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Contributions of Immunoblotting, Real-Time PCR, and the Goldmann-Witmer Coefficient to Diagnosis of Atypical Toxoplasmic Retinochoroiditis▽

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Ocular toxoplasmosis is a major cause of posterior uveitis worldwide. The diagnosis is based mainly on ophthalmological examination. Biological diagnosis is necessary in atypical cases, and this requires aqueous humor sampling by anterior chamber paracentesis. We evaluated real-time PCR targeting the *Toxoplasma gondii* 529-bp repeat element, the Goldmann-Witmer coefficient (GWC), and immunoblotting for the diagnosis of toxoplasmic retinochoroiditis in 54 patients with atypical uveitis. The results of these biological tests, applied to paired aqueous humor–serum samples, were compared to the clinical findings. Combining either PCR or the GWC with immunoblotting increased the sensitivity to 73% or 70%, respectively. Together, PCR and the GWC had 80% sensitivity. If feasible, sensitivity can be increased by combining the three methods (85% sensitivity). The interval between symptom onset and anterior chamber paracentesis strongly influenced the detection of specific intraocular antibody synthesis. The sensitivity of the GWC increased from 45% to 56% when sampling was performed 10 days after symptom onset, and that of immunoblotting increased from 53% to 72% when puncture was performed 30 days after symptom onset. PCR analysis of aqueous humor samples detected toxoplasmic DNA in 55% of patients. In contrast to the results of immunoblotting and the GWC, the results of PCR were not influenced by the interval between symptom onset and paracentesis. PCR was more informative than the GWC and immunoblotting for immunocompromised patients. Acute necrotizing retinal lesions were significantly larger in PCR-positive patients, with a mean of 3.5 optic disc diameters, than in PCR-negative patients, with a mean of 1.5 optic disc diameters.

Toxoplasmic retinochoroiditis is a major cause of posterior uveitis worldwide (15). It is a complication of both acquired acute (12, 19) and reactivated congenital (5) toxoplasmosis. The diagnosis is usually based on ophthalmological examination. The clinical hallmark is unilateral, whitish, fuzzy-edged, round, focal lesions surrounded by retinal edema. The discovery of healed pigmented retinochoroidal scars facilitates the diagnosis. Ocular toxoplasmosis is also confirmed by a favorable clinical response to specific therapy. However, diagnosis and treatment can be delayed in patients with atypical lesions (unusual and complicated forms) or an inadequate response to antimicrobial therapy, and particularly in elderly or immunocompromised patients (13, 14). In such cases, rapid identification of the causative agent requires aqueous humor sampling by anterior chamber paracentesis.

Laboratory diagnosis is based on the comparison of antibody profiles in ocular fluid and serum samples in order to detect intraocular specific antibody synthesis, based on the Goldmann-Witmer coefficient (GWC) (6) or on the observation of qualitative differences by immunoblotting (IB) (16, 17). The GWC is based on the comparison of the levels of specific antibodies to total immunoglobulin in both aqueous humor and serum. Recent studies have shown the usefulness of PCR applied to aqueous humor, in combination with serologic tests, for the diagnosis of ocular toxoplasmosis (1, 3, 4, 8, 9, 20, 21). However, although this combined approach improves diagnostic sensitivity, the volume of the ocular fluid sample may not be adequate for all three methods.

The aim of this study was to evaluate these three laboratory methods for the diagnosis of atypical toxoplasmic uveitis, by comparison with clinical findings.

MATERIALS AND METHODS

Patients and sample collection. From March 2002 to October 2007, 54 patients presented to the Ophthalmology Department of Cochin University Hospital (Paris, France) with ocular lesions compatible with atypical toxoplasmic retinochoroiditis (large or multiple acute foci, no acute lesions or old scars, extensive lesions, vascular complications, and vitritis or chronic uveitis with no clear cause). Aqueous humor and serum were sampled in 51 cases. Vitreous humor was sampled in three cases (vitrectomy was required to remove an epiretinal membrane in one case, and to rule out intraocular lymphoma/leukemia in two cases). The ocular fluids (about 0.2 ml) were analyzed for signs of viral, bacterial, and parasitic infections. All the patients were seropositive for *Toxoplasma gondii*, and all samples underwent PCR, Goldmann-Witmer coefficient calculation, and IB for the diagnosis of ocular toxoplasmosis. Ocular fluids were centrifuged at 13,000 × *g*, and the supernatant was used for serologic tests and the pellet for PCR.

