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Multicenter evaluation of the *Toxoplasma gondii* Real-TM (Sacace) kit performance for the molecular diagnosis of Toxoplasmosis

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Abstract

The molecular detection of *Toxoplasma gondii* DNA is a key tool for the diagnosis of disseminated and congenital toxoplasmosis. This multicentric study from the Molecular Biology Pole of the French National Reference Center for toxoplasmosis aimed to evaluate *Toxoplasma gondii* Real-TM PCR kit (Sacace). The study compared the analytical and clinical performances of this PCR assay with the reference PCRs used in proficient laboratories. PCR efficiencies varied from 90 to 112%; linearity zone extended over four log units ($R^2 > 0.99$) and limit of detection varied from 0.01 to ≤ 1 Tg/mL depending on the center. Determined on 173 cryopreserved DNA from a broad range of clinical specimens, clinical sensitivity was 100% (106/106; 95CI: 96.5-100%) and specificity 100% (67/67; 95CI: 94.6-100%). The study revealed two potential limitations of the Sacace PCR assay: The first was the inconsistency of the internal control when added to the PCR mixture. This point was not found under routine conditions when the IC was added during the extraction step. The second is a lack of practicality, as the mixture is distributed over several vials, requiring numerous pipetting operations. Overall, this study provides useful information for the molecular diagnosis of toxoplasmosis; the analytical and clinical performance of the Sacace PCR kit were satisfactory, the kit having a sensitivity and specificity similar to those of expert center methods and being able to detect low parasite loads, at levels where multiplicative analysis gives inconsistently positive results. Finally, the study recommends multiplicate analysis especially for amniotic fluids, aqueous humor and other single specimens.

Introduction

Toxoplasmosis is a parasitic infection due to a protozoan, *Toxoplasma gondii*, mainly acquired through the consumption of cysts in undercooked meat or oocysts in contaminated food or water¹. Infected people with *T. gondii* are most often asymptomatic, but in pregnant women, toxoplasmosis may be severe as tachyzoites are able to cross placenta, to infect the fetus and cause congenital toxoplasmosis¹. Infected individuals carry *T. gondii* cysts in their muscular and cerebral tissues. If immunosuppression occurs, reactivation of bradyzoites induces a life-threatening severe toxoplasmosis such as cerebral or disseminated toxoplasmosis¹. Detection of *T. gondii* forms in amniotic fluid (AF), cerebrospinal fluid (CSF), blood by microscopic examination, cell culture and/or mice inoculation is poorly sensitive and molecular diagnosis has revolutionized the management of congenital and opportunistic toxoplasmosis^{2,3}. Today, a number of laboratory-developed/in-house PCRs and commercial real-time PCR assays have been described^{4,5}. *Toxoplasma gondii* Real-TM (Sacace, Como, Italy) is a real-time PCR kit, targeting the Rep529 non-coding repetitive DNA sequence, for *T. gondii* DNA detection in clinical specimens including white blood cells of peripheral and umbilical cord blood, biopsy specimen, CSF, and AF. It should be stressed here that parasitic load is often extremely low in clinical specimens. Thus in AF, median was found <10 T/mL⁶. Knowledge of performance and especially sensitivity of the different existing method is important. A wide range of laboratory-developed and commercialized methods are available for diagnosing toxoplasmosis⁴. The aim of the French National Reference Center for Toxoplasmosis (<http://cnrttoxoplasmose.chu-reims.fr/?lang=en>, last accessed January 8th, 2024) is to evaluate PCR tests for the molecular diagnosis of toxoplasmosis, in order to standardize performance at national level. To achieve this, it organizes external quality controls, practice surveys and method

comparisons^{4,7-13}. Here, a multicenter study involving eight laboratories (Center 1-8) was performed to evaluate analytical and clinical performances of *T. gondii* real-TM (Sacace) for the diagnosis of congenital and opportunistic toxoplasmosis. We also report on the experience of a center (Center 9) that has been using this kit routinely since 2019.

Materials and Methods

Study design.

