kept frozen at −20°C until freeze-drying was performed by the use of Lyofal (Salon de Provence, France), and freeze-dried samples were kept at +4°C until qualification. These 2-ml aliquots containing 2×10^5 tachyzoites are termed "stock suspensions" here. Standards were obtained from the freeze-dried stock suspensions by adding 2 ml of sterile water (molecular biology 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.

Process of qualification 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 (15); briefly, it is based on 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 [1 M; pH 7.2], 0.5% $MgCl_2$ [1 M], 1% NaCl [1 M]). After a second centrifugation, the pellet was resuspended in 100 μl of TNN buffer and heated at 100°C for 10 min. A final centrifugation was performed, and the supernatant was carefully collected. DNA extracts were stored at +4°C, and all the PCR tests were performed within the next 15 days.

PCR method used in the coordinating center. The PCR assay targeted the noncoding repetitive DNA termed rep529 (16). *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. (17) except that we used (i) a LightCycler 480 (LC480) apparatus (Roche Applied Science) instead of a capillary LightCycler, (ii) the highest ramp of 2.2 to 4.4°C/s in this PCR device instead of the 20°C/s used in the Reischl study, (iii) LightCycler 480 Probes master mix instead of LightCycler FastStart master hybridization probes, and (iv) Sigma instead of TibMolBiol primers and probes. Good laboratory practices were followed; in particular, a positive control and a negative control were added in each experiment. A DNA extraction tube control 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. (18), revealed in SYBR green (LightCycler 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 to 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 equivalents (Tgg) per PCR tube. Each batch of the standard was extracted three times in three independent experiments, and all dilutions were assayed by PCR 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 to +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 remaining 200-μl 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 all participating centers, following strictly identical dilution protocols according to the directions 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) (22–25). As a consequence, in each participating center, the concentrations of 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 was

TABLE 1 Overview of the methods used for the molecular detection of *Toxoplasma gondii* in this study

Center	DNA extraction apparatus	qrt-PCR device[a]	Amplicon detection method[b]	Fluorophores[c]	DNA target and reference for primers[d]	DNA extraction control	PCR inhibition control
A	Qiagen QIAmp DNA minikit	LC2 Roche	FRET	FL/RED640	rep529 (17)	ß-Globin gene	Plasmidic competitive internal control
B	Qiagen QIAmp DNA minikit	LC2 Roche	FRET	FL/RED640	rep529 (17)	Optical density measure	ß-Globin gene
C	Qiagen QIAmp DNA minikit	LC1 Roche	FRET	FL/RED640	rep529 (17)	ß-Globin gene	ß-Globin gene
D	Tween-Noninet-NaOH method[e]	LC480 Roche	FRET	FL/RED640	rep529 (17)	ß-Globin gene	*T. gondii* DNA internal control
E	Qiagen QIAmp DNA minikit	Applied 7000	TaqMan	FAM/BHQ1, LNA probe	rep529 (19)	Optical density measure	Plasmidic internal control
F	Qiagen QIAmp DNA minikit	Applied 7500	TaqMan	FAM/TAMRA	rep529 et B1 (20)	Albumin gene	*T. gondii* DNA internal control
G	Qiagen QIAmp DNA minikit	LC1 Roche	FRET	FL/RED640	rep529 (17)	Two extractions with the same method	*T. gondii* DNA internal control
H	Roche High Pure PCR template kit	LC2 Roche	FRET	FL/RED640	rep529 (21)	ß-Globin gene	ß-Globin gene

[a] LC, LightCycler.
[b] FRET, fluorescence resonance energy transfer hybridization DNA probes; TaqMan, hydrolysis DNA probes (TaqMan technology; Applied Biosystems).
[c] FL, fluorescein; FAM, 6-carboxyfluorescein, BHQ, black hole quencher; TAMRA, 6-carboxytetramethylrhodamine.
[d] rep529, a 200- to 300-fold-repetitive 529-bp DNA sequence in the *Toxoplasma gondii* genome.
[e] Hohlfeld et al. (15).

tested in quadruplicate, and the concentration of 0.01 Tgg per reaction tube was tested in sextuplicate.

