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Comparison of Enzyme-Linked Immunosorbent Assay, Immunoblotting, and PCR for Diagnosis of Toxoplasmic Chorioretinitis

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Ocular toxoplasmosis is the major cause of posterior uvetis in European populations. The clinical diagnosis of toxoplasmic chorioretinitis is based upon ophthalmoscopic findings, which are often but not always typical. Laboratory testing is therefore important to confirm the etiology of the disease. In the present 2-year prospective study, the relative diagnostic sensitivities of the three analytical techniques (enzyme-linked immunosorbent assay [ELISA], immunoblotting, and PCR) were compared by using a group of patients ($n = 19$) with suspected ocular toxoplasmosis. The relative specificities of the three techniques were assessed by including two control groups of patients: one with nontoxoplasmic and noninflammatory ocular disease ($n = 48$) and the other with nontoxoplasmic and inflammatory ocular disease ($n = 20$). All 19 of the clinically suspect patients had serological evidence of exposure to *Toxoplasma gondii*: 17 had been previously infected, and 2 had current infection. The analysis of paired aqueous humor and serum samples by ELISA and immunoblotting revealed the local production of specific antibodies of the immunoglobulin G type in 63% (12 of 19) and 53% (10 of 19) of patients, respectively. PCR analysis of aqueous humor samples confirmed the presence of *T. gondii* DNA in 28% (5 of 18) of cases. When combined, ELISA, immunoblotting, and PCR findings confirmed the toxoplasmic origin of retinal lesions in 83% (15 of 18) of patients. The relative specificities of the three techniques were 89% for ELISA and immunoblotting and 100% for PCR.

Toxoplasma gondii, an opportunistic parasite, is responsible for 30 to 50% of posterior uveitis cases in immunocompetent individuals (4). Ocular toxoplasmosis is a potential complication of both acquired acute (6, 23, 24) and reactivated congenital (29) toxoplasmosis. In France, systematic screening for seroconversion during pregnancy and aggressive antenatal and postnatal treatment policy for affected children has led to a marked decline in the incidence of severe congenital toxoplasmosis. Currently in France, >70% of cases of congenital toxoplasmosis are subclinical, i.e., they can be identified only by laboratory testing (8).

The main and lifelong risk for children with subclinical congenital toxoplasmosis is chorioretinitis (15), which occurs in nearly 85% of untreated cases (11) usually during the first few years of childhood.

The clinical diagnosis of toxoplasmic chorioretinitis is based upon ophthalmoscopic findings. The typical fundus abnormalities are focal retinal necrosis and choroidal edema, the active lesion being frequently located close to a pigmented scar (10, 31). However, atypical lesions also occur, and their toxoplasmic origin can be demonstrated only by laboratory testing or by a positive response to specific treatment (17).

Laboratory diagnosis is based upon the detection of local specific antibodies within aqueous or vitreous humor by enzyme-linked immunosorbent assay (ELISA) (15). More recently, immunoblotting (19, 28) and gene amplification techniques (5, 20) have been implemented.

Although the sensitivity of these methodologies is fairly well established (9), their specificity remains questionable. We therefore conducted a 2-year prospective study to evaluate these two parameters in relation to the diagnosis of toxoplasmic chorioretinitis.

MATERIALS AND METHODS

Patients. (i) Suspected cases of ocular toxoplasmosis. This population consisted of 19 consecutive patients (7 [37%] females and 12 [63%] males) aged between 12 and 80 years of age (mean, 31 years), who presented at the University of Strasbourg's Department of Ophthalmology, between January 1999 and January 2001 with recent acute decline in visual acuity due to an inflammatory chorioretinal condition that was clinically typical of ocular toxoplasmosis. Each patient underwent a fundus examination, which revealed chorioretinal lesions in 16 cases (84%). In eight of these (53%), the lesions were associated with scar tissue whose appearance suggested a healed lesion. The remaining four patients manifested generalized uveitis, papillitis, and retinal detachment (Table 1).

Samples of aqueous humor (vitreous humor for patient #12) and serum were withdrawn from each subject for the analysis of specific antibodies at the time of clinical diagnosis.

Two patients were immunodepressed: one due to a bone-marrow transplantation (patient 4) and the other one due to the presence of a lymphoma (patient 16).

