Molecular Diagnosis of Toxoplasmosis : Multicenter Evaluation of the Toxoplasma RealCycler Universal PCR Assay on 168 Characterized Human Samples

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Abstract

Real-time PCR (qPCR) plays a crucial role in the diagnosis of toxoplasmosis. In this multicenter study, the *Toxoplasma* RealCycler® Universal assay (Progenie Molecular, Valencia, Spain) was assessed for the diagnosis of toxoplasmosis by eight reference laboratories.

DNAs from diverse clinical samples were included: 141 characterized samples from patients with different clinical forms of proven toxoplasmosis and 27 from patients without toxoplasmosis were tested in duplicate with the commercial assay. Final diagnosis was affirmed by each center by up. Calibrated *T. gondii* standards and 11 external quality control samples (EQCs) were also included. Discrepant results observed after the first run of commercial PCR were controlled by both reference and commercial PCR assays.

Using the commercial assay, the detection threshold varied from 0.01 to 1 Tg/mL depending on the center. The relationship between Cp and DNA concentration was linear over 4 log units (r^2>0.99) and PCR efficiencies were satisfactory (89% to 104%). The results of the 11 EQCs were concordant after one retesting but those for 3 clinical samples remained discrepant. Sensitivity and specificity were calculated at 97.8 % [97.8-100] and 100 % [87.2-100], respectively.

Provided that PCRs are performed at least in duplicate to detect low parasitic loads, *Toxoplasma* RealCycler® Universal PCR showed suitable performances to diagnose the different forms of toxoplasmosis.

Introduction

Toxoplasmosis is a worldwide endemic protozoan disease; 30% of the global human population is chronically infected with large disparities across the world.[1] *Toxoplasma gondii* infection is usually asymptomatic or mildly symptomatic except in immunocompromised patients, and congenital patients.[2] Congenital toxoplasmosis (CT) occurs in infants following infection

during pregnancy and may result in neurologic and neurocognitive deficits, chorioretinitis, fetal abortion and death. Several forms of toxoplasmosis can be observed due to primary infection or to reactivation of chronic toxoplasmosis in the context of immunodepression. Disseminated and pulmonary toxoplasmosis is a life-threatening opportunistic infection that mainly affects hematopoietic stem cell transplant (HSCT) and solid organ transplant (SOT) recipients.[3,4] Cerebral toxoplasmosis is a common cause of central nervous system infection in patients with HIV/AIDS who do not take appropriate prophylaxis and in patients with other immune deficiencies. Lastly, ocular toxoplasmosis occurs in immunocompromised and immunocompetent patients; toxoplasmic retinochoroiditis is the most common form of posterior uveitis in many countries, particularly in South America.[5]

Molecular detection of *T. gondii* plays a crucial role in the diagnosis of these different forms of toxoplasmosis and PCR has significantly improved the management of this parasitic disease. Initially, *Toxoplasma* PCR methods (conventional PCR and real-time PCR (qPCR)) used in medical diagnostic laboratories were laboratory-developed or 'in-house' PCR assays.[6,7] Although they represent sensitive methods[6,8,9] they suffer from lack of standardization.[7] Since several years the growing number of commercially available methods has been facilitating the much-needed standardization, but not all techniques are equal, and it is essential to correctly evaluate them before their implementation in routine diagnosis.[8,10-15] To this aim, available commercialized PCR assays are evaluated by the "Molecular biology" group of the French National Reference Centre for Toxoplasmosis (http://cnrtoxoplasmose.chu-reims.fr, last access December 28, 2021).[8,10,11,15]

Here, analytical performances, clinical sensitivity and specificity of the *Toxoplasma* RealCycler® Universal assay (Progenie Molecular, Valencia, Spain) were assessed for the diagnosis of toxoplasmosis using 168 characterized human samples in 8 referent centers. In this study, the analytical detection threshold of this commercial qPCR assay was first assessed using calibrated lyophilized *T. gondii* samples; then, its performances in medical diagnosis were compared with qPCR methods used in routine molecular diagnosis in distinct clinical settings: CT (amniotic fluid, placenta and umbilical cord blood), toxoplasmic chorioretinitis (aqueous and vitreous humor), and toxoplasmic infection in immunocompromised patients (blood sample, cerebrospinal fluid, bronchoalveolar lavage fluid and miscellaneous samples). To our knowledge, this is the first evaluation of this kit in a clinical context.

