JCM
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A 10-Year Retrospective Comparison of Two Target Sequences, REP-529 and B1, for *Toxoplasma gondii* Detection by Quantitative PCR

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This study aimed to evaluate the repeated sequence REP-529 compared to that of the B1 gene in the molecular diagnosis of toxoplasmosis by quantitative PCR (qPCR) in routine diagnosis. Over a 10-year period (2003 to 2013), all patients prospectively diagnosed with a positive REP-529 qPCR result for toxoplasmosis were included. All DNA samples (76 samples from 56 patients) were simultaneously tested using the two qPCR methods (REP-529 and B1). The mean cycle threshold (C_T) obtained with the B1 qPCR was significantly higher (+4.71 cycles) than that obtained with REP-529 qPCR ($P < 0.0001$). Thirty-one out of 69 extracts (45.6%) positive with REP-529 qPCR were not amplified with the B1 qPCR (relative sensitivity of 54.4% compared to that with REP-529), yielding false-negative results with 15/28 placenta, 5 cord blood, 2 amniotic fluid, 4 cerebrospinal fluid, 1 aqueous humor, 2 lymph node puncture, and 1 abortion product sample. This defect in sensitivity would have left 20/56 patients undiagnosed, distributed as follows: 12/40 congenital toxoplasmosis, 4/5 cerebral toxoplasmosis, 2/8 patients with retinochoroiditis, and 2 patients with chronic lymphadenopathy. This poor performance of B1 qPCR might be related to low parasite loads, since the mean *Toxoplasma* quantification in extracts with B1 false-negative results was 0.4 parasite/reaction. These results clearly show the superiority of the REP-529 sequence in the diagnosis of toxoplasmosis by PCR and suggest that this target should be adopted as part of the standardization of the PCR assay.

Toxoplasmosis is a worldwide parasitic infection due to the intracellular parasite *Toxoplasma gondii*. The infection is usually asymptomatic in immunocompetent patients and more rarely results in fever, lymphadenopathy, or retinochoroiditis. In contrast, immunocompromised patients can experience severe neurologic, ocular, pulmonary, or disseminated disease (1). Yet, toxoplasmosis is well known for its pathogenicity during pregnancy. Indeed, when primary infection occurs in pregnant women, it can lead to congenital toxoplasmosis, with a frequency of transmission and a severity of fetal infection depending on the stage of pregnancy at which infection occurs (2). The diagnosis of toxoplasmosis is routinely based on serology. In some countries, such as France, seronegative pregnant women are monitored monthly by serology. In case of seroconversion, the detection of *T. gondii* DNA by PCR is a major diagnostic method for congenital toxoplasmosis and is performed on amniotic fluid (prenatal diagnosis) (3–5) and placenta or cord blood samples at birth (postnatal diagnosis) (6–8). In immunocompromised patients, DNA can also be found in cerebrospinal fluid (CSF), bronchoalveolar lavage (BAL) fluid, or other samples, as guided by clinical signs. The 35-fold repeated B1 gene (9) has commonly been used for this molecular diagnosis since 1989, with acceptable sensitivity (3, 5, 10), but another sequence (REP-529, GenBank accession no. AF146527) was described more recently as being repeated 200 to 300 times (11), which leads to a better detection of low parasite loads using spiked specimens (12). Our objective was to evaluate the diagnostic gain resulting from the routine use of a quantitative PCR (qPCR) targeting REP-529 compared to a qPCR targeting the B1 gene for the diagnosis of toxoplasmosis in various clinical settings. In this study, we analyzed retrospectively the qPCR results over a 10-year period of all *T. gondii*-positive DNA samples from patients who benefited from molecular diagnosis.