Data and statistical analysis. Threshold cycle (C_T) values were determined by each center using the analysis software of the PCR device (Table 1). For example, in the coordinating center, C_T values were calculated using the "absolute quantification/second derivative maxima" algorithm by the use of LightCycler 480 software (release 1.5.0.39). C_T values 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 determination of the PCR efficiency (EFF) in conformity with the formula $\text{EFF} = 10^{[-1/\text{slope}]} - 1$. Spreadsheet (Microsoft Excel) was used for all statistical calculations, including means, SDs, coefficients 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 of 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 practice in the coordinating center (Montpellier) using these samples. This assay adapted from Reischl et al. (17) has been published previously and is highly sensitive and specific (26). The analytical sensitivity threshold was 0.01 Tgg/PCR tube, equivalent to 0.1 Tgg/ml of AF (27). In addition to the specificity testing done by Reischl et al., we could not find any cross-signal in tests of DNA of other Apicomplexa (*Plasmodium falciparum*, *P. ovale*, and *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 >7,500 negative clinical samples.

For qualification of this *T. gondii* standard, three independent serial dilution assays were performed using 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 gave positive results, thus allowing us (i) to assess the sensitivity threshold of the PCR assay (22–25) 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 Fig. 1. The PCR assay showed a high R^2 value (>0.99) and a slope at −3.38, corresponding to a PCR efficiency of >97.5%. These values are satisfactory for all three regression lines; the PCR assay is therefore linear, allowing correct quantification of the DNA target.