Materials and methods

Proficiency of the participating laboratories

The eight participating laboratories are proficient in detecting *T. gondii* in clinical specimens; they are members of the "Molecular biology" group of the French National Reference Centre for Toxoplasmosis (http://cnrtoxoplasmose.chu-reims.fr, last access December 28, 2021) and participate to the external quality assessment (EQA) for *Toxoplasma*- PCR.[7,16] Moreover, seven of them hold an agreement from the Ministry of Health (Regional Health Agency) to perform prenatal diagnosis for congenital toxoplasmosis.

Samples

Toxoplasma Lyophilized standards and External Quality Control samples

Four participating centers (centers C, E, G, H) used a calibrated *Toxoplasma* (type II) suspension produced by the Molecular Biology Study Group of the NRCT (University Hospital of Montpellier).[9] DNA was extracted from the *Toxoplasma* suspension in each center using their routine method for molecular diagnosis. The four centers compared the sensitivities of

detection of serial dilutions (from 10,000 to 0.1 or 0.01 tachyzoites (Tg)/mL) after amplification using their own in-house qPCR method (here after termed 'reference' PCR method) and the *Toxoplasma* RealCycler® Universal kit (Progenie Molecular, Valencia, Spain), following the manufacturer's instructions. All dilution points were amplified at least in triplicate. Additionally, three of these centers also evaluated at least three samples of External Quality Control samples (EQCs) from QCMD® (Glasgow, UK).[17]

Clinical samples.

DNAs extracted from clinical samples, were used to evaluate the commercial kit, as previously described.[18] Overall, 168 clinical samples were included in the study, collected within the frame of routine practice in the eight participating centers for the diagnosis of congenital, ocular, cerebral, disseminated and acute toxoplasmosis. They comprised 58 amniotic fluid (AF), 15 placenta, 14 umbilical cord blood, 1 foetal biopsy, 20 cerebro-spinal fluid (CSF), 6 brain biopsy, 2 cerebral abscess, 21 aqueous humor, 1 vitreous humor, 15 blood, 9 bronchoalveolar lavage fluid (BALF), 1 bone marrow, 1 serum, 1 plasma and 1 lymph node biopsy samples. In total, 141 positive and 27 negative samples were included (Table 1). To be included in the study, all samples had to be characterized and associated to a clinical diagnosis. Congenital toxoplasmosis (CT) cases were clinically classified as defined by the European Research Network on Congenital Toxoplasmosis group.[19] Cases of toxoplasmosis in immunocompromised patients were assessed according to the European Group for Blood and Marrow Transplantation Infectious Diseases Working Party classification.[3] The diagnosis of toxoplasmosis disease was excluded for the 27 negative PCR results.

Molecular techniques.

DNA extraction

Pre-analytic steps and DNA extraction procedures were adapted to the specimens and differed among laboratories (Supplemental Table 1). Commercial manual extraction kits, used according to the manufacturer's specifications, were QIAamp DNA Micro, Mini, FPPE DNA tissue Kits (Qiagen, Courtaboeuf, France) (centers A, B, D, E). Four automated DNA extraction methods were used : the MagNA Pure 96 DNA with Viral NA Small Volume Kit (Center C) or with High Pure PCR template (Roche Molecular Biochemicals, Meylan, France) (Center G), the MagNA Pure Compact with (Center F), and the Nuclisens easyMag system using the NucliSens Magnetic kit (bioMérieux, Craponne, France) which was optimized by a pretreatment step using proteinase K (Center B).[20,21] Center H used manual methods, the Tween-Nonidet-NaOH (TNN; 0.5% Tween 20, 0.5% Nonidet P40, 10 mM NaOH) lysis buffer method for paucicellular fluids[22] and a treatment by proteinase K (Euromedex, Souffelweyersheim, France) in Tris-SDS (2 to 3 µL/100µL of final solution) (56˚C, 12 hours), followed by boiling at 100˚C for 10 min and protein precipitation solution kit (Protein precipitation A7951, Promega, Charbonnieres, France) for tissues or cellular samples (Table 2).[15,23]

Reference PCR methods

The laboratory-developed qPCR used by six participants targeted the non-coding repetitive DNA sequence *rep*529 (GenBank accession numbers AF146527 and AF146550) and have been previously evaluated and published (Centers B, C, D, E, F, G,H).[6, 9, 10,11,15, 24,25,26] One center used an 'in-house' real-time PCR targeting the B1 region (center A).[27] Center F used a commercialized *Toxoplasma*-PCR kit : TIB MolBiol® assay (LightMix® Kit *Toxoplasma gondii* (EC) order no. 40-0217-32).[12,15] Two centers included samples analyzed with different qPCR methods because they switched from in-house PCR assays targeting *rep*529 and the B1 gene to

