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Use of Fluorescence Resonance Energy Transfer Hybridization Probes To Evaluate Quantitative Real-Time PCR for Diagnosis of Ocular Toxoplasmosis

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***Toxoplasma gondii* infection is an important cause of chorioretinitis in Europe and the United States. Ophthalmological examination and a good clinical response to adequate therapy mainly support ocular toxoplasmosis diagnosis. However, clinical diagnostic may be difficult in some atypical cases. In these cases, laboratory confirmation, based on detection of local specific antibodies and parasite DNA by conventional PCR, is therefore important to confirm the disease etiology. More recently, real-time PCR has been developed to improve prenatal congenital toxoplasmosis diagnosis. We therefore examined the diagnostic value of quantitative real-time PCR for the detection of *T. gondii* in aqueous humor samples, associated with quantification of human β-globin to control sample quantitative quality, by using a double fluorescence resonance energy transfer hybridization probes system with a double fluorescence reading. Of the 23 the clinically toxoplasmosis suspect patients, 22 showed serological evidence of exposure to *Toxoplasma*; one had a serological profile indicative of active infection. The analysis of paired aqueous humor and serum samples revealed an intraocular antibody production in 9 of 23 cases (39.1%). The quantitative real-time PCR revealed positive and high parasite numbers and high *Toxoplasma*/human genome ratios in three cases. Furthermore, PCR was the only positive confirmatory test in two cases (11.1%). None of the patients included in the control group ($n = 7$) had evidence of either local specific antibody production or *T. gondii* DNA detection, suggesting a good relative assay specificity. On the whole, quantitative real-time PCR appears to be useful for diagnosing atypical ocular toxoplasmosis presentations.**

Ocular toxoplasmosis is the most common cause of posterior uveitis in immunocompetent individuals (3, 32). Although this disease has long been considered as the reactivation of a congenital infection (33), there is now clear evidence that acquired toxoplasmosis can also induce ocular lesions (11, 30, 40).

The diagnosis of toxoplasmic chorioretinitis is based mainly on the typical clinical aspects and upon typical ophthalmoscopic features. The characteristic fundus lesion consists of a focal retinal necrosis associated with a retinochoroidal inflammatory focus. In recurrent ocular toxoplasmosis, an active lesion may be located at the margin of an old pigmented scar (43). However, clinical findings may often be far from typical, particularly in elderly or immunocompromised patients, and their toxoplasmic origin can be achieved only by laboratory analysis or by a positive response to specific antitoxoplasmic treatment (23).

Laboratory confirmation of ocular toxoplasmosis may be asserted in 50 to 80% of patients by analyzing paired samples of aqueous or vitreous humor and serum for the detection of local specific antibodies (23, 26, 45) or by using conventional gene amplification techniques (4, 30, 45). More recently, real-time PCR has been developed to improve *Toxoplasma* infection diagnosis (6, 9, 10, 29, 31). We therefore examined the diagnosis value of quantitative real-time PCR with fluorescence resonance energy transfer hybridization probes for the detection of *Toxoplasma gondii* in aqueous humor samples from a large group of patients with or without ocular toxoplasmosis.

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MATERIALS AND METHODS

Patients. (i) Ocular toxoplasmosis group. Twenty-three consecutive episodes of ocular toxoplasmosis in 23 patients who for the most part manifested the typical clinical aspect were included in the present study from the time of their first presentation at the Lille Hospital Department of Ophthalmology between February 1998 and February 2002. Patients meeting the criteria for acute retinal necrosis syndrome, with special attention to rapid progression and circumferential spread of disease (24), and patients with symptoms that were not obviously attributable to newly reactivated ocular toxoplasmosis were excluded from the study.