MATERIALS AND METHODS

Patients and sample collection. During the study period (2003 to 2013), all patients with a diagnosis of congenital toxoplasmosis who benefited from a molecular diagnosis with at least one sample (amniotic fluid, placenta, or cord blood) were included. Congenital toxoplasmosis was defined by a positive prenatal diagnosis for *T. gondii* (molecular diagnosis and/or mouse inoculation) and/or serologic evidence of antibody synthesis by the newborn at birth or during the 1-year serologic monitoring period (specific IgM or IgA detected by ISAGA [bioMérieux, Marcy l'Etoile, France] or neosynthesized IgG detected by Western blot [Ldbio, Lyon, France]). During the study period, routine qPCR molecular diagnosis relied on both gene targets (B1 and REP-529) from 2003 to 2005, and then REP-529 only was used until now. All positive samples were reanalyzed with both gene targets in the same qPCR run to homogenize parasite quantification, as the standard curve had changed in the meanwhile. Additionally, all samples from proven congenitally infected infants that had tested negative in routine diagnosis with REP-529 were also retested in parallel with the B1 PCR.

Samples from adult patients with clinical signs compatible with toxo-

Received 14 October 2014 **Returned for modification** 19 November 2014
Accepted 31 January 2015

Accepted manuscript posted online 4 February 2015

Citation Belaz S, Gangneux J-P, Dupretz P, Guiguen C, Robert-Gangneux F. 2015. A 10-year retrospective comparison of two target sequences, REP-529 and B1, for *Toxoplasma gondii* detection by quantitative PCR. J Clin Microbiol 53:1294–1300. doi:10.1128/JCM.02900-14.

Editor: P. H. Gilligan

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doi:10.1128/JCM.02900-14

plasmosis (lymph nodes with specific anti-*Toxoplasma* IgM, uveitis, retinochoroiditis, or cerebral abscess) and a positive DNA detection with REP-529 from 2009 to 2013 were also included, and the DNA samples were reanalyzed with both qPCRs.

Molecular diagnosis. On reception of the placenta (PL) samples, several samples were taken from different sites and mixed in a solution containing 2.5 mg/ml trypsin, 500 IU/ml penicillin, and 3.3 μg/ml gentamicin. The preparation was digested for 2 h at 37°C under agitation, filtered through gauze, and centrifuged at 1,000 × *g* for 10 min. The supernatant was discarded, and the pellet was washed 3 times. Next, 200 μl of prepared placenta sample and other crushed biopsy samples (lymph node or cerebral) were digested overnight with proteinase K at 56°C prior to extraction. Amniotic fluid (AF) samples (10 ml) were centrifuged at 1,500 × *g* for 10 min, the supernatant was discarded, and two 200-μl samples of the pellet were used for DNA extraction. Other fluids (CSF and aqueous humor [AH]) were centrifuged at 1,500 × *g* for 10 min, and 200 μl of pellet was used for DNA extraction. Routinely, DNA extraction of all samples was performed using QIAamp DNA minikit columns (Qiagen, Courtaboeuf, France) and eluted in 100 μl, except for blood (peripheral blood [PB] and cord blood [CB]) samples, which were processed using 1 to 2 ml of whole blood, extracted using QIAamp DNA midi kit columns (Qiagen), and eluted in 400 μl. Two extracts were performed for the amniotic fluid and placenta samples (only one for other samples), and amplification was run in duplicate for all samples.

The primers and probe used to amplify a 98-bp fragment of the B1 gene were 5′-GAA AGC CAT GAG GCA CTC CA-3′ (forward) and 5′-TTC ACC CGG ACC GTT TAG C-3′ (reverse), and FAM-5′-CGG GCG AGT AGC ACC TGA GGA GAT ACA-3′-TAMRA (FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine) (13), respectively, and the primers and probe targeting REP-529 were those described previously (6). All samples were reanalyzed by both qPCRs in the same run, using the following conditions: 25 μl of reaction mixture containing primers and probe at final concentrations of 600 nM and 200 nM, respectively, 12.5 μl of TaqMan Universal PCR master mix (Applied Biosystems, Courtaboeuf, France), and 5 μl of DNA sample. Amplification was performed on a StepOnePlus device (Applied Biosystems) for 40 cycles (15 s at 95°C and 1 min at 60°C), preceded by 10 min at 55°C for a uracil *N*-glycosylase (UNG) reaction and 10 min of denaturation at 95°C. The appropriate controls were included in each run (positive and negative controls and an internal control of inhibition). The positive samples were quantified using a standard curve obtained by serial dilutions of a standardized control (10^5 *Toxoplasma* RH strain) provided by the National Reference Center for Toxoplasmosis. With the aim of comparing quantification data over the study period, all positive samples were reanalyzed for quantification using the same standard curve. The cycle threshold (C_T) of the positive samples was recorded for the comparison of the REP-529 and B1 qPCRs. All C_T results previously acquired during routine analysis and the results acquired after retesting for parasite quantification for the purpose of this study were compared to ensure that long-term storage did not alter DNA.