Case definition. Clinical diagnosis of toxoplasmic retinochoroiditis was based retrospectively on (i) an expert ophthalmologist's diagnosis, (ii) a successful outcome after specific anti-*Toxoplasma* treatment, and (iii) elimination of other causes.

Ophthalmologic examination. The following ophthalmologic findings were recorded at the time of paracentesis: the number and extent of acute retinal

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foci (expressed as optic disc diameter), the presence of old scars, the degree of anterior segment inflammation, immune status, and the interval between symptom onset and anterior chamber paracentesis. Clinical findings were compared with the biological test results.

GWC. Titers of anti-*Toxoplasma* immunoglobulin G (IgG) and IgM and total IgG in serum and ocular fluids were determined as previously described (17). Anti-*Toxoplasma* IgG in ocular fluid was first measured by an immunofluorescence assay (Toxo-Spot IF; bioMérieux, France). When a sufficient volume of sample was available, an enzyme-linked immunosorbent assay method was also used (Platelia IgG; Bio-Rad, France).

The GWC was calculated as described by Desmonts et al.: [(anti-*Toxoplasma* IgG titer in OF)/(total IgG in OF)]/[(anti-*Toxoplasma* IgG titer in serum)/(total IgG in serum)], where OF is ocular fluid; anti-*Toxoplasma* IgG titers are measured in IU per milliliter; and total IgG is measured in grams per liter (6). The coefficient was considered positive (in favor of intraocular antibody synthesis) when it exceeded 2 (17).

IB. A commercial *Toxoplasma* Western blotting IgG/IgM kit (LDBio Diagnostics, Lyon, France) was used to compare specific IgG profiles in serum and ocular fluids as previously described (17).

IB was considered positive when bands were recognized only in ocular fluid or when bands were more intense on the ocular fluid strip (16).

Real-time PCR. DNA was extracted from 25 to 50 μl of the ocular fluid pellet with the QIAmp DNA minikit (Qiagen, France) by using the manufacturer's biological fluid protocol, except that DNA was eluted in 50 μl of sterile distilled water. Amplification and amplimer detection were performed with an ABI Prism sequence detection system, model 7000 (Applied Biosystems, France) targeting the *T. gondii* 529-bp element, repeated roughly 200 to 300 times (GenBank accession number AF146527). The reaction mixture (25 μl) included 5 μl of DNA extract, 0.8 μM each primer (forward, 5′-TGGTTGGGAAGCGACGAG AG-3′; reverse, 5′-CATCACCACGAGGAAAGCGTC-3′), 0.2 μM Locked Nucleic Acid-substituted TaqMan probe (5′-6-carboxyfluorescein-AG[+A]GA[+C] AC[+C]GG[+A]ATGCG[+A]T-BHQ1-3′), and 1× TaqMan Universal Master Mix with uracil *N*-glycosylase (Applied Biosystems). The assay began with a step at 50°C for 2 min, followed by a step at 95°C for 10 min and then by 55 amplification cycles of 95°C for 15 s and 60°C for 1 min. Each DNA extract was tested pure in the *Toxoplasma* PCR assay. PCR inhibitors were detected by amplifying 5 μl of pure and 1:2-diluted DNA extract with a noncompetitive internal control composed of an exogenous DNA inserted in a plasmid (PCR 2.1 vector; Invitrogen, France). One to 10 copies of plasmid, 0.4 μM primers, and 0.05 μM TaqMan probe targeting the internal control were added in the *Toxoplasma* PCR assay. Internal-control amplification (expressed as the threshold cycle) of DNA extracts from ocular fluid was compared to that of DNA extracts from negative controls. A delay of more than three PCR cycles (1 log unit) was considered to represent significant inhibition. In brief, the DNA extract from one ocular fluid sample was tested three times by *Toxoplasma* PCR (undiluted without the internal control, and undiluted and diluted with the internal control). Each PCR run included a negative extraction control (sterile distilled water), a positive extraction control diluted to obtain 0.5 and 5 *Toxoplasma* genome equivalents per reaction, a negative PCR control (a DNA extract previously found to be negative), and a positive PCR control (a DNA extract previously found to be positive).

Statistical analysis. We used Fisher's exact test and the Wilcoxon test. *P* values below 0.05 were considered to denote significant differences.