This multicentric study involved eight Parasitology-Mycology laboratories from French University hospitals with an expertise in the diagnosis of toxoplasmosis. All participating centers are accredited according to the ISO15189 norm. The eight centers performing prenatal diagnosis of congenital toxoplasmosis are accredited by the French Ministry of Health (Regional Health Agency). All centers participate in the external quality assessment (EQA) for *T. gondii*-PCR organized by the molecular biology group of the French *Toxoplasma gondii* National Reference Center⁴. In a first phase, four expert centers of this group (Centers 1 to 4) evaluated (i) the analytical sensitivity and PCR efficiency of the *T. gondii* Sacace PCR assay using a serial dilution from a calibrated *T. gondii* lyophilized standard and (ii) the technical agreement between their reference PCR and the *T. gondii* Sacace PCR using external quality control (EQC) assessments. In a second phase, Centers 1 to 8 included clinical specimens from hospital collections to evaluate clinical performance of the *T. gondii* Sacace PCR assay. In a third phase we report the experience of one center (Center 9) that uses this kit in routine practice. As the other centers, this center has an expertise in the diagnosis of toxoplasmosis, has an agreement from the Ministry of Health (Regional Health Agency) to perform prenatal diagnosis for congenital toxoplasmosis is accredited according to the ISO15189 norm. This center responded with complete accuracy to all EQA sessions in which it has taken part.

Spiked-samples, external quality control samples and clinical specimens.

Lyophilized standards consisting in 2 mL of a 10,000 *T. gondii* genome/mL suspension¹² were extracted by centers 1 to 4. Extracted DNA (see Table 1 for extraction methods) was then 10-fold diluted to obtain a range of concentrations from 10,000 to 0.01 *T. gondii* genome/mL. The highest concentration at 10,000 *T. gondii* genome/mL was assessed in duplicate, the three following concentrations at 1,000, 100 and 10 *T. gondii* genome/mL were assessed in triplicate and the three lowest concentrations, until 0.01 *T. gondii* genome/mL were analyzed in six wells each (See Supplementary Data). Five EQC samples (TGDNA21-C1) provided by QCMD (Quality Control for Molecular Diagnosis, Scotland, United Kingdom) were assessed in this study and analyzed in duplicate by centers 1 to 4. One was negative, one was a positive amniotic fluid with a concentration estimated at 32 ± 13 *T. gondii* genome/mL, the three others were replicates of a positive amniotic fluid with a concentration estimated at 4 ± 3 *T. gondii* genome/mL. To check the absence of competitive effect induced by the internal control (IC) included in the *T. gondii* Sacace PCR assay, these samples were extracted as recommended by the manufacturer with and without the IC added before the extraction step. For the clinical specimens, since this retrospective study was based on cryopreserved previously extracted DNA and in absence of manufacturer recommendations, we decided to add 0.1 μ L of IC per reaction in the *T. gondii* Sacace PCR mix, after preliminary tests (not shown). In addition, eight centers retrospectively included a total of 173 clinical specimens that were preserved at -20°C as extracted DNA. In a previous work we have demonstrated that this storage provides adequate preservation of *T. gondii* DNA for retrospective molecular analysis¹⁴. Before inclusion in the study, patients and specimens were carefully categorized according to European Group for Blood and Marrow Transplantation Infectious Diseases Working Party classification¹⁵ and to European Research Network on Congenital

Toxoplasmosis group¹⁶. All specimens were checked to have enough volume for the whole study.

Reference PCR methods

Laboratories processed specimens according to their own routine procedures for DNA extraction and PCR methods (Table 1 and Supplementary Data). Briefly, every center used a PCR method targeting Rep529, among which four centers used laboratory-developed PCR, and four used the Bio-Evolution® PCR kit.

Toxoplasma gondii Real-TM PCR kit (Sacace)

The *Toxoplasma gondii* Real-TM PCR kit (Ref: P1-50FRT, Sacace Biotechnologies, Como, Italy) was performed according to the manufacturer's instructions. Of note, these instructions have some peculiarities: (i) the master mix consists in mixing three reagents: PCR-Mix-1-FRT, PCR-mix-2-FRT and the Polymerase TaqF. The kit also provides positive control (Pos Control DNA *T. gondii* /STI (C+)), negative control (C-) and Internal Control STI-87 (IC) vials. Each PCR reaction consists in 25 μ L of master mix and 10 μ L of eluted DNA; (ii) the amplification program includes a first step of five repeats without fluorescence detection, followed by 40 repeats with fluorescence detection. Clinical specimens were assessed in duplicate using the *T. gondii* Sacace PCR assay having 0.1 μ L of IC per reaction. In case of discrepant results between duplicates, the specimen was regarded as positive, and the clinical file was investigated.