Statistical analysis. Before comparing the sensitivities of the REP-529 and B1 targets, we verified the quality of the DNA samples stored at −20°C by using a Wilcoxon paired test to compare the C_T newly obtained with REP-529 qPCR to the C_T previously recorded in routine diagnosis.

For the statistical analysis of the relative sensitivities between the B1 and REP-529 qPCRs, paired C_T values obtained with both qPCRs were compared using the Wilcoxon test. The mean C_T values obtained with B1 or REP-529 qPCR per type of sample were also compared using the Mann-Whitney test. Only newly acquired quantitative results obtained with both PCRs simultaneously were taken into account for statistical analysis. The absolute sensitivity of each PCR for the diagnosis of congenital toxoplasmosis was evaluated on the whole cohort of congenitally infected infants diagnosed over the study period who benefited either from prenatal diagnosis or neonatal diagnosis by PCR.

Statistical analysis was made using GraphPad Prism version 5 (GraphPad software, USA). A P value of <0.05 was considered significant.

RESULTS

Patients and samples. Samples from 41 cases of congenital toxoplasmosis were included, consisting of 20 amniotic fluid, 1 abortion product, 35 placenta, and 5 cord blood samples. Additionally, samples from 7 cases of reactivation toxoplasmosis in immunocompromised patients (2 blood, 4 CSF, 1 vitreous fluid, and 1 cerebral biopsy sample) and 7 cases of symptomatic toxoplasmosis in immunocompetent patients were included (2 lymph node biopsy and 5 aqueous humor samples). Overall, 76 samples from 56 patients were analyzed. The clinical and biological data of the patients are detailed in Table 1.

Lack of impact of storage on qPCR C_T. Forty-nine samples were retrospectively reanalyzed with B1 qPCR in this work; thus, it was essential to verify that long-term storage at −20°C after initial diagnosis had not altered the DNA. The mean C_Ts ± standard errors of the means (SEMs) of REP-529 qPCR obtained on samples before (at time of diagnosis) and after storage were similar (31.9 ± 0.8 versus 32.1 ± 0.8, $P = 0.8347$). Additionally, the C_T results were compared using a Wilcoxon paired test, which confirmed that they did not differ significantly ($P = 0.1631$, data not shown), thus making possible the interpretation of the data.

Comparison of B1 qPCR versus REP529 qPCR. The performances of the two qPCR targets were analyzed on 76 samples. Overall, 31 of 69 extracts (45%) tested positive with the REP-529 qPCR were not amplified by the B1 qPCR; thus, the relative sensitivity of the B1 qPCR was 55% compared to the REP-529 qPCR (Table 2). Seven false-negative results (7 placenta samples) were also observed with REP-529; none were positive with the B1 qPCR. Thus, over the study period, the absolute sensitivities of REP-529 and B1 qPCRs on placenta samples were 80% and 37%, respectively (Tables 1 and 3). Their absolute sensitivities for prenatal diagnosis were 100% and 90%, respectively (Table 3). False-negative results with the B1 qPCR compared to REP-529 qPCR were observed on all sample types, yet mainly on placenta samples (15/28 [54%]), blood samples (6/7 [86%]), and CSF (4/4 [100%]) (Table 2). Overall, the mean C_T obtained with REP-529 qPCR when B1 qPCR was negative was 36.84 ± 0.36 (corresponding to 0.37 ± 0.3 parasites/reaction), underlining the need for a very sensitive qPCR assay. For the 38 samples that were able to be amplified with both qPCRs, the mean C_T obtained with the B1 qPCR was significantly higher (+4.7 ± 0.3 C_T) than that obtained with REP-529 qPCR ($P < 0.001$) (Table 4). The mean gain in amplification C_T ranged from 3.65 to 4.98, according to the type of sample, and was highly significant for amniotic fluid and placenta samples ($P < 0.001$, Table 4). The difference was not statistically significant for the aqueous humor samples, probably because of a lack of statistical power.

