



Diagnosis of Toxoplasmic Chorioretinitis: Moving Towards Standardized Assays

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Abstract

Ocular toxoplasmosis (OT) is the most common retinal infection. Its prevalence varies depending on the geographic area: from 2.8 % -4.2% in Europe, 17% in South America and up to 38.3% in Africa. Despite the advances in diagnostic technologies, current gold standards for OT diagnosis remain mainly based on clinical examinations, which can be misleading when signs are masked by the inflammation or might present under atypical forms, especially in the elderly and immunocompromised patients. Laboratory tests are normally used to support clinical diagnosis in dubious cases and are mainly represented by Goldmann-Witmer Coefficient (GWC) determination and IgG and IgM comparative immunoblot (IB). These methods can detect anti-toxoplasma specific IgG and IgM antibodies synthesized in the ocular compartment, as serology in peripheral blood samples is not predictive of active OT. In addition, the production of specific anti-Toxoplasma antibody may remain under detectable concentrations in certain immunocompromised patients. In the last decade, PCR has been proposed as an additional confirmation test, but its applicability is still debated. Here we retrospectively evaluated our 9 years' experience in OT diagnosis by real time PCR and IgG/IgM IB.

Ocular samples from patients undergoing routine diagnostic tests for ocular infections between 2010 and 2019 were retrospectively tested and results were compared with the clinical diagnosis. The experimental procedures were according to Ethical Requirements of San Matteo Hospital Foundation, Pavia. In this comparison with the clinical diagnosis, real time PCR on ocular fluids showed 93.33% diagnostic accuracy. Sensitivity was estimated at 90% and specificity was 96.67%. The false positive rate was 3.3% and the false negative rate 10%. We propose that real time PCR can be used as first line test in laboratory confirmation of OT in ocular fluid samples independently from the type of patient, age, sex and immunocompetence status. When larger volumes of ocular fluid are available (more than 200µL) IB can be performed in order to obtain the best diagnostic efficacy.

Keywords: Ocular toxoplasmosis; Real time PCR; IgG -IgM immunoblot

Introduction

Ocular toxoplasmosis (OT) is the most common retinal infection. Its prevalence varies depending on the geographic area: from 2.8 % -4.2% in Europe, 17% in South America [1] up to 38.3% in Africa [2].

However, the disease seems to be largely underestimated. The increasing reporting of asymptomatic lesions [3] and the emerging high frequency of postnatal infections suggest that the disease is often acquired by postnatal infection, although for a long time the first cause of OT was considered the congenital transmission that occurs during pregnancy from the mother to the child. In postnatal toxoplasmosis the infection onset is

often asymptomatic, and the active infection occurs because of cysts reactivation or of the parasite present in the blood, especially in at-risk groups such as elderly, children and immunocompromised [4]. Clinical signs may appear long after first infection and symptoms may manifest late, in an advanced disease stage, when the retina damage is severe. In addition, recurrence occurs likely within two years after the first infection and the time between recurrences shortens with the disease progression [5]. Treatments efficacy and regression of lesions strictly depend on the time of therapeutic intervention. Therefore, accurate and timely diagnosis is essential for an early treatment that may reduce the risk of permanent damage of the retina. Despite the advances in diagnostic technologies current gold standards for OT diagnosis remain mainly based on clinical examinations, which can be misleading when signs are masked by the inflammation or might present under atypical forms, especially in elderly and immunocompromised patients. Laboratory tests are normally used to support clinical diagnosis in dubious cases and are mainly represented by Goldmann-Witmer Coefficient (GWC) determination and IgG and IgM comparative immunoblot (IB) [6,7]. These methods measure the presence of specific IgG and IgM synthesized in the ocular compartment, however because such antibodies may persist long after infection, both GWC and IB are prone to false positive results [8]. Serology in blood samples is not predictive, due to the high seroprevalence in the population. In addition, the production of

Submitted: 01 June 2019 | **Accepted:** 30 August 2019 | **Published:** 31 August 2019

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Citation: Francesca G, Rosa Maria AE, Alfonso C, Guglielmo F, Enrico B, et al. (2019) Diagnosis of Toxoplasmic Chorioretinitis: Moving Towards Standardized Assays. JSM Clin Pathol 4: 5.