Fourteen (60.8%) of the patients were female, 9 (39.1%) were male, and their ages ranged from 14 to 73 years (mean age, 35.4 years). Each patient underwent a fundus examination, which revealed a unilateral posterior uveitis with active retinitis or retinochoroiditis in all cases. Clinical features were recorded at the time of diagnosis, including the history and the grade of the uveitis, the size and the location of the active lesion (25), and the status of the vitreous humor, especially its posterior face (Table 1). All patients were evaluated by the same physician (P. Labalette). Fundus photographs were obtained to assess the course of the disease in all cases, associated with fluorescein angiography in selected cases. Samples of aqueous humor and serum were drawn for the quantification of specific antibodies and molecular analysis at the time of clinical diagnosis (prior to the onset of treatment). All patients received a standard therapy. A combination of pyrimethamine (50 mg/day), sulfadiazine (50 to 75 mg/kg/day), or clindamycin (20 to 30 mg/kg/day) supplemented with folinic acid (5 mg/day) was the first-line therapy (30). Alternative antibiotic therapy (clindamycin alone or azithromycin at 250 to 500 mg/day or roxithromycin at 300 mg/day) was used or

TABLE 1. Clinical features and laboratory test results for 23 patients suspected of having ocular toxoplasmosis at presentation

Patient[a] (no., sex, age [yr])	Status[b]	Ocular data				Laboratory data									
				Flare[d]		Serum *Toxoplasma*-specific antibody result (interpretation[e])				Aqueous humor					
										Ocular production of specific antibodies			Quantitative real-time PCR[f]		
		Lesion size[c] (DA)	Posterior detachment of vitreous humor	AS	PS	IgG ELISA (UI/ml)	IgM ELISA	ISAGA IgM	ISAGA IgA	IgG	IgM	IgA	*T. gondii*	β-Globin	Ratio
1, F, 14	IC	5	Absent	1	1	160	0.1 (N)	6/12 (E)	6/12 (E)	P	N	P	0	0.1	0
2, F, 15	IC	0.5	Partial	1	2	63	0.06 (N)	ND[g]	ND	N	E	N	0	6	0
3, F, 17	IC	0.25	Absent	0	1	39	0.06 (N)	0/12 (N)	0/12 (N)	N	N	N	0	0	ND
4, M, 18	IC	2	Partial	0	2	170	0.04 (N)	6/12 (E)	0/12 (N)	N	N	E	0	2	0
5, M, 19	IC	0.75	Absent	1	1	ND	ND	ND	ND	N	N	N	0	0	ND
6, M, 24	IC	0.5	Partial	1	1	170	0.08 (N)	3/12 (N)	3/12 (N)	N	N	E	0	0.1	0
7, F, 25	IC	0.33	Absent	1	2	72	0.08 (N)	0/12 (N)	3/12 (N)	N	N	N	0	0	ND
8, F, 26	IC	0.5	Absent	2	1	18	0.08 (N)	0/12 (N)	3/12 (N)	N	N	E	0	0.4	0
9, M, 26	IC	2	Complete	2	2	85	0.1 (N)	0/12 (N)	0/12 (N)	N	N	N	0	27	0
10, F, 27	IC	1	Absent	2	1	30	0.07 (N)	6/12 (E)	0/12 (N)	N	N	N	0	6	0
11, F, 28	IC	1.5	Absent	1	1	140	0.11 (N)	0/12 (N)	0/12 (N)	N	N	N	0	0	ND
12, F, 31	IC	3	Partial	4	3	74	0.12 (N)	3/12 (N)	6/12 (E)	P	N	N	0	31	0
13, F, 32	IC	ND	ND	ND	ND	200	0.11 (N)	6/12 (E)	0/12 (N)	N	N	N	0	6	0
14, F, 32	IC	2	Partial	0	1	17	0.16 (N)	3/12 (N)	0/12 (N)	P	N	N	0	1	0
15, M, 43	IC	1	Complete	0	1	76	0.11 (N)	3/12 (N)	3/12 (N)	P	N	N	0	8	0
16, F, 44	IC	1	Partial	2	3	180	0.12 (N)	0/12 (N)	0/12 (N)	P	N	N	0	20	0
17, M, 45	IC	ND	ND	ND	ND	44	0.1 (N)	3/12 (N)	3/12 (N)	P	N	N	0	3	0
18, F, 49	IC	1.5	Complete	3	3	48	0.8 (P)	12/12 (P)	12/12 (P)	P	N	P	0	95	0
19, M, 51	IS	15	Absent	2	1	360	0.03 (N)	0/12 (N)	0/12 (N)	ND	ND	ND	1,330	77	17.3
20, M, 58	IC	0.5	Complete	2	1	33	0.12 (N)	0/12 (N)	0/12 (N)	N	N	ND	0	0	ND
21, M, 58	IC	1.5	Partial	1	2	480	0.09 (N)	12/12 (P)	12/12 (P)	P	N	E	0	3	0
22, F, 59	IS	5	Partial	2	3	13	0.02 (N)	0/12 (N)	0/12 (N)	N	N	N	50	0.01	5,000
23, F, 73	IS	12	Partial	1	3	120	0.02 (N)	3/12 (N)	9/12 (P)	P	N	N	128	5	25.6