Regarding congenital toxoplasmosis, the B1 qPCR failed to amplify any of the available samples (AF, PL, or CB) in 11 out of 40 cases (27.5%) that were positive with REP-529 qPCR. This defect in sensitivity would have had variable consequences according to the results of the other biological techniques used. Importantly, prenatal diagnosis would have been falsely negative in two cases (19 and 31), and fetal infection would have remained unproven in a case of fetal loss (case 17). Of note, the B1 target also yielded a false-negative result on the placenta sample from case 19 (Table 1). In three other cases (cases 20, 26, and 27), for which no prena-

TABLE 1 Clinical characteristics and qPCR results for 41 mother-child pairs of congenital toxoplasmosis, 8 cases of ocular toxoplasmosis, 5 cases of cerebral toxoplasmosis, and 2 cases of chronic lymphadenopathy

Case no.	Clinical setting[a]	Clinical signs (pregnancy trimester at maternal infection for CoT)[b]	Sample type[c]	qPCR result for:		Mouse inoculation	Other biological criteria for infection
				B1	Rep-529		
1	CoT	Abnormal neurodevelopment (ultrasound): medical termination of pregnancy (T1)	AF	+	+	+	Positive qPCR (REP) on fetal biopsy
			PL	+	+	ND[d]	
2	CoT	Asymptomatic (T3)	AF	+	+	+	IgM detection at 3 mo in infant (in another hospital), PL positive by mouse inoculation
3	CoT	Asymptomatic (T3)	PL	+	+	+	Positive prenatal diagnostic in another hospital; neosynthesized IgG/IgM by WB; IgM and IgA detection at birth[e]
			CB	−	+	ND	
4	CoT	Asymptomatic (T3)	PL	+	+	ND	Neosynthesized IgG/IgM by WB; IgM and IgA detection at 1 mo
			CB	−	+	ND	
5	CoT	Asymptomatic (T3)	PL	+	+	+	IgM at birth
6	CoT	Asymptomatic (T2)	AF	+	+	−	Persisting IgG at 1 yr of life
			PL	−	+	+	
7	CoT	Ventriculomegaly (ultrasound): medical termination of pregnancy (T1)	PL	+	+	+	*Toxoplasma* detection in AF by mouse inoculation
8	CoT	Asymptomatic (T3)	PL	−	+	+	Neosynthesized IgG/IgM by WB; IgM and IgA detection at birth
9	CoT	Intracerebral calcifications (ultrasound) (T2–3)	AF	+	+	+	Persisting IgG at 1 yr of life, *Toxoplasma* detection in PL by mouse inoculation
10	CoT	Asymptomatic (T2)	PL	+	+	+	Neosynthesized IgG by WB
11	CoT	Seizure after birth (T3)	PL	−	+	+	Positive prenatal diagnostic in another hospital, neosynthesized IgM (WB) at birth
12	CoT	Unknown (T3)	PL	+	+	+	Neosynthesized IgG/IgM by WB
13	CoT	Intrauterine fetal death (T2)	AF	+	+	+	
14	CoT	Asymptomatic (T3)	AF	+	+	+	Neosynthesized IgG/IgM, IgM and IgA detection at birth
			PL	−	+	+	
15	CoT	Asymptomatic (T3)	PL	−	+	−	IgM and IgA detection at 1 week of life
			CB	−	+	ND	
16	CoT	Unknown (T3)	PL	+	+	+	Neosynthesized IgG/IgM by WB; IgM and IgA detection at birth
			CB	−	+	ND	
17	CoT	Termination of pregnancy (T1)	AP	−	+	ND	
18	CoT	Chorioretinitis (T2)	AF	+	+	+	IgM detection at birth
			PL	−	−	−	
19	CoT	Asymptomatic (T3)	AF	−	+	+	Neosynthesized IgG/IgM by WB; IgM and IgA detection at birth
			PL	−	+	+	
20	CoT	Asymptomatic (T3)	PL	−	+	+	IgM (in another hospital) at birth
21	CoT	Asymptomatic (T3)	AF	+	+	+	Neosynthesized IgG/IgM by WB at birth
22	CoT	Asymptomatic (T3)	PL	−	+	+	Positive prenatal diagnostic in another hospital
23	CoT	Asymptomatic (T2)	AF	+	+	+	Neosynthesized IgG/IgM by WB; IgM and IgA detection at birth
			PL	−	−	+	
24	CoT	Chorioretinitis (T2)	AF	+	+	+	IgM and IgA detection at birth and neosynthesized IgG/IgM by WB at 1 mo
			PL	−	+	+	
25	CoT	Asymptomatic (T1)	AF	+	+	+	
			PL	−	−	−	
26	CoT	Unknown (T3)	PL	−	+	+	Persisting IgG at 1 yr of life
27	CoT	Asymptomatic (T3)	PL	−	+	+	Neosynthesized IgG by WB at 3 mo of life
28	CoT	Chorioretinitis (T3)	AF	+	+	+	IgM and IgA detection at birth and neosynthesized IgG (WB) at 1 mo of life
			PL	−	+	+	
29	CoT	Asymptomatic (T2)	AF	+	+	+	Neosynthesized IgG at 3 mo (WB)
			PL	−	−	−	
30	CoT	Asymptomatic (T3)	PL	−	+	−	Neosynthesized IgG/IgM by WB; IgM and IgA detection at birth
31	CoT	Asymptomatic (T2)	AF	−	+	−	DNA detection by REP-529 qPCR in cord blood (B1 not determined)
			PL	−	−	−	