Data and statistical analysis

In the first phase of the study, analytical performances were evaluated by four centers and relied on the results obtained in the serial dilution assay, as well as EQC assessment. To calculate the PCR efficiencies, we used the Cq values obtained in serial dilutions assays and plotted them on a logarithmic scale along with their corresponding concentrations. We generated a linear regression curve and calculated the slope of the trend line. PCR efficiency is calculated using the equation:

Table 1: Details of specimens, PCR methods and signal analysis used in each center in routine diagnosis of Toxoplasmosis.

Center	DNA extraction protocol [†]	Number of specimens analyzed		<i>Toxoplasma gondii</i> PCR in routine diagnosis (Reference PCR)			Sacace PCR
		Calibrated curves	EQC	Clinical Specimens	Target PCR assay	PCR apparatus	PCR apparatus
1	TNN Proteinase-K, boiled and protein precipitation (Promega)	yes	5	14	rep529 In-house Fret PCR ²¹	LC480 (Roche)	LC480 (Roche)
2	QIAamp DNA Micro Kit (Qiagen) Nuclisens easyMag (bioMérieux)	yes	5	14	rep529 Bio-Evolution	CFX 96 (BioRad)	CFX 96 (BioRad)
3	QIAamp DNA Mini Kit (Qiagen)	yes	5	48	rep529 In-house Fret PCR ²¹	LC480 (Roche)	LC480 (Roche)
4	QIAamp DNA Mini Kit (Qiagen) QIAamp FPPE DNA tissue kit (Qiagen) QIASymphony (Qiagen)	yes	5	22	rep529 Bio-Evolution	LC480 (Roche)	MX3005 (Stratagene)
5	QIAamp DNA Mini Kit (Qiagen) EZ1 (Qiagen)	No	0	42	rep529 In-house PCR ²²	StepOne Plus (Applied Biosystems)	StepOne Plus (Applied Biosystems)
6	MagNA Pure 96, MagNA Pure Compact (Roche) Maxwell (Promega) EZ1 (Qiagen)	No	0	12	rep529 Bio-Evolution	Rotor Gene Q (Qiagen)	Rotor Gene Q (Qiagen)
7	QIAamp DNA Mini Kit (Qiagen)	No	0	17	rep529 In-house PCR ²¹	LC2.0	LC480 (Roche)
8	EMAG (BioMerieux)	No	0	14	rep529 Bio-Evolution	ABI7500	ABI7500 (Applied Biosystems)
9	QIAamp DNA Mini Kit (Qiagen)		In routine practice		rep529 <i>T. gondii</i> Sacace	Rotor Gene Q (Qiagen)	Rotor Gene Q (Qiagen)

[†] Center 1, 2, 4, 5 and 6 had several extraction protocols according to matrixes (see Supplementary Table 1), EQC: External Quality Control, TNN: Tween-Nonidet-NaOH (0.5% Tween 20, 0.5% NP40, and 10 mmol/L NaOH) lysis buffer method²³

Eff = (10(-1/slope)-1) x100. This also allowed us figuring out the zone of linearity (regression analysis, R²). Limit of detection was approached and defined as the lowest concentration where >50% of the reactions remain positive¹⁷. In the second phase, clinical performances were assessed by comparing qualitative results and quantitative Cq values obtained for clinical specimens between the commercial kit and the reference PCR of each lab. Qualitative results were analyzed through sensitivity and specificity calculation. Quantitative Cq values were compared

performing Bland-Altman plots¹⁸ (using Prism Software).