RESULTS

Forty of the 54 patients had a clinical diagnosis of toxoplasmic retinochoroiditis. Their ages ranged from 16 to 81 years (median, 34 years), and there were 18 women and 22 men. Eleven patients were immunocompromised: six had AIDS (CD4 cell count, $<0.178 \times 10^9$/liter), one was a bone marrow transplant recipient, three had lymphoma, and one was on high-dose steroids. The total size of the acute retinal foci was larger in immunocompromised patients (mean, 4.9 optic disc diameters) than in immunocompetent patients (mean, 1.7 optic disc diameters). Among the 14 patients with atypical uveitis who had no clinical diagnosis of toxoplasmic retinochoroiditis, 10 had an etiological diagnosis, consisting of three cases of ocular toxocariasis, three cases of varicella-zoster virus infection, and one case each of herpes simplex virus infection, syphilis, Epstein-Barr virus infection, and birdshot chorioretinopathy. No significant difference was observed between the toxoplasmic retinochoroiditis group and the nontoxoplasmic retinochoroiditis group with respect to clinical features (Table 1).

TABLE 1. Clinical features of patients with and without toxoplasmic retinochoroiditis[a]

Characteristic	Patient group	
	Toxoplasmic retinochoroiditis (n = 40)	Nontoxoplasmic retinochoroiditis (n = 14)
Median age (yr) (range)	34 (16–81)	43.5 (16–82)
Sex ratio (% male/% female)	55/45	57/43
Median interval (days) between symptom onset and paracentesis (range)	22.5 (1–730)	29.5 (4–1,095)
% with acute retinal foci (no. positive/no. tested)	86 (25/29)	86 (12/14)
Median size (optic disc diam) of acute retinal foci (range)	1.5 (0.5–15)	1 (0.5–15)
% with pigmented scar (no. positive/no. tested)	41 (16/39)	29 (4/14)
% with anterior chamber inflammation (no. positive/total)	73 (29/40)	71 (10/14)

[a] Differences between these groups of patients were nonsignificant for all characteristics.

The results of the biological tests for the 40 patients with toxoplasmic retinochoroiditis are shown in Table 2.

The GWC had 45% sensitivity and 93% specificity. Sufficient ocular fluid was available in eight cases to determine the GWC by both an immunofluorescence assay and an enzyme-linked immunosorbent assay, and no significant difference was observed between the two techniques. The interval between symptom onset and paracentesis was significantly longer for patients with a positive GWC (median, 30 days) than for those with a negative GWC (median, 15.5 days) ($P < 0.04$). The GWC was always negative for samples obtained during the 10 days following symptom onset, whereas at least 56% of subsequent samples were positive. The degree of anterior segment inflammation, evaluated in terms of the cellular reaction in aqueous humor, was significantly higher for patients with a positive GWC ($P < 0.05$). A pigmented chorioretinal scar was observed in 67% of patients with a positive GWC and in only 29% of patients with a negative GWC ($P < 0.03$). Five of the 11 immunocompromised patients with toxoplasmic retinochoroiditis had a positive GWC.

IB had a sensitivity of 53% and perfect specificity. As with the GWC, the interval between symptom onset and paracentesis was significantly longer for patients with positive IB results (median, 100 days) than for those with negative IB results (median, 15 days) ($P < 0.04$). The sensitivity of IB increased with the interval between symptom onset and sampling, from 36% in the month following symptom onset to 72% thereafter.

Five immunocompromised patients with toxoplasmic retinochoroiditis had positive IB results.

PCR had 55% sensitivity. Unlike the results of the serologic tests, the PCR results were not influenced by the interval

TABLE 2. Characteristics of the 40 patients with toxoplasmic retinochoroiditis and results of the GWC, IB, and real-time PCR