(Continued on following page)

TABLE 1 (Continued)

Case no.	Clinical setting[a]	Clinical signs (pregnancy trimester at maternal infection for CoT)[b]	Sample type[c]	qPCR result for:		Mouse inoculation	Other biological criteria for infection
				B1	Rep-529		
32	CoT	Asymptomatic (T3)	PL	+	+	+	Neosynthesized IgG/IgM by WB; IgM and IgA detection at birth
			CB	−	+	ND	
33	CoT	Asymptomatic (T3)	AF	+	+	+	IgM and IgA detection at birth, neosynthesized IgG by WB during follow-up
			PL	−	+	+	
34	CoT	Unknown (T3)	PL	+	+	ND	Positive prenatal diagnostic in another hospital
35	CoT	Chorioretinitis and intracerebral calcifications (T2)	AF	+	+	+	Persisting IgG at 1 yr of life
			PL	−	−	−	
36	CoT	Asymptomatic (T3)	AF	+	+	+	Neosynthesized IgG/IgM by WB; IgM and IgA detection at birth
			PL	−	+	−	
37	CoT	Asymptomatic (T3)	PL	+	+	+	Neosynthesized IgG/IgM by WB; IgM and IgA detection at birth
38	CoT	Asymptomatic (T3)	AF	+	+	+	
			PL	+	+	+	
39	CoT	Unknown (T3)	AF	+	+	+	Positive serological monitoring in another hospital
40	CoT	Asymptomatic (T3)	PL	+	+	+	Positive prenatal diagnostic in another hospital, IgM at birth
41	CoT	Asymptomatic (T3)	PL	−	−	+	IgM and IgA detection at birth and neosynthesized IgG (WB)
42	OT	Uveitis	AH	+	+	ND	
43	OT	Uveitis	AH	+	+	ND	Neosynthesized IgG in AH (WB)
44	OT	Uveitis and chorioretinitis	AH	+	+	ND	
45	OT	Uveitis	AH	+	+	ND	Neosynthesized IgG in AH (WB)
46	OT	Hyalitis, not treated for toxoplasmosis	AH	−	+	ND	
47	OT	Uveitis, HIV^+ patient; treated for toxoplasmosis	VF	+	+	ND	Neosynthesized IgG in VF (WB)
48	OT	Uveitis, HIV^+ patient; treated for toxoplasmosis	PB	−	+	ND	Serological reactivation (high levels of IgG and IgM)
49	OT	Retinochoroiditis, HIV^+ patient; treated for toxoplasmosis	PB	+	+	ND	
50	CeT		CSF	−	+	ND	
51	CeT	Neurological symptoms, HIV^+ patient	CSF	−	+	ND	
52	CeT	Immunocompromised patient, 5 cerebral lesions on CT scan[f]	CeB	+	+	ND	DNA detection in CSF in another hospital
53	CeT	Heart transplant patient, neurological symptoms	CSF	−	+	ND	IgM detection in serum
54	CeT	Cerebellar syndrome	CSF	−	+	ND	Serological reactivation (high levels of IgG and IgM)
55	CL	Chronic toxoplasmosis, asthenia	LNP	−	+	ND	History of seroconversion (persisting IgM)
56	CL	Chronic toxoplasmosis, asthenia	LNP	−	+	ND	History of seroconversion 6 mo before