Samples	Number of samples	Toxoplasmosis	Absence of toxoplasmosis
Congenital toxoplasmosis			
Amniotic fluid (AF)	58	48	10
Placenta	15	14	1
Umbilical cord blood	14	11	3
Fœtal biopsy	1	1	0
Cerebral toxoplasmosis			
Cerebro-spinal fluid (CSF)	20 (19)*	19 (18)	1
Brain biopsy	6	6	0
Cerebral abcess	2	2	0
Ocular toxoplasmosis			
Aqueous humor (AH)	21(19)	18 (16)	3
Vitreous humor	1	1	0
Disseminated/pulmonary toxoplasmosis			
Blood/buffy-coat	15	12	3
Broncho alveolar lavage fluid (BALF)	9 (8)	5 (4)	4
Bone marrow aspiration	2	1	1
Serum	2(1)	1 (0)	1
Plasma	1	1	0
Acute toxoplasmosis			
Lymph node biopsy	1	1	0
Total of human samples	168 (163)	141 (136) [84% (83.5%)]	27 [16% (16.5%)]

Table 1 : Description of human clinical samples and type of toxoplasmosis diseases. *Number in brackets : number of samples included in statistical analysis according the flow chart (Figure 1).

the commercialized BioEvolution® test (BioEvolution®, Bussy-Saint-Martin, France) (Centers A, B).[10] PCR apparatuses used in each center are described in table 2. Depending on the center and the type of sample, DNA extracts were analyzed in one to four wells. Among the 168 included samples, 16 (9.5%) were analyzed in simplicate at the time of routine clinical diagnosis (centers A and D). After qPCR, DNA samples were then frozen and stored at −20 °C or at −80°C as recommended elsewhere.[18] DNA samples should not have been thawed prior to inclusion in this study.

***Toxoplasma* RealCycler® Universal method**

The *Toxoplasma* RealCycler® Universal assay (Progenie Molecular, Valencia, Spain) was performed according to the manufacturer's instructions, using 6 µL of DNA extract and 17 µL of mix. In the manual of this kit, the use of ABI 7500 (Thermo Fisher Scientific, Waltham, MA, USA) and CFX96™ (Bio-Rad, Marne-La-Coquette, France) PCR devices were proposed. In this study, *Toxoplasma* RealCycler® Universal amplification was also performed using StepOnePlus and StepOne (Thermo Fisher Scientific, Waltham, MA, USA), LighgtCycler 480 (Roche Diagnostic, Meylan, France) and MX3005 (Stratagene, La Jolla, CA, USA) as suggested by the manufacturer.

Toxoplasma PCR in routine diagnosis							Commercial PCR
Center	DNA extraction protocol	Target/ PCR assay	PCR Apparatus	Samples provided by the center (168)	EQC samples (11)	Calibrated curves (4)	PCR Apparatus
A	QIAamp DNA Mini Kit QIAamp FPPE DNA tissue kit QIAsymphony (Qiagen®)	B1/In-house PCR rep529/Bio Evolution®	LC 480 (Roche)	8			MX3005 (Stratagene)
B	QIAamp DNA Micro Kit (Qiagen®) Nuclisens easyMag (bioMérieux®)	rep529/In-house PCR rep529/Bio Evolution®	CFX 96 (BioRad) ABI 7000 (ThermoFischer)	17			MX3005 (Stratagene)
C	MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche®)	rep529/In-house PCR	LightCycler 2.0 (Roche)	23	4	yes	ABI 7500 (ThermoFicher)
D	QIAamp DNA Mini Kit (Qiagen®)	rep529/In-house PCR	StepOne Plus (ThermoFischer)	32			StepOne plus (ThermoFischer)
E	QIAamp DNA Mini Kit (Qiagen®)	rep529/In-house PCR	LightCycler 480 (Roche)	28	3	yes	StepOne (ThermoFischer)
F	MagNA Pure 96, MagNA Pure Compact (Roche®)	rep529/TIbMolBiol®	LightCycler 480 (Roche)	19			CFX 96 (BioRad)
G	MagNA Pure 96 DNA High Pure PCR template preparation kit (Roche®)	rep529/In-house PCR	LC2.0 or LC480 (Roche)	22	4	yes	CFX 96 (BioRad)
H	TNN* Proteinase-K, boiled and protein precipitation† (Promega®)	rep529/In-house PCR	LightCycler 480 (Roche)	19		yes	LC480 (Roche)

Table 2 : Details of DNA extraction, PCR methods used in each center in routine diagnosis of toxoplasmosis, and PCR apparatuses used with the *Toxoplasma* RealCycler® Universal PCR assay. * TNN : Tween-Nonidet-NaOH (0.5% Tween 20, 0.5% Nonidet P40, 10 mM NaOH) lysis buffer method[22]

† DNA extraction technique described in Sterkers Y *et al,* 2012[23]

Each clinical samples as well as the QCMD® EQC samples were analyzed in two wells.