(ii) Nontoxoplasma control group. In order to assess the specificity of the various techniques used, two control groups were included in the study. The first consisted of 48 patients presenting with noninflammatory ocular pathologies. Of these, 37 patients (77% [mean age, 74 years]) had a cataract, 10 (21% [mean age, 45 years]) had a keratoconus, and 1 (2% [age, 34 years]) had a chemical burn.

The second control group consisted of 20 non-human immunodeficiency virus-infected patients presenting with nontoxoplasmic uveitis. The accepted etiologies

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TABLE 1. Analysis of 19 patients suspected for ocular toxoplasmosis

Patient[a] (no., age, sex)	Status[b]	Sample[c]	*Toxoplasma*-specific antibodies					PCR[d]	Ophthalmic sign(s)[h]
			IgG UL (serum)	IgM (serum)[d]	IgG avidity[e]	Intraocular presence (IgG)	IB IgG (different band)[i]		
1, 20, F	IC	AH	87	Neg	CI	Yes	No	Pos	CHR with scars
2, 18, M	IC	AH	48	Neg	CI	No	No	Pos	CHR with retinal edema, no scars
3, 80, F	IS?	AH	1,293	Pos	AI	No	No	Pos	CHR with hyalites, no scars
4, 41, M	IC	AH	17	Pos	AI	No	No	Pos	Panuveitis with scar
5, 17, M	IC	AH	45	Neg	CI	Yes	No	Neg	CHR, no scars
6, 31, M	IC	AH	8	Neg	CI	Yes	Yes	Neg	Papillitis, no scars
7, 37, M	IC	AH	80	Neg	CI	Yes	Yes	Neg	CHR with scars
8, 28, F	IC	AH	55	Neg	CI	Yes	Yes	Neg	CHR with scars
9, 30, M	IC	AH	135	Neg	CI	Yes	Yes	Neg	CHR with scars
10, 23, F	IC	AH	38	Neg	CI	Yes	Yes	Pos	Retinal detachment papilla edema, hyalitis
11, 32, M	IC	AH	19	Neg	CI	Yes	Yes	Neg	CHR with scars
12, 27, M	IC	VH	39	Neg	CI	Yes	Yes	Neg	CHR with scars
13, 34, M	IC	AH	103	Neg	CI	Yes	Yes	ND[g]	CHR with scars
14, 12, M	IC	AH	70	Neg	CI	Yes	Yes	Neg	CHR
15, 59, F	IC	AH	126	Neg	CI	Yes	No	Neg	CHR
16, 33, F	IS	AH	372	Neg	CI	Yes[f]	No	Neg	CHR, cutaneous and ocular lymphoma
17, 35, M	IC	AH	21	Neg	CI	Yes[f]	No	Neg	CHR with scars
18, 20, M	IC	AH	55	Neg	CI	Yes[f]	Yes	Neg	CHR, no scars
19, 58, F	IC	AH	54	Neg	CI	No	No	Neg	CHR

[a] M, male; F, female.
[b] IC, immunocompetent; IS, immunosuppressed.
[c] AH, aqueous humor; VH, vitreous humor.
[d] Neg, negative; Pos, positive.
[e] CI, chronic infection; AI, acute infection.
[f] Hemato-ocular barrier was disrupted.
[g] ND, not determined.
[h] CHR, chorioretinitis.
[i] IB, immunoblotting.

were as follows: herpesvirus infection ($n = 6$), sarcoidosis ($n = 4$), autoimmune pathology ($n = 3$), infection with the varicella-zoster virus ($n = 3$), cytomegalovirus infection ($n = 1$), infection with mycobacterium ($n = 1$), helminthiasis ($n = 1$), and unknown origin ($n = 1$). In case of corneal transplantation, sera were obtained on the day before surgery to document the recipient's infectious state according to national transplantation laws; in the cataract cases, paired samples, including serum from the serum collection, was available if blood had been drawn for unrelated serum analysis on the day before surgery or on the day of surgery. All manipulations were done strictly in accordance with national laws and with the Declaration of Helsinki.

Detection of toxoplasma-specific antibodies. The levels of anti-*T. gondii*-specific IgM and IgG in serum were determined by using the SFRI ELISA technique (2). The immunoglobulin G (IgG) positivity cutoff was set at 10 IU/ml. If IgM was detected, the IgG avidity index was determined, also by SFRI ELISA (33), in order to rule out the possibility of chronic toxoplasmosis with persisting IgM (avidity index > 0.7, chronic toxoplasmosis; avidity index < 0.3, acute toxoplasmosis).