Highly similar results were obtained for RH strain- and type

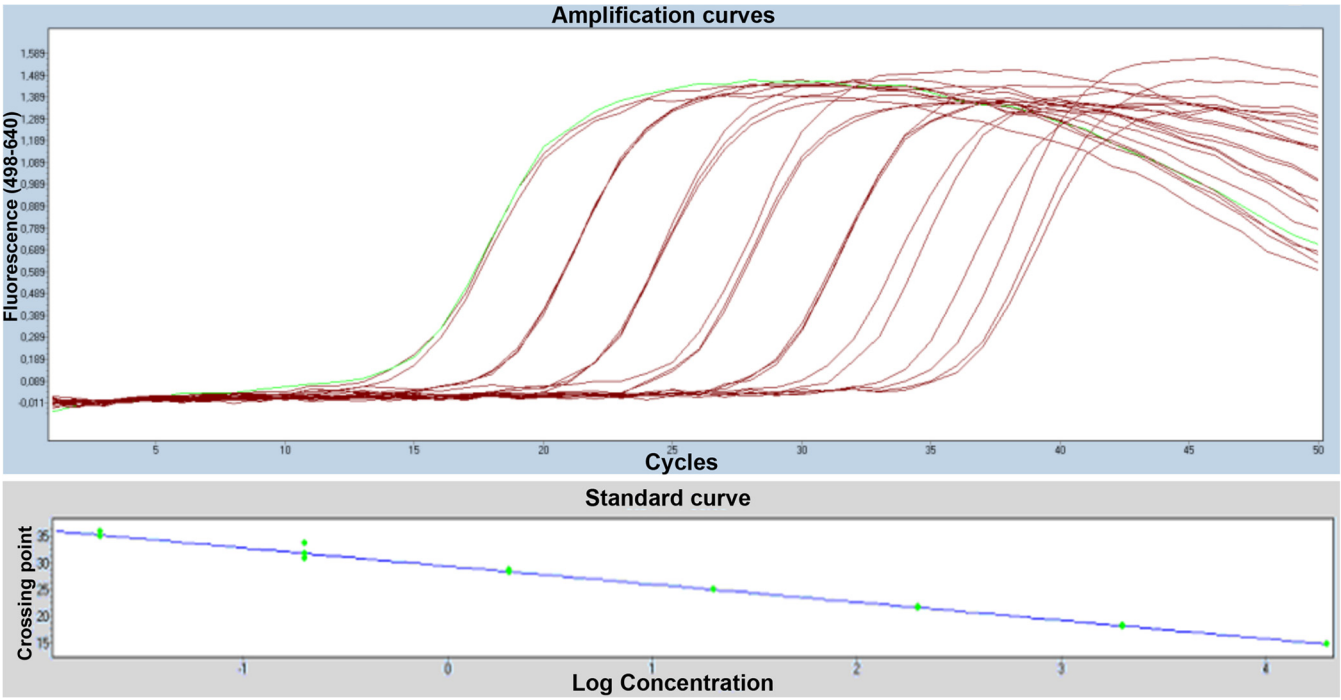

FIG 1 LightCycler real-time PCR amplification of *Toxoplasma gondii* DNA performed during qualification of the *T. gondii* standard in one serial dilution assay (Batch TgRHJun11, vial 1). (Top) Amplification curves based on real-time fluorescence measurements during PCR. All tests were performed in triplicate 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 the logarithm of the copy number and the C_T value. 498-640, 498 to 640 nm.

II-based standards (Table 2). All replicates at each concentration down to 0.1 Tgg/PCR tube were found to give positive results; at the lowest concentration (0.01 Tgg/PCR tube), 6/9 and 8/9 reactions were found to be positive using the RH-based standard and the type II-based standard, respectively. Individual C_T values for *T. gondii* detection were in the range of 16 to 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 interassay comparisons yielded various C_T SDs of from 0.01 to 0.16 and from 0.12 to 0.39, respectively, that logically increased 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 con-

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

Strain and/or batch	Parameter[b]	Value at indicated concn (Tgg[a]/PCR tube)						
		10^4	10^3	10^2	10	1	0.1	0.01
RH strain batch TgRHJun11	No. of positive tubes/total no. of test tubes	9/9	9/9	9/9	9/9	9/9	9/9	7/9
	Mean C_T 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]
	Interassay SDs	0.26	0.16	0.17	0.23	0.52	0.41	0.54
RH strain batch TgRHJul11	No. of positive tubes/total no. of test tubes	9/9	9/9	9/9	9/9	9/9	9/9	6/9
	Mean C_T 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]
	Interassay SDs	0.18	0.14	0.12	0.13	0.44	0.69	0.53
Type II clinical strain TgIIJul11	No. of positive tubes/total no. of test tubes	9/9	9/9	9/9	9/9	9/9	9/9	8/9
	Mean C_T 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]
	Interassay SDs	0.37	0.28	0.22	0.39	0.61	0.96	1.49

[a] Tgg, *T. gondii* genome equivalent.
[b] Mean cycle threshold (C_T) values and standard deviations (SDs) were calculated from three independent serial dilution assays performed using three different vials of each batch.
[c] Data were calculated on the basis of the positive values only.

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

Center (PCR efficiency)	Parameter	Value at indicated concn (Tgg[a]/PCR tube)[b]						
		10^4	10^3	10^2	10	1	0.1[c]	0.01[c]
A (98%)	Nb positives[d]	2/2	2/2	2/2	2/2	3/3	1/4	1/6
	Mean C_T values[e] ± 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 (110%)	Nb positives[c]	2/2	2/2	2/2	2/2	3/3	3/4	1/6
	Mean C_T 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 0.96	33.27
C (90%)	Nb positives[c]	2/2	2/2	2/2	2/2	3/3	4/4	0/6
	Mean C_T 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 (93%)	Nb positives[c]	2/2	2/2	2/2	2/2	3/3	4/4	4/6
	Mean C_T 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 (100%)	Nb positives[c]	2/2	2/2	2/2	2/2	3/3	2/4	0/6
	Mean C_T 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 (92%)	Nb positives[c]	2/2	2/2	2/2	2/2	3/3	2/4	0/6
	Mean C_T 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 (93%)	Nb positives[c]	2/2	2/2	2/2	2/2	3/3	2/4	0/6
	Mean C_T 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 (94%)	Nb positives[c]	2/2	2/2	2/2	2/2	3/3	3/4	0/6
	Mean C_T 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] At these concentrations, standard deviations (SDs) were calculated on the basis of the positive values only.
[d] Nb positives, number of positive reactions/total number of reactions performed.
[e] C_T, cycle threshold.

taining 10 Tgg/PCR tube, ranged from 0.3% to 0.42%. The interassay CV, calculated by testing a sample containing 10 Tgg/PCR tube in five separate assays over a 2-week period, ranged from 0.76% to 1.47%. Thus, reproducibility and repeatability were high for both intra- and interassay experiments.