Ethical approval and informed consent

Clinical specimens were collected through the routine clinical activity of each participating center. As recommended in the ethical standards of the French Ethics Committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008, in agreement with the French Health Public Law (CSP Art L1121-1.1) for non-interventional retrospective study, no written or verbal informed consent to participate in the study

from the patient was necessary. However, according to the law, written consent was obtained before any prenatal AF sampling. Patients were anonymized; the study does not include potentially identifying patient/participant information.

Results

Analytical performances of T. gondii

Sacace PCR assay

All PCR efficiencies measured in this study were ≥ 90 . No difference was seen between the reference PCRs and the Sacace PCR assays performed in Center 1 to 4 (See Table 2 and Supplementary Data). With a R^2 varying from 0.9954 to 1, linearity zone were similar whatever the center and the PCR methods used, The lowest concentration where $>50\%$ of the reactions remain positive¹⁷ was similar and determined at ≤ 1 *T. gondii* genome/mL for all the centers except Center 1, which retrieved it at 0.01 *T. gondii* genome/mL (Table 2 and Supplementary Data). All qualitative results of EQC samples were concordant (Table 2). Regarding quantitative results (quantitative cycle (Cq) values), comparison of Cq values obtained using the *T. gondii* Sacace PCR assay and obtained using each reference PCR showed good agreement with ΔCqs ranging from 2 to 5.4, according to the center (Table 2 and Supplementary Data).

Validation of internal control introduced in the PCR mix instead of in the specimen before extraction.

No impact of the addition of the IC in the PCR mixes as compared to adding it before the extraction procedure was seen in serial dilution assays and EQC testing (Table 2 and Supplementary Data). This allowed us continuing the evaluation using this procedure in clinical specimens.

Clinical specimens

Among the 173 clinical specimens assessed, 106 were sampled from patients diagnosed with congenital (n=41), disseminated (n=32), cerebral (n=20), ocular (n=12) or acute (n=1) toxoplasmosis and 67 were sampled from patients without any *T. gondii* infection. More

than a dozen of distinct matrixes was included in this study (Table 3 and Supplementary Data). Among the 173 clinical specimens assessed, IC amplification failed in at least one of the duplicates from 15/173 (9 %) specimens, corresponding to 24 out of 346 (6.9%) wells (Supplementary Data, highlighted). Of these 15 specimens, seven were positive and eight negative specimens. Puzzlingly, a second run of the Sacace PCR assay allowed recovering a positive IC in both duplicate. Thus, 173/173 specimens presented interpretable results, and all presented qualitative results of *T. gondii* Sacace PCR assay in agreement with the reference PCR of each participating centers. Thereby, all qualitative results obtained with the reference PCRs and the *T. gondii* Sacace PCR test in each center were concordant. Of note, among the 106 positive clinical specimens, eight specimens were found to be inconsistently positive by four centers which corresponded to specimens with a low parasite load close to the detection limit of the method, four with their reference PCR and six with the Sacace PCR kit, two of which were inconsistently positive with both methods (Center 2- clinical specimen 13 and Center 6- clinical specimen 10) (See Table 3 and highlighted in Supplementary Data). As compared to reference PCR, sensitivity and specificity of the *T. gondii* Sacace PCR were calculated at 100% (95CI: 96.5-100%) and 100% (95CI: 94.6-100%), respectively. Cq values obtained with both reference and commercial PCRs revealed an excellent concordance, excepted for 3/106 specimens in two centers which were outliers (Figure 1). Bland-Altman analysis revealed bias ranging from 3.5 to 5.5 according to the centers (Figure 1).

Table 2: Analytical performance of *T. gondii* Sacace PCR kit: Determination of PCR efficiency and limit of detection, and EQC exactitude.