If the result of *Toxoplasma* RealCycler® Universal PCR was discordant with that of the previous routine diagnosis, the DNA sample was re-tested with the reference PCR. The sample was excluded from the statistical analysis if this PCR reference result did not confirm the initial result or if the sample DNA was not in sufficient quantity to be re-tested. If the initial result obtained with the reference qPCR at the time of diagnosis was confirmed by this new run of reference qPCR, the *Toxoplasma* RealCycler® Universal PCR was performed again with this sample. This flow chart is reported in figure 1.

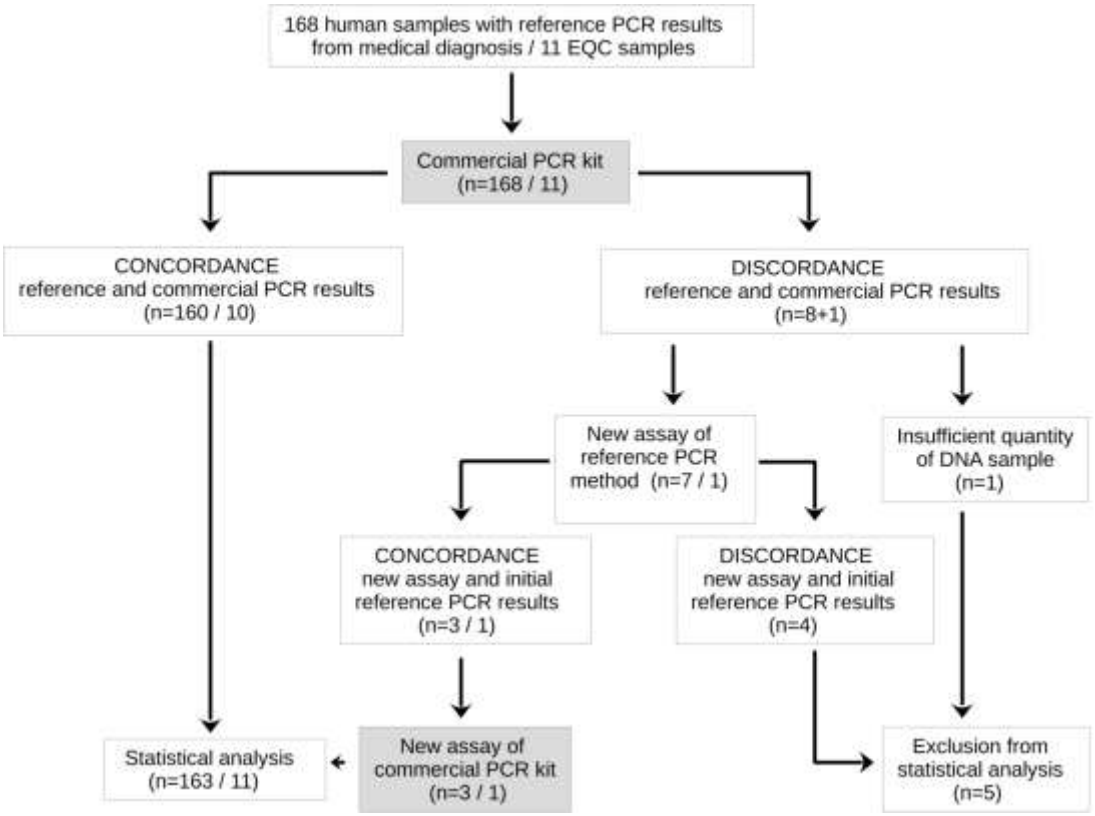

Figure 1 : Flow chart of the 168 initially included human samples and 11 External Quality Control (EQC) samples. * after DNA freezing and storage at −20 °C. In grey: positioning of the commercial PCR kit in the flow chart.

Ethical approval and informed consent

Clinical samples were obtained from the participating centers in compliance with the Quality Assurance system and legal policies. This work was carried out in accordance with the French guidelines and regulations; it does not include potentially identifying patient/participant information. The study corresponds to a non-interventional retrospective study and is in agreement with the French Health Public Law (CSP Art L1121-1.1). Written consent was obtained before any prenatal AF sampling.

Data and statistical analysis

The results were expressed as a "PCR performance score", corresponding to the number of positive amplifications over the total number of PCR reactions performed [6,10,11,28] and as a mean of the crossing point values ± standard deviation (Cp ± SD). Results were analyzed using Fisher's exact test; a p value of 0.05 or less was considered to be significant. For serial dilutions of *Toxoplasma* standard, the performances of the PCR were also evaluated by the linear measuring range

(regression analysis, R^2), and the efficiency (%) [$E=(10^{(-1/slope)}-1) \times 100$].