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 (15) and then modified by Payeur et al. (25) and Turunen et al. (32). Specific IgG levels were estimated by SFRI ELISA, for this analysis serum was diluted 1/300, and aliquots of aqueous humor were diluted 1/10. The specific IgG ratio was calculated as the quotient of serum optical density divided by the aqueous humor optical density and is expressed as a coefficient. A value of <2 was taken to be indicative of the ocular production of anti-*T. gondii* antibodies. In such cases, the integrity of the blood-retina barrier was tested in order to rule out the possibility of passive serum transudation. This was achieved by monitoring aqueous humor sample for the presence of anti-mumps virus antibodies (Captia Mumps IgG; Trinity Biotech, New York, N.Y.). Since the mumps virus does not cause chorioretinitis, the presence of local antibodies was assumed to reflect passive transudation through the blood-retina barrier. Patients who registered as seronegative for mumps were monitored for the presence of anti-measles antibodies.

For this purpose, an immunoblotting anti-*T. gondii* IgG detection kit was used (LDBIO Diagnosis, Lyon, France). Then, 25-μl aliquots of aqueous humor and 10-μl portions of serum were each diluted with 1 ml of sample buffer. The procedure was performed in accordance with the manufacturer's instructions. The detection of a supplementary band in the analyzed aqueous humor relative to the pattern elicited by the paired serum sample was considered to be indicative of the ocular production of anti-*T. gondii* antibodies (22) (Table 2).

PCR of *T. gondii* DNA. DNA was extracted from samples of aqueous humor by its binding to silica gel membranes (QIAamp DNA Mini Kit; Qiagen SA, Courtaboeuf, France). Prior to DNA extraction, aliquots of aqueous humor were centrifuged at 20,000 × *g* for 3 min; the pellet was resuspended in 190 μl of Tris-EDTA (10:1) buffer and then purified according to the manufacturer's recommendations. DNA was recovered by elution in a final volume of 200 μl of elution buffer.

T. gondii DNA was detected by PCR amplification of part of the B1 gene (26) and part of the 18S rRNA gene (7). PCR amplification was performed within a 50-μl reaction volume by using a hot-start *Taq* DNA polymerase (HotStar*Taq*; Qiagen SA). The reaction mixture consisted of 10 μl of template DNA (DNA purified from aqueous humor); 5 μl of 10× HotStar*Taq* PCR buffer (including 1.5 mM $MgCl_2$); 5 μl of dATP, dTTP, dGTP, and dCTP (each at 200 μM); 1 μl of each primer (1 μM); and 0.25 μl of HotStar*Taq* DNA polymerase (1.25 U).

An 88-bp segment was amplified with the B1 gene primers (5′-AAGGGCTG ACTCGAACCAGATGT-3′ and 5′-GGGCGGACCTCTCTTGTCTCG-3′),

TABLE 2. Comparative analyses of sensitivity and specificity

Analysis[a]	% (no. of strains positive/total no. of strains)	
	Sensitivity	Specificity
ELISA IgG	63 (12/19)	89 (39/45)
IB IgG	53 (10/19)	89 (39/45)
PCR	28 (5/18)	100 (45/45)
ELISA + IB	68 (13/19)	89 (39/45)
ELISA + PCR	78 (14/18)	89 (39/45)
ELISA + IB + PCR	83 (15/18)	89 (39/45)

[a] IB, immunoblotting.

and a 301-bp segment was amplified with 18S rRNA gene primers (5′-CCTTG GCCGATAGGTCTAGG-3′ and 5′-GGCATTCCTCGTTGAAGATT-3′).

The thermal cycling conditions were as follows: (i) for the B1 gene, 95°C for 15 min (DNA polymerase activation and DNA denaturation); 40 cycles at 95°C for 30 s (denaturation), 61°C for 30 s (annealing), and 72°C for 30 s (polymerization); and 72°C for 5 min (additional extension step) with storage at 4°C; and for the 18S rRNA gene, 95°C for 15 min (DNA polymerase activation and DNA denaturation); 40 cycles at 95°C for 30 s (denaturation), 55°C for 30 s (annealing), and 72°C for 30 s (polymerization); and 72°C for 5 min (additional extension stage), followed by storage at 4°C.