specific anti-Toxoplasma antibody may stay under detectable concentrations in certain immunocompromised patients [9]. PCR has been proposed as additional confirmation test for a decade, but its applicability is still debated. While in blood has been demonstrated an unreliable marker for OT [10,11], the detection of *T.gondii* DNA in ocular fluid is considered necessary for biological diagnosis [11,12]. However, several independent studies on ocular fluid, either aqueous humor (AH) or vitreous humor (VH), reported suboptimal sensitivity of PCR tests, in spite of the high specificity, especially with laboratory developed assays and nested PCR methods [8,13,14]. Sampling time after onset of inflammation, ocular sample volume, low DNA extraction efficiency, low sensitivity related to the PCR target gene as well as cross contamination have been major limitations in routine OT molecular diagnosis [8,14,15]. None the less, reduced hands-on time and rapid result reporting still make PCR an appealing and cost-effective method for an effective diagnosis and timely intervention [9]. Infact, improvement of analytical performance in real time PCR commercial assays and progressive automation of methods may now overcome those issues. Based on the analysis of available molecular technologies, a national survey in France recently highlighted the advantages of the new real time PCR assays and endorsed molecular diagnosis as a reference technique by outlining standardised guidelines that encompass all analytical steps, sampling, extraction and PCR choice [15].

In our laboratory commercial real time PCR has long been the test routinely applied in diagnosis of fetal infection transmission

and other *T. gondii* infections. PCR and serological test in OT diagnosis, performed in the the last 9 years were tested to evaluate their reliability and results were compared with the clinical diagnosis.

We retrospectively compared real time PCR and IgG/IgM IB resultswith clinical diagnosis in ocular fluid in samples collected over a 9-years period from patients undergoing vitreum or aqueous humor sampling for ocular infections routine diagnostic tests for ocular infections.

Material and Methods

To evaluate the reliability of PCR in OT diagnosis, samples form patients undergoing routine diagnostic tests for ocular infections were tested and results were compared with the clinical diagnosis.

The experimental procedures were according to Ethical Requirements of San Matteo Hospital Foundation, Pavia.

Clinical samples

Sixty-one (61) ocular fluid samples were collected between 2010 and 2019 at the Microbiology and Virology Unit of San Matteo Hospital Foundation in Pavia from patients with ocular infections referred as probable ocular toxoplasmosis. The patients underwent routine confirmatory test for diagnosis on blood and ocular samples, including 53 aqueous humor (AH) and 8 vitreous humor (VHs). Leftovers from test were stored at -80°C for further examination.