In each center, the agreement between the commercial PCR assay and the reference PCR method (methods A and B) was analyzed using the Bland and Altman plot,[29] this consists in a graph presenting on the abscissa the average of the values obtained by both methods, *i.e.* (A + B)/2 and on the ordinate the difference between the values obtained by both methods A and B, *i.e.* (A - B). The upper and lower limits of agreement are estimated by the bias ± 1.96 SD and represented on the plot. The Bland-Altman plots were produced using GraphPad Prism 8.0.2.

Results

Evaluation of the detection threshold using calibrated lyophilized T. gondii samples

Four centers (C, E, G, H) compared the *Toxoplasma* RealCycler® Universal method and their reference diagnostic PCR method by testing serial dilutions of the common standard of the NRC-T network ("TG25040, 10^4 parasites per mL, type II strain, Montpellier 2014").[9] At least three reactions were used for testing each of the standard's concentrations (10,000, 1000, 100, 10, 1, 0.1 Tg/mL and 0.01 Tg/mL for center H) (Table 3).

The threshold of *Toxoplasma* DNA detection - defined as the lowest concentration where >50% of the reactions remain positive[6] varied depending on the center from 0.01 to 1 Tg/mL for the *Toxoplasma* RealCycler® Universal method and from 0.1 to 10 Tg/mL for the reference PCR assays (Table 3). For two centers (E, G), this threshold was 1 Tg/mL and was identical between the two PCR methods. For centers C and H, the threshold using the *Toxoplasma* RealCycler® Universal method was 1 Tg/mL and 0.01 Tg/mL, respectively, which was lower by one dilution than the thresholds obtained by the reference PCR methods used by these same centers (10 Tg/mL and 0.1 Tg/mL respectively). The relationship between the Cp and the DNA concentration was linear over 4 log units (r^2>0.99) for the commercial PCR kit; PCR efficiencies were satisfactory (Eff=89% to 104%) and were slightly better than those of the reference PCR methods for two centers (centers B and G) (Table 3).

Furthermore, according to the center, the performance scores were 73% to 81.5% and 70% to 92.6% for reference PCRs and commercial PCR, respectively (Table 3).

Clinical performances of the Toxoplasma RealCycler® Universal PCR method from characterized human samples analyses

One hundred and sixty-eight characterized human samples were included; 160 of those were analyzed in duplicate by the *Toxoplasma* RealCycler® Universal PCR method and 8 were so in simplicate. The commercial qPCR assay results were concordant with those of the reference qPCR methods for 160 out of the 168 initial samples, whereas the results obtained for 8 DNA samples from patients with toxoplasmosis were discrepant. Among these, 7 were re-analyzed with the reference qPCR methods in each center involved and one sample was in insufficient amount to be re-tested and was excluded (Figure 1). Among these 7 initially discrepant DNAs, 4 were excluded from statistical analysis, since the qPCR results were negative after re-testing with the reference qPCR, suggesting DNA degradation. For the 3 remaining samples, the control results using the reference qPCR assay were concordant with the initial diagnosis even though PCR reactions were inconstantly positive with delayed amplification (Cp>35) (Table 4). These 3 samples -amniotic fluid, brain biopsy and plasma- from 3 different centers were still negative after re-testing in duplicate with the commercial qPCR method (Table 4). No false positive results were observed using the commercial method (0/27 samples).

