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Comparison of Immunoblotting, Calculation of the Goldmann-Witmer Coefficient, and Real-Time PCR Using Aqueous Humor Samples for Diagnosis of Ocular Toxoplasmosis▽†

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We compared three biological methods for the diagnosis of ocular toxoplasmosis (OT). Paired aqueous humor and serum samples from 34 patients with OT and from 76 patients with other ocular disorders were analyzed by three methods: immunoblotting or Western blotting (WB), the calculation of the Goldmann-Witmer coefficient (GWC), and PCR. WB and GWC each revealed the intraocular production of specific anti-*Toxoplasma* immunoglobulin G in 81% of samples (30 of 37). PCR detected toxoplasmic DNA in 38% of samples (13 of 34). Nine of the 13 PCR-positive patients were immunocompetent. Combining the techniques significantly improved the diagnostic sensitivity, to 92% for the GWC-WB combination, 90% for the WB-PCR combination, and 93% for the GWC-PCR combination. The combination of all three techniques improved the sensitivity to 97%.

Ocular toxoplasmosis (OT) is the main cause of posterior uveitis worldwide and is a frequent cause of vision loss (14, 15, 21). The current gold standard for the diagnosis of OT is ophthalmologic examination, but the findings may be equivocal for patients with atypical lesions. In particular, toxoplasmic retinochoroiditis can mimic acute retinal necrosis syndrome (1). Therefore, laboratory methods often are necessary to confirm the diagnosis of OT. The most reliable sample type is that of aqueous humor, which can be tested for local specific antibody (Ab) production or for *Toxoplasma gondii* DNA by PCR.

Local Ab production can be detected with an immunoblotting method and quantified by calculating the Goldmann-Witmer coefficient (GWC) (3). In both cases, the specific Ab profiles of serum and aqueous humor samples are compared. Specific Abs can be detected by enzyme-linked immunosorbent assay (ELISA) and/or by immunofluorescence (IF) assay.

The aim of this study was to compare the sensitivities and specificities of these three biological methods for the diagnosis of OT. The design of this study is that of a prospective case series.

MATERIALS AND METHODS

Patients and methods. We analyzed data from a series of 110 patients diagnosed with various ocular disorders during a 15-month period (December 2004 to February 2006) at the Department of Ophthalmology, Pitié-Salpêtrière Hospital, Paris, France. In most cases, the clinical findings were suggestive of atypical *Toxoplasma gondii* retinochoroiditis but were inconclusive. In order to confirm the diagnosis, anterior-chamber paracentesis was performed, and aqueous humor was sampled (vitreous humor for 18 patients). Blood was sampled simultaneously. Some patients were tested two or three times during the study, yielding a total of 120 samples. Clinical findings suggestive of *T. gondii* retinochoroiditis (i.e., focal retinal necrosis and choroidal edema with possible old scars) associated with successful outcomes of specific treatments were considered the gold standard. Considering these findings, a final diagnosis of OT was made for 34 patients (39 samples). The controls consisted of nontoxoplasmic ocular infection (see Table S1 in the supplemental material) and noninfectious ocular disorders. Among control patients, 64% (50 out of 78) had serological evidence of chronic toxoplasmic infection.

Laboratory tests. Aqueous humor samples were centrifuged for 10 min at 10,000 × *g*. The supernatant was used for Ab analysis, and the pellet was used for real-time PCR detection of parasite DNA.

GWC. We used both a commercial ELISA kit (Platelia Toxo IgG; Bio-Rad, Marnes la Coquette, France) and an in-house IF method (using antigens extracted from the virulent RH strain) to measure *Toxoplasma*-specific immunoglobulin G (IgG) in serum and aqueous humor samples. Aqueous humor and serum samples were diluted 1:101 for both procedures. The levels of total IgG in the two samples were determined by using an immunodiffusion technique (NOR-Partigen IgG-HC and LC-Partigen IgG for serum and aqueous humor samples, respectively [Dade-Behring, France]). The GWC was calculated as (anti-*Toxoplasma* IgG in aqueous humor/total IgG in aqueous humor)/(anti-*Toxoplasma* IgG in serum/total IgG in serum). A value of 2 was considered evidence of intraocular Ab synthesis.