[a] F, female; M, male.
[b] IC, immunocompetent; IS, immunosuppressed.
[c] DA was determined as described in the text.
[d] AS, anterior segment; PS, posterior segment.
[e] N, negative; E, equivocal; P, positive.
[f] Parasite and β-globin are quantified as numbers, respectively, of *T. gondii* and human genomes per microliter. Quantitative real-time PCR results are expressed as the *Toxoplasma* genome/human genome ratio.
[g] ND, not done.

substituted in patients when classic therapy was contraindicated, had failed or had induced side effects. Antitoxoplasmic treatment was continued until complete resolution of the active lesion occurred. No corticosteroid therapy was added whenever possible; if such treatment was required, a short course of oral prednisone was used unless ocular inflammation or obvious complications required its continuation. Close information and follow-up were mandatory to limit the threat of adverse events.

Three patients were immunosupressed: two due to the presence of a lymphoma (Table 1, patients 22 to 23) and the third one due to human immunodeficiency virus infection (Table 1, patient 19).

(ii) Control group. In order to assess the quantitative real-time PCR specificity, a control group of 7 patients presenting with nontoxoplasmic uveitis was included in the study. The accepted etiologies were viral uveitis ($n = 4$), autoimmune uveitis ($n = 2$), and glaucomatous cyclitis ($n = 1$). The mean age of the patients (five women and two men) was 46.7 years (age range, 4 to 79 years).

Each sample was obtained and analyzed after patient informed consent.

Analyses of blood and aqueous humor samples. Serum was obtained from centrifuged blood samples and was stored for a maximum of 24 h at 4°C prior to the analysis of immunoglobulins. Aliquots of aqueous humor (100 to 200 μl withdrawn after anterior chamber paracentesis) were centrifuged at 10,000 × *g* for 10 min. Supernatants were used for the analysis of immunoglobulins; the pellets were dissolved in 100 μl of phosphate-buffered saline (PBS) prior to DNA extraction. The DNA was then purified by using the QIAamp DNA minikit (Qiagen SA, Courtaboeuf, France) according to the manufacturer's recommendations. DNA was recovered by elution in a final volume of 100 μl of elution buffer. The solution was used for the amplification of both *T. gondii* DNA and human β-globin DNA.

Immunoassay procedures. For each patient, serum and aqueous humor samples were paired and tested on the same day under the same protocol with similar dilutions. Anti-*T. gondii* antibodies were determined by enzyme-linked immunosorbent assay (ELISA) for immunoglobulin G (IgG) (Enzygnost IgG; Behring, Marburg, Germany) and by ISAGA for IgM and IgA (Department of Parasitology, Lille, France) (30). Briefly, our immunocapture method derived from those published by Pinon and coworkers (35, 36). Specific IgM and IgA were detected by ISAGA with a suspension of formalin-treated *Toxoplasma* (bioMérieux, Marcy l'Étoile, France). Microtiter plates were sensitized with 100 μl of antihuman IgM (TIBI 82, anti-μ chain [final concentration, 5 μg/ml]; Argène Biosoft, Varhiles, France) or 100 μl of antihuman IgA (CHAFI 1 to 4, anti-α chain [Argène Biosoft]; final concentration, 10 μg/ml). After 18 h of incubation at 4°C, the microtiter plates were washed and saturated in a storage solution containing PBS, sodium azide, and 1% bovine serum albumin. The plates were stored at 4°C for a maximum of 3 weeks. Just before use, they were washed twice in PBS; the tested sera and aqueous humor specimens were diluted 1:21 in PBS and distributed (100 μl) in two consecutive wells. After incubation for 2 h at 37°C, the plates were washed in PBS. A suspension (100 μl) of formalin-treated *T. gondii* (bioMérieux) was added to each well. After incubation for 3 h at 37°C, the plates were read against a black background, both immediately and after centrifugation at 750 × *g* for 10 min. In each assay, positive and negative controls were included.