	Slope	Efficiency	R ²	LoD (T/mL)	EQC (N=5)	ΔCq
Center 1						
Reference PCR	3.1	108	0.9954	0.01	Concordant	3 ^a
Sacace wo IC	3.1	112	0.9981	0.01	Concordant	0.5 ^b
Sacace w IC	3.1	112	0.9986	0.01	Concordant	
Center 2						
Reference PCR	3.4	98	1.0000	<1	Concordant	2 ^a
Sacace wo IC	3.6	90	0.9994	<1	Concordant	0.3 ^b
Sacace w IC	3.5	94	0.9981	<1	Concordant	
Center 3						
Reference PCR	3.5	94	0.9999	<1	Concordant	2.6 ^a
Sacace wo IC	3.5	94	0.9997	<1	Concordant	2 ^b
Sacace w IC	3.6	91	0.9986	<1	Concordant	
Center 4						
Reference PCR	3.5	94	0.9999	≤1	Concordant	5.4 ^a
Sacace wo IC	3.3	100	0.9997	<1	Concordant	0.6 ^b
Sacace w IC	3.5	92	0.9979	<1	Concordant	

R²: regression analysis, LoD: limit of detection, EQC: external quality assessment, ΔCq^a: mean difference between EQC Cq values obtained using reference method and using *T. gondii* Sacace PCR with IC introduced during extraction procedure, ΔCq^b: mean difference between EQC Cq values obtained using *T. gondii* Sacace PCR with IC either introduced in the PCR mix or during extraction procedure, w: with, wo: without.

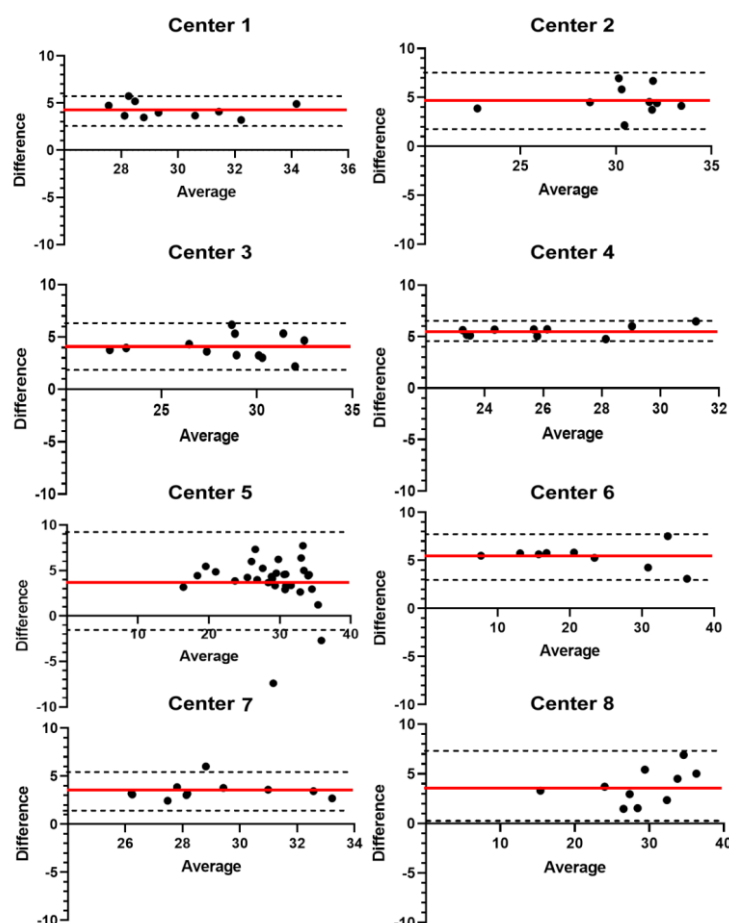


Figure 1: Bland-Altman plots comparing the Cq values of the routine method and the Sacace PCR for each of the eight centers and for clinical specimens.

Table 3: Characteristics of 173 clinical specimens included in this study.

Specimen nature	Number of specimens				Final results
	Toxoplasmosis	Positive Sacace results*	Absence of toxoplasmosis	Negative Sacace results*	
Congenital toxoplasmosis					Concordant
Amniotic fluid (AF)	17 (<i>1</i>)	17 (2 , <i>1</i>)	6	6 (1)	100%
Placenta	19	19 (1 , <i>1</i>)	5	5 (1)	
Umbilical cord blood	4	4	1	1	
Fetal biopsy	1	1	0	0	
Cerebral toxoplasmosis					
Cerebro-spinal fluid (CSF)	16 (<i>1</i>)	16 (2 , <i>2</i>)	10	10	100%
Brain biopsy	2	2	0	0	
Blood	2	2	0	0	
Ocular toxoplasmosis					
Aqueous humor (AH)	12	12 (2)	3	3 (1)	100%
Disseminated toxoplasmosis/ Acute toxoplasmosis					
Blood/buffy-coat	26 (<i>2</i>)	26 (<i>2</i>)	25	25 (4)	
Broncho alveolar lavage fluid (BALF)/ sputum	2	2	11	11 (1)	
Bone marrow	2	2	0	0	100%
Lymph node biopsy	1	1	2	2	
Biopsy/Abscess	1	1	2	2	
Other	1	1	2	2	
Total of clinical specimens	106	106	67	67	100%