Center		Concentration (Tg/mL)	10^4	10^3	10^2	10	1	0.1	0.01	PCR performance scores (%)
C	Reference PCR Eff = 78% R^2 = 0.989	N* Cp mean ±sd	3/3 21.84±0.15	3/3 24.91±0.13	3/3 28.90±0.6	**3/3** 33.85±1.51	1/3 34.30	0/3 /	ND	13/18 (72.2%)
	Commercial PCR Eff = 89% R^2 = 0.999	N Cp mean ±sd	3/3 21.24±0.29	3/3 24.80±0.03	3/3 28.63±0.29	3/3 31.98±1.25	**2/3** 38.03±0.29	0/3 /	ND	14/18 (77.7%)
E	Reference PCR Eff = 98% R^2 =0.998	N Cp mean ±sd	3/3 20.59 ± 0.14	3/3 24.02±0.21	3/3 27.45±0.51	3/3 30.86±1.18	**2/4** 36.19±1.60	1/4 32.18	ND	15/20 (75%)
	Commercial PCR Eff = 93% R^2 = 0.999	N Cp mean ±sd	3/3 23.51±0.09	3/3 27.24±0.21	3/3 30.32±0.36	3/3 33.75±1.12	**2/4** 37.46±1.01	0/4 /	ND	14/20 (70%)
G	Reference PCR Eff = 95% R^2 = 0.999	N Cp mean ±sd	3/3† 24.76±0.04	3/3 27.05±0.02	3/3 30.58±0.15	3/3 33.95±0.71	**2/3** 38.05±2.76	0/3 /	ND	14/18 (77.7%)
	Commercial PCR Eff = 91% R^2 = 0.998	N Cp mean ±sd	3/3 b 20.64±0.07	3/3 22.94±0.16	3/3 26.22±0.17	3/3 30.04±0.44	**2/3** 35.26±1.01	0/3 /	ND	14/18 (77.7%)
H	Reference PCR Eff = 120% R^2 = 0.991	N Cp mean ±sd	3/3 18.66±0.02	3/3 22±0.12	3/3 25.51±0.10	3/3 28.22±0.11	3/3 30.77±0.90	**6/6** 33.59±4.11	1/6 38.82	22/27 (81.5%)
	Commercial PCR Eff = 104% R^2 = 0.997	N Cp mean ±sd	3/3 21.34±0.14	3/3 24.83±0.02	3/3 27.79±0.08	3/3 30.10±0.08	3/3 32.36±0.18	6/6 34.76±1.16	**4/6** 37.67±1.57	25/27 (92.6%)

Table 3 : ***comparison of the reference PCR method and commercial PCR kit results using serial dilutions of the*** **T. gondii** ***standard from the National Reference Center for Toxoplasmosis.*** * Nb positives, number of positive reactions/total number of reactions performed ; † 5.10^3 Tg/mL

Cp: crossing point ; sd : standard deviation. In grey : Cp used to calculate the efficiency (%) [$E=(10^{-1/slope}-1)x100$]

Centre	Discordant (n)	Sample	Disease	Diagnosis by reference PCR method		Commercial PCR kit		New analysis by reference PCR method		New analysis by Commercial PCR kit	
				Results	Ct	Results	Ct	Results	Ct	Results	Ct
C	1	AF	Congenital toxoplasmosis	Positive	41.7/40.1/-	Negative	-/-	Positive	42.4/-	Negative	-/-
D	1	Brain biopsy	Cerebral toxoplasmosis	Positive	34/33.1	Negative	-/-	Positive	-/35.4	Negative	-/-
G	1	Plasma	Disseminated toxoplasmosis	Positive	39.5	Negative	-/-	Positive	40/-	Negative	-/-
	1	EQC	/	Positive	39.01/34.91	Negative	-/-	Positive	38.37/37.9	Positive	-/36.6

Table 4 : Description of samples showing discrepant results between the *Toxoplasma* RealCycler® Universal assay and the reference qPCR method.

Ultimately, the statistical analysis of this study was performed on 163 clinical samples; 136 from patients with toxoplasmosis and 27 from patients without toxoplasmosis (Table 1). The sensitivity and the specificity of *Toxoplasma* RealCycler® Universal PCR method were 97.8 % [97.8-100] (133/136) and 100 % [87.2-100] (27/27), respectively.

For each center, Bland-Altman analysis was used to study the agreement between commercial PCR and reference qPCR assays, considering that Cp is the quantitative measured parameter. It included from 7 to 30 concordant positive samples according to the center. The agreement between the reference method of each center and the commercial method is good, with no sample (centers A, B, C and E), or only 1 (centers D, F and H) or 2 (center G) samples outside the confidence intervals (Figure 2).

Furthermore, 11 ECQ samples were analyzed in 3 centers (centers C, E, G) and in 10/11 cases, the results of the Toxoplasma RealCycler® Universal method were concordant with the results obtained in the first run (reference qPCR and QCMD® results). Following the protocol, this discrepant sample was re-tested in duplicate with both qPCR methods (Figure 1): the two PCR reactions were positive with the reference PCR method while only 1 out of 2 was positive with the commercial qPCR assay (Table 4).