The positive cutoff for the *T. gondii*-specific IgG test corresponds to 4 IU/ml, according to the manufacturer's instructions. Ocular production of specific IgG was determined from the antibody levels in paired serum and aqueous humor samples by using the technique first described by Goldmann and Witmer (21) and then modified and adapted (30, 38, 44, 45). A cutoff coefficient value of 2 was considered suggestive of intraocular antibody synthesis related to active *T. gondii* replication. ISAGA results were scored as 0 to 12 arbitrary units as previously described (46): anti-*T. gondii* IgM and IgA were considered positive when scores were higher than 6 in serum. A score of 6 was considered equivocal in serum. In aqueous humor, anti-*T. gondii* IgM and IgA ocular values of at least 6 and 3 were considered positive and equivocal, respectively, as aqueous humor specimens were tested at the same dilution as corresponding sera (30). These borderline agglutinations have currently been used for newborns (46). An equivocal IgA score of 3 in aqueous humor associated with a positive score of 12 in serum was considered inconclusive (30) (Table 1, patient 21).

Molecular diagnostic procedures. The real-time quantitative PCR was targeted at the *T. gondii* B1 gene and based on LightCycler technology (Roche Molecular Biochemicals, Meylan, France), as previously described (9, 10). Briefly, the primers used for a 126-bp fragment amplification were the 23-mer (forward) primer 5′-GGAGGACTGGCAACCTGGTGTCG-3′ and the 25-mer (reverse) primer 5′-TTGTTTCACCCGGACCGTTTAGCAG-3′. The two hybridization probes (Genset, Paris, France) were the 25-mer probe 5′-ACGGG CGAGTAGCACCTGAGGAGAT-3′ labeled at the 5′ end with LC-Red 640 and phosphorylated at the 3′ end and the 27-mer probe 5′-CGGAAATAGAA AGCCATGAGGCACTCC-3′ labeled at the 3′ end with fluorescein.

For quantification, one 10-fold serial dilution of *Toxoplasma* genomic DNA was made that ranged from 5,000 to 0.5 parasites per μl (according to the evaluation that one parasite corresponded to 0.1 pg of DNA (10). The parasite quantification for each sample was calculated by interpolation from the standard curve included in the same run, realized in triplicate from independent experiments, and expressed as the number of *T. gondii* organisms per microliter (mean of independent experiments).

PCR runs were performed by using the LightCycler instrument (Roche) in a final volume of 20 μl consisting of 0.5 μM concentrations of each primer, 0.25 μM concentrations of each probe, 2 μl of Fast Start DNA master hybridization probe reaction mixture (Roche), 3 mM $MgCl_2$, and 10 μl of *Toxoplasma* genomic DNA or clinical specimen DNA. Each test included enzymatic prevention of contamination using UNG (uracyl *N*-glycosylase) (Roche).

The quantitative β-globin PCR was performed by using control kit DNA (Roche) in order to control for DNA integrity, as described previously (42). Results were expressed as number of human genome per microliter, according to the manufacturer's instructions.

Moreover, to control variables in the quality of samples, data were expressed as a *Toxoplasma* genome/human genome ratio (Table 1).