DNA extracted from tachyzoites of the *T. gondii* RH strain was used as a positive control. A defined number of tachyzoites was suspended in Tris-EDTA (10:1) buffer to obtain a final density of one tachyzoite per 200 μl. The positive control was purified and amplified as described above. PCR revealed only one tachyzoite in the positive control sample. Negative amplification controls (reaction mixture without template DNA) were also tested.

To detect the presence of PCR inhibitors in samples of aqueous humor, a plasmid containing the B1 target sequence with a deletion of 59 bp (26) was added to the reaction mixture. Failure to detect the corresponding 242-bp DNA band would indicate the presence of an amplification inhibitor in the template DNA, and the results in the corresponding aqueous humor sample would be consequently considered inconclusive. Then, 20-μl aliquots of the reaction mixture were analyzed by electrophoresis on a 2% agarose gel, and the DNA fragments were visualized by staining with ethidium bromide.

Statistical analysis. Differences in sensitivity between ELISA, PCR, and immunoblotting analyses were compared by using a Fisher exact test. *P* values of <0.05 were considered statistically significant.

RESULTS

Clinical, serological, and PCR results of the patients with suspected toxoplasmic chorioretinitis are presented in Table 1. All 19 clinically suspect patients were seropositive for *T. gondii*. Of 19 cases, 17 (89%) had an IgG-positive and an IgM-negative serological profile and an avidity index of >0.7. These findings were suggestive of chronic toxoplasmosis. The other two patients were IgG and IgM seropositive and had an avidity index of <0.3, a result that is indicative of active infection.

Specific ocular IgG was detected by ELISA in 15 of 19 (79%) patients. The blood-retina barrier was found to be compromised in 3 of the 15 IgG-positive patients. In patients 16 and 17 and in patient 18, whose blood-retina barrier was compromised, the ocular presence of specific IgG may have been due either to passive transudation or to local synthesis. When we take into account the integrity of the blood-retina barrier, the ELISA technique had a sensitivity level of 63% (12 of 19), with a positive predictive value of 67% and a negative predictive value of 85% (Table 2).

The immunoblot test detected a supplementary toxoplasma-specific IgG band in aqueous humor compared to serum in 10 of 19 (53%) patients (a positive predictive value of 63% and a negative predictive value of 81%). These 10 samples were also ELISA positive. In the three patients with compromised blood-retina barriers, one patient had a supplementary band but the other two did not. When immunoblotting findings were combined with ELISA results, a definitive diagnosis of retinal toxoplasmosis did not increase significantly (68 to 63%, $P = 0.55$).

Of the 19 aqueous humor samples, 18 were analyzed by PCR; the volume obtained from patient 17 was insufficient for this purpose. PCR revealed the local presence of *T. gondii* DNA in five of these samples (28%). The positive predictive value and negative predictive value were 100 and 78%, respectively. Of the five PCR positive samples, three were PCR positive but ELISA and immunoblot negative, one was PCR and ELISA positive but immunoblot negative, and one was PCR and immunoblot positive but ELISA negative. When ELISA and PCR findings were combined, the definitive diagnosis of retinal toxoplasmosis growth increased significantly from 63 to 78% ($P < 0.001$).

When ELISA, immunoblotting, and PCR findings were combined, the toxoplasmic nature of retinal lesions could be demonstrated in 83% of cases (15 of 18) (Table 2). When ELISA and immunoblotting findings were combined, the sensitivity decreased to 68% (13 of 19).

Assay specificities were determined from the results of specimens from patients with nontoxoplasmic ocular pathologies. In the group of patients with nontoxoplasmic and noninflammatory ocular disease, serological analyses revealed 71% (34 of 48) of these to have had a previous toxoplasmic infection. The local presence of specific IgG was detected by ELISA in 6% (3 of 48) of patients, but in each case the blood-retina barrier was compromised. Immunoblotting revealed no evidence of local specific IgG production in these instances or any other. *T. gondii* DNA was not detected in any of the patients of this group by PCR.

In the group of patients with nontoxoplasmic pathology and no inflammatory ocular disease, 11 of 20 (55%) had serological evidence of previous infection with toxoplasma.