Discussion

The molecular diagnosis of toxoplasmosis still relies on a targeted diagnostic approach performed in proficient laboratories, since no syndromic molecular panels include the detection of *Toxoplasma* DNA. In the absence of a syndrome-based approach, it remains important to continue the optimization of the preanalytical and analytical steps and to evaluate the performances of the laboratory-developed methods and commercialized kits of qPCR assays targeting specifically *T. gondii*.[8,10-15,18,20,21,28,30] These are the objectives of the "Molecular biology" group of the French National Reference Centre for Toxoplasmosis (http://cnrtoxoplasmose.chu-reims.fr, last access December 28, 2021). Initially, the laboratory-developed methods (reference PCR in this study) of several participants had been compared to detect low amounts of *T. gondii* from simulated specimens,[6] and used to validate a common standard for *T. gondii*.[9] To date, 4 commercialized PCR kits have been evaluated by our group or others authors: AMS94/F; Clonit, (Clonit, Milan, Italy), Bio-Evolution® kit (Bio-Evolution, Bussy-Saint-Martin, France), ELITe MGB Real-Time PCR assay (Elitech, Puteaux, France) and LightMix® Kit *Toxoplasma gondii* assay (TIB MolBiol®, Berlin, Germany). [8, 10-15]

The *Toxoplasma* RealCycler® Universal assay was evaluated for the first time in this multicentric study. Four calibrated *T. gondii* standards, 11 ECQ and 168 clinical samples drawn for diagnosis of congenital, ocular, cerebral, disseminated and acute toxoplasmosis during routine practice of eight laboratories were used. For routine molecular diagnosis of toxoplasmosis, these centers used laboratory-developed PCR methods[6,7] or two other commercial kits of qPCR which have been previously evaluated.[10,12,13,15] All centers involved in the present study participate to the national external quality assessment on a regular basis with consistent results.[7,17]

The analytical detection threshold for the *Toxoplasma* RealCycler® Universal assay complied with the usual requirements for this molecular diagnosis: it varied from 0.01 to 1 Tg/mL depending on the center and was below to the target threshold proposed previously.[6] Costa *et al* reported that the parasitic loads are low during toxoplasmosis infection and sometimes less than 1 Tg/mL of amniotic fluid during CT.[31] Therefore, the "Molecular biology" group of the French National Reference Centre for Toxoplasmosis recommended that, for a best sensitivity of the molecular diagnosis of *T. gondii*, the detection threshold should tend toward 0.5 Tg per reaction tube, corresponding to 0.75 to 2.5 Tg/ml of AF.[6] With the same methodology, this threshold was 1 Tg/mL for the TIB MolBiol assay (TIB MolBiol®, Berlin, Germany) and varied from 1 Tg/mL to 10 Tg/mL depending