[a] CoT, congenital toxoplasmosis; OT, ocular toxoplasmosis; CeT, cerebral toxoplasmosis; CL, chronic lymphadenopathy.
[b] T1, first trimester of pregnancy; T2, second trimester of pregnancy; T3, third trimester of pregnancy.
[c] AF, amniotic fluid; PL, placenta; CB, cord blood; AP, abortion product; AH, aqueous humor; VF, vitreous fluid; PB, peripheral blood; CSF, cerebrospinal fluid; CeB, cerebral biopsy; LNP, lymph node puncture.
[d] ND, not determined.
[e] WB, Western blot.
[f] CT, computed tomography.

tal diagnosis had been performed because of late maternal infection during the third trimester of pregnancy, the B1 qPCR would have left the newborns undiagnosed, since no serological evidence of infection was observed until several months later (Table 1). For the five remaining cases (cases 8, 11, 15, 22, and 30), the consequences of the reduced sensitivity of B1 qPCR would have been negligible, as serological markers of congenital infection (specific IgM or IgA detection, neosynthesized IgG on Western blots) were observed in newborns at birth or during the first week of life. In case 15, B1 qPCR was falsely negative in both placenta and cord blood samples. In the remaining cases, the lack of sensitivity of B1 on the placenta samples was moot, since the diagnosis had been made previously on AF.

Regarding ocular toxoplasmosis, one aqueous humor sample from an immunocompetent patient for whom Western blot was not contributive and one blood sample from an HIV+ patient

TABLE 2 Relative sensitivities of B1 qPCR on samples tested positive with REP-529 qPCR, according to sample type

Sample	B1 qPCR result (no. positive/ total no. [%])
Amniotic fluid	18/20 (90)
Placenta	13/28 (46.4)
Abortion product	0/1 (0)
Cord blood	0/5 (0)
Blood	1/2 (50)
Ocular fluids	5/6 (83)
Cerebrospinal fluid	0/4 (0)
Biopsy (cerebral or lymph node)	1/3 (33)
All samples	38/69 (55)

TABLE 3 Sensitivity of B1 qPCR and REP-529 qPCR for the diagnosis of congenital toxoplasmosis (2003 to 2013)

Sample source	No. positive/total no. (%) for qPCR result with:	
	B1	REP-529
Prenatal diagnosis (amniotic fluid)	18/20 (90)	20/20 (100)
Neonatal diagnosis (placenta)	13/35 (37)	28/35 (80)

who did not undergo aqueous humor puncture (Table 1) were negative with B1, which would have left 2 patients undiagnosed. Additionally, the B1 target would have left 4 out of 5 (80%) cerebral toxoplasmosis cases undiagnosed, as well as the two patients with chronic lymphadenopathy (Table 2).