Immunoblotting. Immunoblotting was done with a commercial test (Toxoplasma Western blot IgG-IgM [LDBIO, Lyon, France]) by following the manufacturer's recommendations. The method detects Abs to antigens in the 20- to 120-kDa range. Briefly, 10 μl of serum or aqueous humor sample was incubated in the appropriate buffer with nitrocellulose strips. After 2 h at room temperature on a rocking platform, the strips were washed three times with phosphate-buffered saline (PBS) and then incubated with a polyclonal rabbit anti-human IgG-alkaline phosphatase conjugate for 1 h. The washing step in PBS was repeated, and the strips were further incubated with nitroblue tetrazolium to visualize bound secondary Ab. The reaction was terminated by adding water, and then the strips were air dried and pasted on paper.

Immunoblot interpretation. Two independent observers blinded to the results of the calculation of the GWC and PCR compared the band patterns of paired aqueous humor and serum samples with the assistance of a magnifying glass. The immunoblot was considered positive for intraocularly specific Ab production when the aqueous humor sample yielded either a unique band or at least three bands that were more intense than those for the corresponding serum sample.

DNA amplification. Real-time PCR using TaqMan technology (Applied Biosystems) was applied to aqueous humor samples. The targets were a portion of

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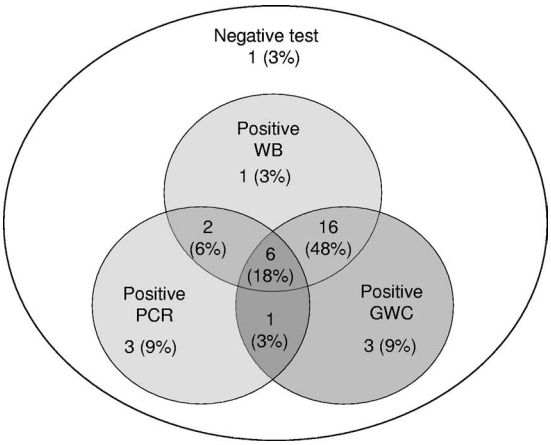

FIG. 1. Venn diagram showing data from patients with OT (n = 33) whose samples were tested by the three methods. Only one sample was negative by all three methods.

the repetitive B1 gene and the 529-bp repeat element (REP; 200 to 300 copies/genome), as reported elsewhere (2, 5, 10, 12, 18). The primers were 5′-AGAG ACACCGGAATGCGATCT-3′ (sense) and 5′-TTCGTCCAAGCCTCCGAC T-3′ (antisense) for the B1 gene and 5′-GAAAGCCATGAGGCACTCCA-3′ (sense) and 5′-TTCACCCGGACCGTTTAGC-3′ (antisense) for the REP sequence. The hybridization probes were 5′-TCGTGGTGATGGCGGAGAGAA TTGA-3′ for the B1 gene and 5′-CGGGCGAGTAGCACCTGAGGAGATAC A-3′ for the REP sequence. Parasite DNA was extracted from 10 μl of aqueous humor by using the QIAmp DNA blood minikit (Qiagen). A decontamination step with uracil DNA-glycosylase was used to prevent carryover contamination. PCR inhibitors were detected by including an internal control in each reaction mixture. In the case of the PCR inhibitor, the sample was diluted before PCR was performed again. Considering the samples from patients with OT, we did not have any problems with PCR inhibitors.

The results obtained for patients with OT and patients with other ocular disorders were compared by using the Wilcoxon test and the chi-square test. The diagnostic yields of the three methods were compared by calculating the kappa index.

RESULTS

On the basis of clinical findings and the outcome of specific treatment, a final diagnosis of OT was made for 34 patients (39 samples) of the 110 patients (120 samples) included in the study. Figure 1 shows the comparison of positive samples tested by all three methods (n = 33). Only one sample from a patient with OT was negative by all three methods. This patient was a 24-year-old woman with typical OT lesions who had no history of congenital toxoplasmosis or immunosuppression. The genotype of the involved strain was not available. Sufficient aqueous humor was not available to perform the three methods for six other patients with OT.

Testing the same patient many times did not give different results throughout the study, except for one patient, whose Western blot (WB) result was first negative and then positive 2 months later (see below). Therefore, the results would not change significantly if each patient was included only once. We compared the results of testing aqueous versus vitreous humor samples and found no major discordance (see Table S3 in the supplemental material). However, for one patient the WB result was positive with the vitreous humor sample and negative with the aqueous humor sample. In this case, the vitreous humor was sampled 2 months after the aqueous humor, and this interval may account for the difference between the results. The GWC was positive for the two samples.