Multicentric assessment of the *T. gondii* 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 (16) but differing with respect to the DNA extraction method, primers, PCR technology, and amplicon detection technique (Table 1). Data for all participating laboratories are shown in Table 3. All methods reliably detected the parasite at concentrations 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 positive reactions, respectively, whereas the five other assays yielded negative results.

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, 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 various PCR-inhibiting properties that depend on the biological fluid examined, the standard should use the same matrix as the one examined in diagnosis (AF, blood, etc.); also, the amount of target present prior to DNA extraction and PCR should be calculated as accurately 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 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 the development of that from blood samples (9, 28). A minor drawback is that AF drawn from hydramnios is often naturally diluted and 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 assumed that this helps improve reproduction of 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, in contrast to the previously described methods that use *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 (E. Varlet-Marie, Y. Sterkers, and P. Bastien, 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 results nearly identical to those obtained with 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, noninfectiousness, and flexibility in use. Freeze-dried samples have proved stable for at least 3 years under conditions of storage at +4°C (not shown). As assessed from PCR testing of the panels, freeze-drying resulted in only a slight reduction of C_T values ranging between 0.2 and 1, regardless of the concentrations of tachyzoites tested (from 1,0000 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 use of this standardized material is fundamentally different from the use of a simple DNA extract, which is often distributed for EQAs (29). 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 to be more "homogeneous" and 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 the participants: indeed, compared with DNA extractions performed independently in each center, using this DNA extract resulted in a reduction in sensitivity of all PCR assays except the one which used in the Roche extraction system (10) (Molecular Biology pole of the NRC-T, unpublished data). This outcome could be explained only by a lack of conjoint optimization between the DNA extraction method of the sender and the PCR methods used by the participants. 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 in 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 (30), and the observed quality criteria were highly satisfactory. The multicentric assessment allowed definite validation of its quality and utility. The data obtained by the eight proficient laboratories cannot be statistically compared since the calculation of C_T values depends on the assays used, in particular, on the PCR device, the software, and the detection method. This being said, at the highest concentrations, the *T. gondii* standard test results were found to be similar among centers: at 10 Tgg/ml, intracenter ΔC_T values were below 1.0 in all centers, and the slopes ranged from −1.554 for laboratory C to −1.310 for laboratory B. In contrast, at reaction mixture concentrations below 10 Tgg/PCR tube, the participants showed more divergent results: at 1 Tgg/PCR tube, intracenter ΔC_T values 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 (22–25).

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 last 23 positive clinical samples recorded in our routine activity exhibited a mean C_T value of 30.5 ± 3.7 (range, 25 to 36), 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 (10).

Multipurpose standards. There are several advantages in using such a standard. (i) It allows a self-assessment of the technical performances (in particular, of the analytical sensitivity) of the *Toxoplasma* PCR assay in each center and hence PCR assay validation. (ii) It allows each center to follow up the performances of its molecular diagnosis, 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 (5, 6) 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 small amounts of parasites, including clinical samples. In our hands, highly similar results were obtained in several centers by 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 to be an essential step toward 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 was helpful for 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 1×10^5 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 multicentric studies correlating parasitic loads in AF and fetal prognoses to be performed. One limitation of our standard is that it can be used only for the diagnosis of congenital toxoplasmosis. The future development of this standard will need to address the implementation of quantification in whole blood, which represents a new challenge and would undoubtedly be useful for the follow-up of immunosuppressed patients.

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