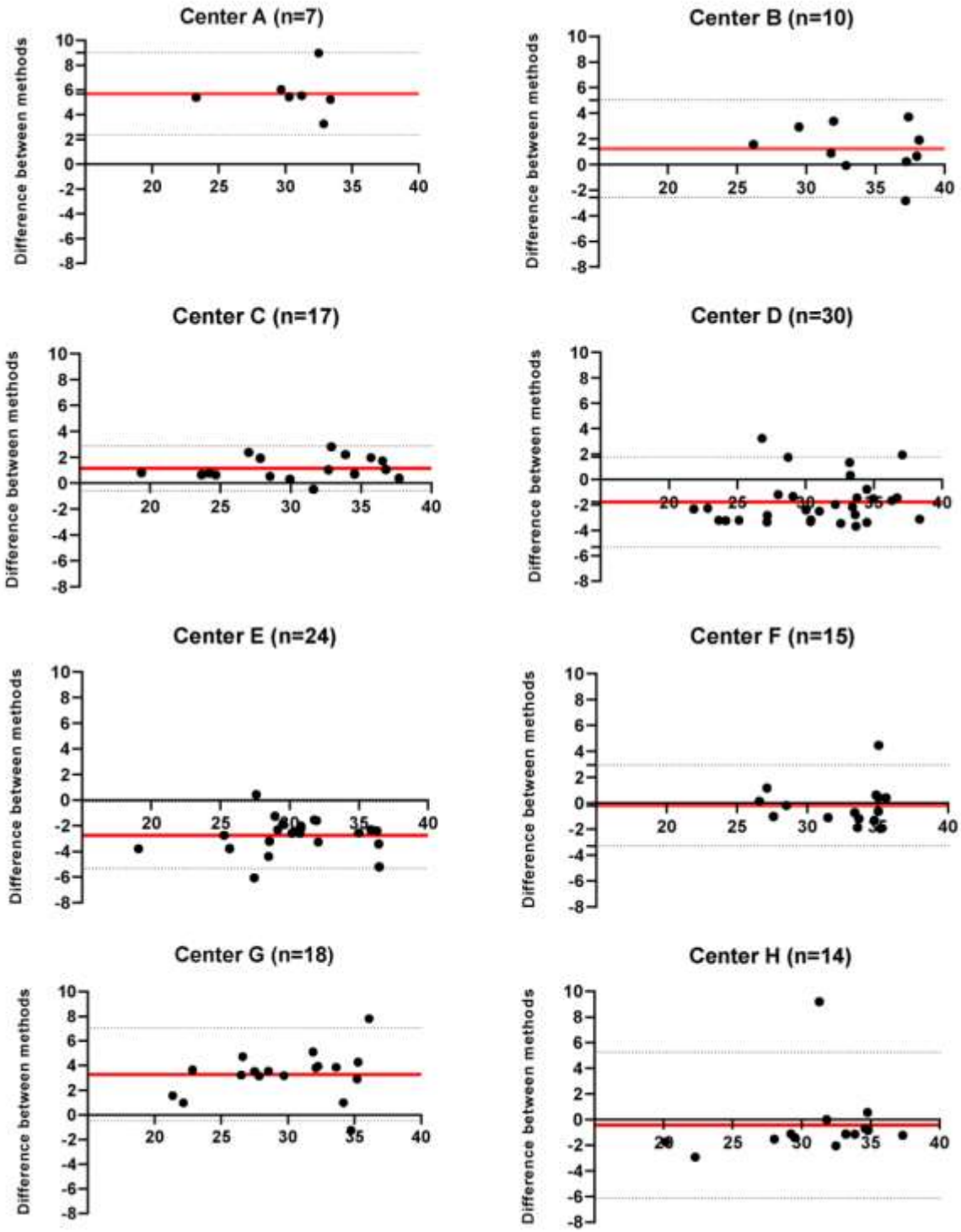

Figure 2 : Bland-Altman plots for each center. The red lines and the dotted black lines represent the means difference (bias) and ±1.96 standard deviations (95%limits of agreement), respectively.

on the center for the ELITe MGB kit (Elitech, Puteaux, France).[11,15]

Initially, 168 DNAs extracted from characterized human samples and 11 ECQs were included and tested with the commercial qPCR method. Nine discrepant results (for eight clinical and one ECQ samples) were observed between the commercial and reference PCR methods; and these samples were re-tested by each center according to the flow chart. Five of these eight samples were excluded from the global analysis. Among them, two samples (AH, BALF) were inconstantly positive using the reference PCR assay at the time of diagnosis, one (serum) sample tested in simplicate had shown a high Cp, and for the remaining sample (CSF), the Cp mean was 32.72.

We previously showed that freezing and storage at −20 °C provides adequate preservation of *Toxoplasma gondii* DNA for retrospective molecular analysis.[18] However, a weak DNA degradation starting from a low parasitic load might explain that these samples were not positive after storage. Furthermore, only DNA extracted from AF was studied in Delhaes *et al* and one cannot rule out that *T. gondii* DNA extracted from human 'cellular' samples may be less stable.[18] Among the four false negative samples re-tested with the commercial PCR assay, the ECQ sample was weakly (Cp>35) and inconstantly positive (1 positive /2 reactions) after this control; and three samples yielded persistently discrepant results. The parasitic loads in these three clinical samples (AF, brain biopsy, plasma) were low using reference PCR assays (Cp>35) which may in part explain the negativity of the commercial qPCR.

In total, the sensitivity and the specificity of *Toxoplasma* RealCycler® Universal PCR assay were 97.8% (133/136) and 100% (27/27), respectively; and the 11 ECQs were correctly detected only if the PCR was performed in quadriplicate. These clinical performances were better or close to those reported for the BioEvolution® kit (Se: 86% and Sp: 100%), ELITe MGB® kit (Se: 89% and Sp: 100%), TIB MolBiol® (Roche®) assay (Se: 98.1% and Sp: 98.5%) with a comparable methodology [10,11,15] Only in the multicentric retrospective evaluation of the BioEvolution® assay during immunocompromised patients follow up, Ait Ammar *et al* reported a higher sensitivity and specificity, at 98.8% and 100%, respectively.[13]

The good performances of this commercial kit are probably partly linked to the use of a number of 'good practices': detection in multiplicate in this study, amplification of the repetitive DNA target rep529, and the use of an internal control for the detection of PCR inhibitors. No PCR inhibitors were detected by the kit, however no DNA samples with PCR inhibitors detected with reference PCR methods were specifically included in this study. It should be noticed that the kit could be used with several PCR apparatuses. The manufacturer proposes some technical recommendations depending on the type of validated samples (amniotic fluid, culture, plasma and serum), DNA extraction or PCR devices, but specifies that this kit is potentially compatible with other options. The only drawbacks noted at the time of the study were the absence of uracil-N-glycosylase in the assay and the lack of CE-IVD marketing yet.

In conclusion, provided that PCR reactions are performed at least in duplicate to detect low parasitic loads, the *Toxoplasma* RealCycler® Universal PCR assay showed suitable performances for the molecular diagnosis of the different clinical forms of toxoplasmosis.

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