*In brackets the numbers in bold corresponded to IC issue, the numbers in italic of inconstantly positive.

Discussion

Molecular diagnosis of *T. gondii* is a highly valuable tool in the diagnosis of congenital and opportunistic toxoplasmosis. A sensitive test is of great importance to promptly start an effective treatment if necessary^{19,20}. Commercial kits are now widely available and the French National Reference Center for *T. gondii* evaluates their performance as a network of independent experts. To our knowledge, no independent evaluation of the Sacace kit has been published; four centers confirmed the good analytical performances of the Sacace PCR assay on serial dilution

assays and EQC samples. Eight centers confirmed the good clinical performances of the test, with sensitivity and specificity at 100% (95CI: 96.5-100%) and (95CI: 94.6-100%) respectively as compared to their reference PCRs. Of note, Cq values obtained with Sacace were "artificially" decreased as compared to those obtained with the reference PCRs, due to the fluorescence measurement, which begins after five amplification cycles in the *T. gondii* Sacace PCR protocol, then mean (\pm SD) delta Cq values were 3.2 (\pm 1.5) and 4.4 (\pm 0.8) concerning EQCs and clinical specimens, respectively. In this study, we worked on specimens from our routine

activities, giving a wide range of parasite loads. Thus, four specimens were found to be at the sensitivity limit by the reference method of four centers. Using the *T. gondii* Sacace PCR, six *Toxoplasma* positive specimens were found inconstantly positive, two of which were also found inconsistently positive with the reference method (Table 3 and highlighted in Supplementary Table 1). This once again stresses the importance of duplicating PCRs to reduce the risk of false-negative results. The high percentage of negative IC seen in this study needs to be raised although the retrospective design of the present study did not allow to integrate the IC as recommended by the manufacturer, *i.e.*, before extraction. However, in routine practice, this is not an uncommon practice, as DNA extractions are often shared between several bacteriology, virology, parasitology and mycology parameters, and it should be noticed that not every IC can be added during the extraction step. One center uses this commercial PCR in routine practice for two years. They introduce the IC directly in the specimen before extraction procedure. In 2022, 697 PCR were realized, of which 53 were *T. gondii* positive and only two specimens presented a negative IC, confirming that adding IC during extraction procedures is reliable. Another limitation of the use of the Sacace PCR assay in routine practice may be the test practicability. Here, the PCR mix consists in addition of three components (PCR-mix-1, PCR-mix-2-FRT and the TaqF Polymerase) multiplying technical manipulations while other commercial kits offer a single ready-to-use master mix.

To conclude, the *T. gondii* Real-TM kit (Sacace) proved high analytical and clinical performances for the diagnosis of toxoplasmosis, but with two potential limitations: inconsistency of IC when added in the PCR mixture and the fractionation of the different components of the master mix.

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Supplementary Data available online: Analytical and clinical performances of T. gondii Sacace PCR kit: Determination of PCR efficiency and limit of detection of T. gondii Sacace PCR kit. Results of T. gondii Sacace PCR kit and reference PCR of all clinical specimens evaluated. This supplementary data includes the supplementary table 1 which is a large spreadsheet holding all the results of the PCRs realized in this study and also supplementary figures 1 to 4 representing the serial dilution assays. IC: internal control, Eff: Efficiency of the PCR, R²: regression analysis, N: number, NA: Not available. Highlighted in grey: Toxoplasma positive PCRs. Highlighted in orange: negative IC. Highlighted in yellow: inconstantly positive specimen.