DISCUSSION

This study included all positive samples obtained by REP-529 qPCR over a 10-year period clearly shows the superiority of the REP-529 target for the diagnosis of toxoplasmosis, whatever the clinical setting. As previously described in other studies, this can be explained by the difference in the number of repetitions of this sequence (about 7- to 10-fold more repeated than B1) (11), which results in a gain of about 4 C_T (12, 14), as observed here ($P < 0.001$), and allows for the detection of 10-fold lower parasite loads (14). However, few studies evaluated the impact in terms of the sensitivity of diagnosis in routine clinical use.

Filisetti et al. (15) compared three PCR methods on 23 selected AF samples and 16 CB samples from 19 cases of congenital toxoplasmosis and found that only 5 out of 9 REP-529-positive AF samples were positive with the B1 PCR. It must be noted that the overall sensitivity in this study was very low, since only 11 out of 19 AF (58%) samples were positive with the reference method used, i.e., rRNA gene PCR (16), which might be due to the use of conventional PCR methods in this study. Cassaing et al. (14) included 33 positive samples (8 AF, 15 PL, 3 AH, 3 CSF, 2 blood, and 2 BAL fluid), of which 13/15 (87%) PL and 8/8 AF (100%) samples that tested positive with REP-529 qPCR were also positive with B1 qPCR. No differences in sensitivity between the two targets were observed for the AH, CSF, and BAL fluid samples, but all samples were tested in duplicate, and in 11 instances, negative results were observed in 1 of the 2 amplifications with B1 qPCR, whereas one sample was amplified by REP-529 qPCR only once. Additionally, they considered a B1 result to be positive, even though it was >40 C_T, in 4 samples. Another study (17) included 135 prospective AF samples, of which 27 and 22 were positive with REP-529 and B1 qPCR, respectively. They declared that 2 of the 5 presumed false-negative B1 results were in fact false-positive REP-529 results; however, no details are provided on the newborn follow-ups and the criteria that led them to this conclusion. Finally, the most recent study (18) evaluated three qPCR methods, mainly on AF samples. They reported 33 positive results with their B1 qPCR compared to 43 with two REP-529 qPCR methods, leading to a relative sensitivity of 77% for B1. Two cord blood samples that tested positive with REP-529 qPCR were negative with B1 qPCR. Additionally, they found that the two REP-529 qPCR methods and devices (Applied Biosystems and Roche) performed equally.

Our study focused on the evaluation of the relative sensitivity of B1 versus REP-529 PCR targets; thus, all PCR-positive samples were included. Additionally, all samples (AF and PL) from congenitally infected children with a negative REP-529 qPCR result were retested. No samples with B1-positive and REP-529-negative results were observed, either prospectively or retrospectively, in cases of proven congenital toxoplasmosis. The overall relative sensitivity of the B1 qPCR was only 55%, and its absolute sensitivity was 37% in the setting of congenital toxoplasmosis (Tables 2 and 3). This defect in sensitivity was particularly crucial for 3 antenatal diagnoses (2 prenatal diagnoses and 1 undiagnosed early fetal loss) and 3 neonatal diagnoses. In the case of fetal loss, the recognition of the role of *Toxoplasma* is important, because it allows for elimination of other causes of spontaneous abortion and it avoids useless investigations. On the other hand, the positivity of prenatal diagnosis usually leads to a change in chemotherapy, using pyrimethamine-sulfonamide combination therapy to treat the mother (1, 2), which would have been missed in the two B1-negative patients described here. Finally, the parasite DNA detection in placenta, even if not yet recognized as a standard criterion for the diagnosis of congenital toxoplasmosis, was shown to have a positive predictive value of >90% in our hands (6); at the least, it is a strong argument for accurately following the infant and increasing the frequency of serologic testing to confirm infection, with the aim of reducing a delay in treatment. We observed here that parasite DNA detection from placenta samples was the earliest biological sign of infection in neonates with neither IgM nor IgA detection, and we recall the interest of this sample in patients for whom antenatal diagnosis was negative or not performed. In 2 cases (23 and 41), both qPCRs were negative on the placenta samples, whereas they were positive with mouse inoculation, still underlining the interest for combining both techniques (Table 1).