GWC. The GWC had a sensitivity of 81% for active OT (30 out of 37 samples) and a specificity of 98.7% (one false-positive value out of 70 determinations). Positive and negative predictive values were 97 and 91%, respectively.

In 23 cases for which at least one of the two methods used to determine the GWC was positive and for which sufficient aqueous humor was available, we compared the GWC values obtained by the two methods. We found that ELISA gave significantly higher values than IF (P = 0.001) (see Table S2 in the supplemental material).

Sufficient aqueous humor samples were available to determine the GWC by both IF and ELISA for 28 patients with OT. Interestingly, the ELISA-based GWC had a sensitivity of 82% (23 out of 28), while the IF-based GWC had a sensitivity of only 50% (14 out of 28).

Immunoblotting. Qualitative immunoblotting was performed on 37 paired serum and aqueous humor samples. Immunoblotting had a sensitivity of 81% for active OT (30 out of 37 samples) and a specificity of 95.5% (two false-positive results out of 43 assays). Positive and negative predictive values were 93.8 and 86.5%, respectively.

PCR. PCR had a sensitivity of 38.2% for active OT (13 out of 34 samples) and a specificity of 100% (no false-positive results out of 72 tests). Positive and negative predictive values were 100 and 78.6%, respectively. The REP and B1 gene targets had sensitivities of 38.2 and 26.5%, respectively.

DISCUSSION

To our knowledge, this is the largest series of consecutive OT cases in which all three available biological diagnostic methods were used. Among the OT patients, four were infected by human immunodeficiency virus (HIV), and the others all were immunocompetent.

PCR detected *Toxoplasma* DNA in 38% (13/34) of patients. Although low, this level is higher than those commonly reported (6, 23), possibly owing to the use of two targets, including the abundant REP sequence. A new method based on nested PCR appears to have better sensitivity (13). PCR was positive in nine cases in which local Ab production was detected and also in three cases with no detectable local Ab production. PCR was positive on 75% of samples with positive results by one or another of the methods used to detect local Ab production. PCR positivity often is associated with a negative WB or GWC result (8, 9, 23) and is the only test that confirms the diagnosis (16). PCR is classically positive for immunocompromised patients, but this was the case for only 4 of the 13 PCR-positive patients in our study. All four of these patients were infected by HIV. The nine other PCR-positive patients were immunocompetent. Our results do not support previous reports that *Toxoplasma* PCR is useless for immunocompetent patients (6, 7, 22).

All of the GWC values were greater than 2. Raising the cutoff from 2 to 3 did not significantly affect the sensitivity or specificity. In our hands, the sensitivity of the GWC was similar to or higher than that found in most previous studies (11, 20).

Interestingly, the GWC was far less sensitive when based on IF than on ELISA, possibly because the two methods detect

different antigens: IF uses total parasites and detects surface antigens expressed by tachyzoites, whereas ELISA detects a mix of surface and intracellular antigens. We no longer use IF to calculate the GWC.

GWC and WB had similar sensitivities, and combining these two methods increased the diagnostic sensitivity to 92%, which compares well with values obtained elsewhere (19). The other two-by-two combinations of the three biological methods had similar sensitivities, and combining the three methods yielded near-perfect sensitivity (97.4%).

GWC values above 2 correlated with WB positivity ($\kappa = 0.714$). In contrast, we found no correlation between GWC and PCR or WB and PCR ($\kappa = 0.231$ and 0.276, respectively).

Eleven control patients (12 samples) had other infections, consisting of herpes simplex virus ($n = 3$), cytomegalovirus ($n = 3$), varicella-zoster virus ($n = 1$), *Mycobacterium* species ($n = 2$), *Treponema pallidum* ($n = 1$), and *Toxocara* species ($n = 1$). None of the three biological methods for diagnosing *Toxoplasma* infection was falsely positive in any of these cases.

Although many authors have reported the value of specific IgA detection in this setting (9, 17, 20), we found that GWC and WB detection of local IgG production was adequate.

The interval between symptom onset and sample collection has been reported to influence the results of biological diagnostic tests (4, 8). Most of our patients were referred by other ophthalmologists and, thus, were sampled some time after onset. This may have improved the sensitivity of GWC and WB but could have reduced that of PCR.

In conclusion, our findings show the usefulness of PCR for the diagnosis of OT in both immunocompetent and HIV-infected patients. As previously reported, combining several biological techniques improved the diagnostic yield.

More generally, our work underlines the importance of biological tests for the confirmation of OT diagnosis, especially in patients with atypical lesions.

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