Patient age (yr)/sex[a]	Result[b] of:			Time between symptom onset and paracentesis (days)	Total size of acute retinal foci (optic disc diam)	No. of acute retinal foci	Pigmented scar	Anterior chamber inflammation (Tyndall beam)
	GWC	IB	PCR					
20/M	−	−	−	4	0.5	3	Yes	1+
18/F	−	−	−	5	1.5	1	No	1+
33/F	−	−	−	5	0.5	1	Yes	0
29/M	−	+	+	6	1	1	No	2+
30/F	−	+	+	7	5	1	Yes	1+
22/M	−	−	−	9	1	1	No	1+
66/F	+	−	−	10	NA[c]	NA	Yes	3+
18/F	+	−	−	10	1	1	No	1+
20/M	+	−	−	10	NA	1	No	2+
30/F	−	+	+	11	2	1	No	1+
62/M	+	+	+	13	3	3	No	3+
45/M	−	+	+	16	2	1	No	1+
37/M	−	−	−	17	0.75	1	No	2+
22/F	−	−	−	17	1	1	No	0
63/F	+	−	+	25	NA	1	No	1+
26/M	+	+	+	26	1.5	1	No	3+
56/M	−	+	+	30	0	0	Yes	2+
81/M	+	−	−	30	1	1	No	2+
50/M	+	+	+	100	0.5	1	Yes	1+
76/F	−	−	+	120	2.5	1	No	0
33/F	−	+	+	120	6.5	3	No	1+
35/M	+	+	−	150	1	1	Yes	1+
57/M	−	−	+	150	2	2	Yes	1+
19/F	−	+	−	150	1	1	No	0
67/M	+	+	−	210	0	0	Yes	0
17/M	+	+	+	365	0	0	Yes	2+
20/M	+	+	+	365	1	6	Yes	0
38/M	+	+	−	365	0	0	No	0
24/M	−	+	−	730	0	0	No	0
30/F (AIDS)	−	+	+	5	15	1	No	0
70/M (lymphoma)	−	−	+	7	0.5	1	No	0.5+
27/F (AIDS)	−	−	+	15	0	0	No	1+
59/M (lymphoma)	−	−	+	15	0	0	No	0
45/M (AIDS)	+	+	+	20	10	1	Yes	1+
16/M (corticotherapy)	+	−	+	20	1.5	1	Yes	2+
32/F (AIDS)	+	−	−	30	4	2	Yes	2+
46/F (AIDS)	−	−	+	45	2	2	Yes	0
39/M (AIDS)	+	+	−	85	2	1	Yes	3+
74/F (lymphoma)	−	+	+	150	NA	NA	NA	1+
47/F (BMT)	+	+	−	180	4	2	No	0.5+

[a] M, male; F, female; BMT, bone marrow transplantation. For immunocompromised patients, the cause of immune system compromise is given in parentheses.
[b] +, positive; −, negative.
[c] NA, not available.

between symptom onset and paracentesis (median intervals, 22.5 days for the PCR-positive group and 23.5 days for the PCR-negative group). Acute necrotizing retinal lesions were significantly larger in PCR-positive patients than in PCR-negative patients (3.5 versus 1.5 mean optic disc diameters; $P < 0.04$). Eight immunocompromised patients with toxoplasmic retinochoroiditis were PCR positive.

Combining the three techniques increased sensitivity to 85%. The GWC combined with PCR had 80% sensitivity (Table 3). IB combined with PCR had 100% specificity. The GWC was positive for a patient with a final diagnosis of varicella-zoster virus uveitis, but simultaneous toxoplasmic retinochoroiditis could not be ruled out.

Finally, six patients with ocular toxoplasmosis had negative results by all three tests. These six patients all had intervals of less than 3 weeks between symptom onset and paracentesis (median, 7 days), no scars, low-level inflammation of the anterior chamber, and small retinal foci.

DISCUSSION

In our study population, a combination of PCR, IB, and the GWC had 85% sensitivity and 93% specificity for the diagnosis of atypical or extensive toxoplasmic retinochoroiditis. Clinical

TABLE 3. Sensitivities and specificities of the GWC, real-time PCR, and IB, alone and in combination

Diagnostic method	No. of samples:		Sensitivity (%)	Specificity (%)
	Positive	Negative		
GWC	18	22	45	93
IB	21	19	53	100
PCR	22	18	55	100
GWC + IB	28	12	70	93
PCR + IB	29	11	73	100
PCR + GWC	32	8	80	93
PCR + GWC + IB	34	6	85	93

diagnosis of such cases is difficult and depends on the ophthalmologist's experience. Paracentesis was performed only for patients with atypical retinochoroiditis and inconclusive clinical findings. Aqueous humor was collected from 51 patients and vitreous humor from 3 patients who required vitrectomy.

The sensitivity of the GWC for atypical uveitis (based mainly on aqueous humor samples) was 45%. Previous studies have shown GWC sensitivities of 39% to 93% (4, 9–11, 17, 18, 21). These discrepancies could be explained by differences in (i) the interval between symptom onset and paracentesis, (ii) the characteristics of the uveitis (typical or atypical), (iii) underlying immunological status, and (iv) the chosen GWC positivity threshold, which ranges from 2 to 8 in the literature. The high sensitivity of the GWC (81%) reported by Fekkar et al. could be due to diagnosis made at the late stages of the disease, but no clinical data are provided in their study (9). The specificity of the GWC was 93%. The GWC was positive for a patient with a final diagnosis of varicella-zoster virus uveitis, but simultaneous toxoplasmic retinochoroiditis could not be ruled out. Most of our patients with a positive GWC (60%) had mild or significant anterior segment inflammation. This local inflammation could lead to a significant increase in local antibody synthesis. A positive GWC was also associated with the presence of old scars.