The B1 qPCR also had poor sensitivity in other clinical settings. The diagnosis of cerebral toxoplasmosis is difficult, and ancient studies have reported the poor sensitivities of conventional PCR methods (19, 20), probably related to low parasite loads, but no recent studies have evaluated new qPCR methods in this setting. The present study was not designed to answer this question, but we noted that four patients would have been undiagnosed using B1 qPCR. Besides, the REP-529 qPCR allowed the diagnosis of two cases of chronic toxoplasmosis in patients with lymphadenopathy and history of *Toxoplasma* seroconversion in the past 12 months, which led us to consider this diagnosis and stop further exploration aimed at diagnosing a hematological malignancy.

TABLE 4 Mean cycle threshold obtained with B1 qPCR and REP-529 qPCR, according to sample type

Sample type (*n*)	Mean ± SEM C_T for:			*P* value by:	
	B1	REP-529	ΔC_T	Wilcoxon test	Mann-Whitney test
Amniotic fluid (18)	34.80 ± 0.58	29.87 ± 0.57	4.93 ± 1.35	0.0002[a]	<0.0001[a]
Placenta (12)	35.86 ± 0.57	30.87 ± 0.58	4.98 ± 2.07	0.0002[a]	<0.0001[a]
Ocular fluid (5)	32.98 ± 2.00	29.84 ± 2.30	3.49 ± 1.1	0.0625	0.3095
All samples (37)	34.51 ± 0.56	29.7 ± 0.53	4.81 ± 0.31	<0.0001[a]	<0.0001

[a] Significant at a *P* value of <0.001.

False-negative results targeting B1 had a C_T value of >34 (mean, 36.84 ± 0.36). This poor performance of B1 qPCR might be related to low parasite loads, a frequently observed situation in congenital toxoplasmosis, particularly in France, where women are treated all throughout pregnancy, which probably decreases the parasite burden. In the study by Romand et al. (21), 88 positive AF samples were included, of which 35 (40%) were shown to contain <10 parasite/ml.

The proportion of undetected samples with the B1 qPCR was high for placenta samples (57% of REP-529-positive samples) and blood samples (50%). In 12 placenta samples, B1 qPCR was negative, whereas mouse inoculation was positive, which is unusual. Poor sensitivity on blood and placenta samples suggests that inhibitors would more likely interfere with this PCR. Indeed, Chabbert et al. (22) nicely showed that the efficacy of PCR on samples spiked with small amounts of parasites was lower in placenta or blood than in AF samples with both sets of B1 primers used.

After the description of the REP-529 sequence, the B1 target has still frequently been used in parallel in most labs, including ours, until it can be demonstrated that the occurrence of B1-positive results and REP-529-negative results was never observed (14, 15, 18), suggesting that this sequence is present in all parasite isolates. However, a recent Brazilian study (23) reported a lower proportion of positive amniotic fluids with the REP-529 target than with B1 (36.5% and 87.3%, respectively), with only 23.8% of samples being positive with both targets. This unusual finding must be verified on other series of clinical samples from South America to check if atypical parasite strains circulating in this area might lack the REP-529 sequence or have mutations or modifications in the number of repetitions that might lead to a decreased sensitivity of this PCR target, which now appears to be the gold standard for European parasite strains.

Therefore, in light of the results obtained in the present study, we suggest the widespread use of the REP-529 qPCR target, which should replace B1 target, at least in Western countries, until its value is confirmed in South America.

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