RESULTS

The ocular histories, initial findings, and biological data for the patients with suspected ocular toxoplasmosis are summarized in Table 1. The mean size of infectious retinitis was 2.5 disk areas (DA), ranging from 0.25 to 15 DA. The lesion was associated with a positive anterior segment flare or posterior segment haze in all cases, and with a partial (9 of 13) or complete (4 of 13) detachment of posterior vitreous in 13 of the 23 cases (56.5%) (Table 1).

All patients but one, for whom a toxoplasmosis serological profile has not been done (Table 1, patient 5), were seropositive for *T. gondii*. Nineteen (86.4%) had a chronic toxoplasmosis serological profile (positive anti-*T. gondii* IgG levels associated with negative or equivocal anti-*T. gondii* IgM and IgA levels). Of the other three patients, two were anti-*T. gondii* IgG and IgA seropositive (patients 21 to 23) and the third, patient 18, had a serological profile indicative of active infection (a positive anti-*T. gondii* IgG level associated with high positive anti-*T. gondii* IgM and IgA levels).

Intraocular antibody production (anti-*T. gondii* IgG associated with anti-*T. gondii* IgA in two cases) was detected in 9 of 23 cases (39.1%). Intraocular anti-*T. gondii* IgA production was borderline in three other cases (13%).

Of the 23 aqueous humor samples analyzed, 5 presented a negative detection of human β-globin by PCR, suggesting a DNA quantity under the detection level, or a deficient integrity of DNA. The quantitative real-time PCR was positive for 3 of the 18 β-globin PCR positive cases (16.7%), with a parasite number ranging from 50 to 1,330 per μl and a quantification expressed as a *Toxoplasma* genome/human genome ratio ranging from 17.3 to 5,000 (patients 19, 22, and 23). Moreover, PCR was the only positive ocular test in two cases (11.1%): patients 19 and 22. Concerning patient 19, the aqueous humor sample volume obtained was insufficient to proceed to both immunoassay and molecular procedures.

None of the patients included in the control group had evidence of local specific antibody production. Despite a positive β-globin PCR with a mean of DNA quantification at 9.4 human genomes/μl (human genome range, 1 to 44 [$n = 7$]), *T. gondii* DNA was not detected in any of these patients by real-time PCR. From these data, the specificity rate was evaluated

to be 100% for the immunoassay and the molecular diagnostic procedure.

DISCUSSION

We report here the results of our experience with real-time PCR for the identification and quantification of *T. gondii* in aqueous humor samples from patients with or without ocular toxoplasmosis. Currently, ocular toxoplasmosis diagnosis is based on ophthalmoscopic findings, response to treatment, and serologic determination (4, 19, 23, 45). However, in cases of atypical lesions or when vitritis hides the fundus, making the establishment of a clinical diagnosis difficult, aqueous humor analysis may be used as a diagnostic tool to confirm the uveitis etiology.

As anticipated (12, 19, 30, 39, 45), analysis of serum samples is only indicative of infection. Detection of intraocular antibody production has been reported previously, but results obtained presented erratic values (3, 4, 12, 19, 20, 30, 45). In the present study, laboratory tests performed on paired samples of aqueous humor and serum at the time of presentation by ELISA and ISAGA revealed the local production of specific antibody in 39.1% of cases, a value similar to published data (3, 12, 15, 19, 38). Among our patients, 39.1% (9 of 23) had intraocular IgG production with a Goldman-Witmer coefficient cutoff value of 2, only one had intraocular IgM production, and 21.7% (5 of 23) had local IgA production at positive or borderline levels, associated with IgG production in 8.7% of cases. In three of these patients, quantification of parasite-specific IgA at a borderline level represented the only positive ocular test. These results point again the importance of this class of antibody in the diagnosis of ocular toxoplasmosis (39) and might indicate local production of this class of antibody at the site of toxoplasmosis infection, perhaps enhanced by the intraocular presence of transforming growth factor β, which promotes switching to IgA (41).