IB had a sensitivity of 53%. Previous studies, using the *Toxoplasma* Western blotting IgG kit (LDBio Diagnostics) on aqueous humor, showed similar sensitivities (50 to 59%) for the diagnosis of atypical (17) and typical (10, 20) ocular toxoplasmosis. A recent study using the same kit showed 81% sensitivity, but the interval between symptom onset and paracentesis was not specified (9). We found that the sensitivity of IB increased with the length of this interval.

We achieved 55% sensitivity with a real-time PCR assay targeting the *T. gondii* repeat element of 529 bp. Two studies of real-time PCR targeting the B1 gene in aqueous humor gave sensitivities of 36% and 40% (4, 21). Real-time PCR has been shown to be more sensitive on a variety of samples when the 529-bp repeat element rather than the B1 gene is used as a target (2). In another study of PCR using the 529-bp repeat element, Fekkar et al. reported a detection rate of 38% in ocular fluid (9). This difference could be explained by the extraction conditions (10 μl versus 25 to 50 μl of aqueous humor and the use of a kit for blood extraction versus a kit for biological fluids) (7). Moreover, the larger proportion of immunocompromised patients in our study could explain why we obtained higher sensitivity. In contrast to the IB and GWC results, the results of PCR were not influenced by the interval between symptom onset and paracentesis. The total size of acute retinal foci was larger in PCR-positive patients, in keeping with previous studies (8, 21). PCR was positive for 8 of the 11 immunocompromised patients and was the only positive test in 4 of these 8 cases. In keeping with previous studies, PCR was more informative than the GWC and IB for immunocompromised patients (3, 8). This could be related to the larger total size of acute retinal foci observed in our immunocompromised patients, with a mean of 4.9 optic disc diameters, compared to 1.7 optic disc diameters in immunocompetent patients. Nevertheless, we confirm the usefulness of PCR for immunocompetent patients as well (9).

We found that only 40% of patients had both positive PCR results and intraocular specific IgG synthesis, in keeping with previous reports that PCR positivity is usually associated with a lack of local IgG synthesis (11, 20).

The rate of detection of specific intraocular antibodies was related to the interval between symptom onset and paracentesis. Early sampling was associated with negative GWC results and with low IB sensitivity. The sensitivity of the GWC increased to 56% when sampling was performed 10 days after symptom onset, and IB was positive for 72% of cases 30 days after symptom onset. Several studies have examined the influence of this interval on GWC results. Fardeau et al. reported that the GWC was useless during the first 2 weeks but that its sensitivity increased sharply when anterior chamber puncture was performed between the 3rd and 8th week after symptom onset (8). Garweg et al. reported that GWC sensitivity increased from 57% to 70% when puncture was performed at 6 weeks instead of 3 weeks (11). As stated above, PCR sensitivity was not influenced by this interval.

Combining the three biological techniques increased the sensitivity to 85%, with a specificity of 93%. Unfortunately, it is not always possible to perform the three tests simultaneously, because of the small aqueous humor sample volume. We propose the following algorithm for choosing the test with the best sensitivity according to ophthalmologic findings. When paracentesis is performed during the 10 days following symptom onset, real-time PCR is most suitable, especially if the patient is immunocompromised or if the total size of the foci is large (> 2 optic disc diameters). Beyond 10 days, the best choice is the GWC if old scars are present and/or if the reaction in the anterior chamber is mild to severe, or PCR if the total size of foci is large (>2 optic disc diameters); IB should be preferred when paracentesis is performed more than 30 days after symptom onset. A combination of PCR and GWC has increased sensitivity.

This study identified certain criteria that should be taken into account in order to improve the biological diagnosis of atypical ocular toxoplasmosis. The interval between symptom onset and anterior chamber paracentesis is an important factor in the detection of specific intraocular antibody synthesis. Strong anterior chamber inflammation and the presence of old scars orients the choice of test toward the GWC. PCR should be preferred for immunocompromised patients, when the interval between symptom onset and paracentesis is less than 10 days, or when there is a large total area of acute retinal foci. If feasible, sensitivity can be increased by combining these methods.

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