Local specific IgG was detected by ELISA in 10% (2 of 20) of individuals, both of whom had a compromised blood-retina barrier. Immunoblotting revealed no evidence of local specific IgG production in these instances or any other. *T. gondii* DNA was no detected in any of the patients of this group by PCR.

From these data, specificity rates were calculated to be 89% for ELISA and immunoblotting and 100% for PCR analysis (Table 2).

DISCUSSION

The diagnosis of toxoplasmic chorioretinitis is essentially based on ophthalmoscopic findings (27, 30). Although the ophthalmologic signs of toxoplasmic chorioretinitis are highly suggestive, they can be mimicked by other infections (4), or the symptoms can be atypical. Hence, laboratory testing is crucial to establish the etiology of the disease. Determination of seropositivity is useful but is only indicative of infection. Demonstration of local specific antibody production furnishes indirect evidence of ocular infection, but detection of parasite DNA in ocular fluids is necessary for definitive proof of ocular infection. In the present study, all 19 patients suspected of ocular toxoplasmosis were seropositive. Of these, the serology results indicated that 17 had had previous infections, so reactivation was the probable cause of clinical symptoms. A total of 53% of the patients had retinal scars but no known history of toxoplasmic infection, a finding that reflects the frequently asymptomatic nature of ocular involvement in this setting. The mean age of the experimental group of patients (31 years) was similar to that reported for another cohort of individuals with suspected reactivated congenital ocular toxoplamosis (5), although this coincidence is not in itself suggestive of a congenital origin. In two patients, chorioretinitis was associated with serological evidence of acute toxoplasmosis (i.e., the presence of IgM and a low IgG avidity index), which suggested that these patients had only recently become infected. One of these

patients was immunodeficient, a situation that is classically associated with toxoplasmic chorioretinitis (18). The other patient was immunocompetent and may have had a severe or atypical form of chorioretinitis (16).

In the present study, the analysis of paired serum and aqueous humor samples by ELISA revealed the local production of specific IgG production in 63% (12 of 19) of cases, a sensitivity that corresponds well to those reported in other studies (9, 14).

However, an analysis of the same samples by immunoblotting yielded a sensitivity of only 53% (10 of 19). When the blood-retina barrier is compromised, immunoblotting was conclusive for only one patient (patient 18). The presence of a different immunoblot profile in aqueous humor may be due to a compartmentalized B-cell response specific to the eye, as suggested by Klaren and Peek (21).

A direct detection of the parasite within aqueous humor samples by PCR formally confirms the diagnosis of either primary or reactivated ocular toxoplasmosis (3). In the present study, PCR revealed the local presence of *T. gondii* DNA in 28% (5 of 18) of patients. Similar low-sensitivity levels were recorded by Figueroa et al. (28%) (13), Aouizerate et al. (30%) (1), and Fardeau et al. (26%) (12) and may be attributable to late sampling. Indeed, PCR positivity was associated with local specific antibody production in only two of the five cases. However, if aqueous humor samples are withdrawn too early after the onset of symptoms, false-negative results may be produced (14, 25). Notwithstanding this drawback, *T. gondii* DNA PCR is a useful tool for the early diagnosis of toxoplasmic chorioretinitis. In the present study, PCR confirmed the toxoplasmic origin of ocular lesions in three patients (17%) who registered negative for local specific antibody production by ELISA.

On the basis of data derived from two control groups of patients, we were able to demonstrate that the specificity of the three techniques used varied between 89% (ELISA and immunoblotting) and 100% (PCR). These findings suggest that surgery or inflammatory processes seldom damage the blood-retina barrier (2 of 68) and are not associated with the release of parasite, as evidenced by PCR. The data stress the value of combining ELISA and PCR for the diagnosis of toxoplasmic chorioretinitis. In the present study, *T. gondii* DNA PCR and the detection of local specific antibody production by ELISA and immunoblotting together confirmed the toxoplasmic origin of retinal lesions in 83% of patients (15 of 18), with a positive predictive value of 71% and a negative predictive value of 93%. The sensitivity of immunoblotting in this series is less than that of ELISA (53% versus 63%) but may be conclusive when the blood-retina barrier is compromised. PCR confirmed the toxoplasmic origin of chorioretinitis in three patients with negative or borderline local antibody production, thereby rendering possible early specific treatment.

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