Assessment of intraocular antibody production furnishes indirect evidence of ocular infection, whereas a direct detection of the parasite within aqueous humor samples categorically confirms the diagnosis of either primary or reactivated ocular toxoplasmosis (2). Whereas the amplification of *T. gondii* DNA is known to be a sensitive tool currently used in the diagnosis of acute fetal infection when it is performed with amniotic fluid samples (2, 9, 29), it has been carried out with aqueous humor samples offering low-sensitivity levels (1, 4, 5, 7, 8, 15, 17, 18, 28, 30, 37, 45). In the present study, quantification of *T. gondii* DNA was associated with quantification of human β-globin gene to control the efficiency of the extraction and PCR steps using a double fluorescence resonance energy transfer hybridization probe system with a double fluorescence reading for each sample (one for the LC-Red 640 used for *T. gondii* probe and analyzed by channel 2 and one for the LC-Red 705 used for human β-globin probe and analyzed by channel 3). Of the 23 aqueous humor samples analyzed, 5 have presented a negative detection of human β-globin, suggesting a DNA quantity under the detection level or a deficient integrity of DNA. Sample storage conditions before the amplification have been reported to have an important influence on the PCR sensitivity values (27). In our study, some of the DNA samples were stored at −20°C before the amplification was performed because immediate processing was impossible. On the other hand, despite the classic and well-established DNA extraction method, the presence of inhibitors in the aqueous humor samples would not be totally ruled out. Thus, the quantitative real-time PCR revealed intraocular presence of *T. gondii* DNA in 3 of the 18 β-globin positive PCR samples (16.7%), a sensitivity level similar to data reported previously (1, 15, 17–19, 45). No false-positive result was noted (which confirms a high specificity). Moreover, in two of these patients the molecular diagnostic procedure represented the only positive confirmatory test, which agreed with published data (15, 45). These three patients, who were immunocompromised (two cases of lymphoma and one case of human immunodeficiency virus infection), presented atypical active retinal lesions with the highest DA values (ranging from 5 to 15 DA, for a mean area at 2.5 DA) associated with both marked anterior and posterior segment inflammation and partial detachment of vitreous (two cases). In fact, in deeply immunosuppressed patients detecting *T. gondii* DNA in aqueous humor could not be a local event since the parasite can be even found in their blood (4, 14, 19).

In this context, quantitative real-time PCR revealed a high parasite number (up to 1,330/μl [Table 1, patient 19]) and high *Toxoplasma* genome/human genome ratios (up to 5,000 [patient 22]), findings which are consistent with a limited immune degradation of parasite DNA (19). Since variable vitreous inflammation is currently associated with ocular toxoplasmosis and is known to reduce the value of the Goldman-Witmer coefficient (34), the cell DNA quantification using the ubiquitous human β-globin gene could be of interest in making a global evaluation of the ocular cellular response, which has been suggested to play a pivotal role in active recurrent ocular toxoplasmosis (16). The quantitative real-time PCR results expressed as *Toxoplasma* genome/human genome ratios could be helpful for the physician to evaluate local infection. Actually, a high ratio may reflect an absence or a low grade of inflammatory response potentially associated with the presence of *Toxoplasma* cysts that are more resistant to therapy (13, 22). Other molecular follow-up studies are needed to explore the importance of quantitative real-time PCR in the management of ocular toxoplasmosis.

In summary, although the diagnosis of ocular toxoplasmosis is usually based on the presence of typical lesions associated with local production of anti-*T. gondii* IgG antibody and a good response to adequate therapy, the results reported here suggest that *T. gondii* quantitative real-time PCR is an efficient detection method potentially helpful for diagnosing atypical presentations of ocular toxoplasmosis. In the past decade, the use of PCR has significantly improved both prenatal diagnosis of congenital toxoplasmosis and diagnosis of acute toxoplasmic disease in the immunocompromised patient, in whom toxoplasmosis clinical presentation is atypical. Nevertheless, like many "in-house" PCR assays, conventional *T. gondii* PCR suffers from a lack of standardization and variable performance according to the laboratory. In contrast, the LightCycler PCR system is a very fast closed-tube system eliminating the risk of PCR contamination by-product carryover, giving reproducible quantitative results (9, 10), and thus making this assay very suitable for a future PCR standardization.

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