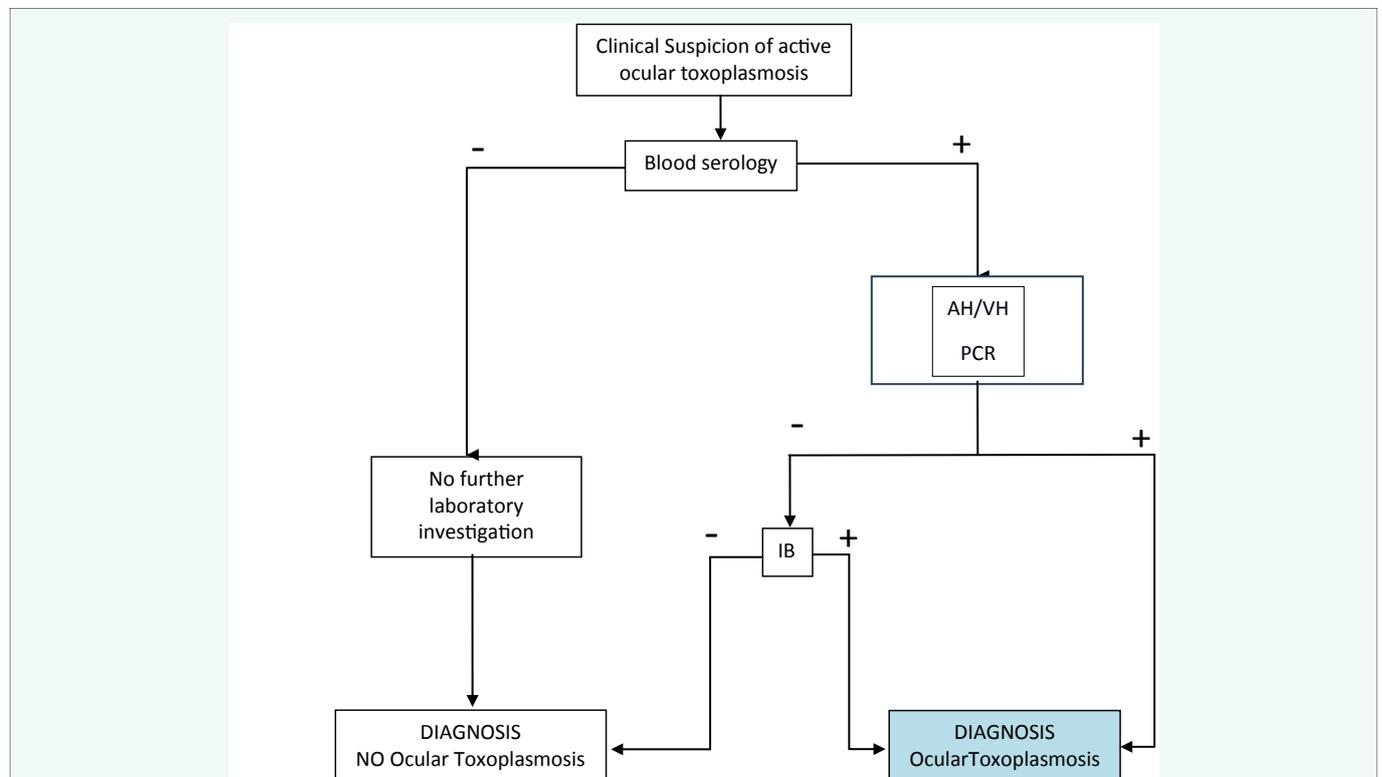


Figure 1 Algorithm for laboratory confirmation of Ocular Toxoplasmosis.

*In AH samples with larger volume. AH= Aqueous Humor. VH=Vitreous Humor PCR= real time PCR; IB = IgG/IgM immunoblot.



Routine test

Routine serological diagnosis was performed on serum by LIAISON® Toxo IgG II / IgM CLIA (DiaSorin, Saluggia, Italy), VidasToxo IgG II (BioMerieux, Marcy-L'Etoile, France) Toxo IgM ISAGA, Toxo IgG Avidity (BioMerieux, Marcy-L'Etoile, France), IgA Elisa (DiaSorin, Saluggia, Italy). In addition, comparative immunoblotting was performed on and serum and ocular fluid samples with larger volume by IgG/IgM Immunoblot (IgG-IgM IB) (LDBIO, Lyon, France). All assays were used according to manufacturer instructions.

Molecular detection of *Toxoplasma gondii*

Molecular detection of *T. gondii* was performed by Toxoplasma ELITE MGB Kit (ELITech Group, Torino, Italy) a commercial real time PCR assay CE marked for *in vitro* diagnostics, that targets the RE repeated region (more than 38 folds). Analytical steps were according to manufacturer's instructions. In brief, DNA was extracted from 200 µL of ocular fluid by EasyMAG (BioMerieux, Marcy-L'Etoile, France), samples with a smaller volume were added with sterile saline up to a final volume of 200 µL. CPE Internal Control (ELITechGroup, Torino, Italy), an extraction and PCR inhibition control, containing an unrelated exogenous control was added to each sample before extraction in order to verify the validity of the analytical procedure, extraction and amplification. Then 10µL of eluted DNA were used for DNA Amplification by Toxoplasma ELITE MGB Kit in association to Applied Biosystems 7300 Real-Time PCR System (ThermoFisher Scientific, US). PCR was set up according to manufacturer's instruction.

Results

Ocular samples of 61 subjects with possible OT were retrospectively investigated by real time PCR. The group included 29 men (47.52%) and 32 women (52.46%), of age between 0 and 88 years old (mean=343; SD= 21.51) (Table 1). According to ophthalmologic symptoms, 50 (81.97%) patients had chorioretinitis, 9 (14.75%) uveitis, 1 (1.64%) panuveitis and 1 (1.64%) vitritis. Ocular samples consisted in 53 aqueous Humour (AH) and 8 vitreous humour (VH). All patients have been tested for anti-Toxoplasma immunity with LIAISON® Toxo IgG II / IgM CLIA. One AH was lost during testing and excluded together with the corresponding serum. Overall the serology and PCR results of 60 samples were evaluated and compared with the diagnosis based on clinical findings (Table 1).

Thirty samples were from OT-positive patients and as per clinical diagnosis they were clinically classified as 25 chorioretinitis, 3 uveitis 1 panuveitis and 1 vitritis. Ocular samples from OT cases included 28 AH and 2 VH. At real time PCR test, 27 samples (23 AH and 2 VH) resulted positive, and 3 (2AH and 1 VH) were negative. Overall, 27 over 30 OT cases were positive at real time PCR on ocular fluid and 3 were false negative. Real time PCR sensitivity was 90% and the rate of false negative was 10 % (Table 2). The serology was positive in all the OT cases (30 out of 30).

Thirty samples were from OT-negative patients classified as 24 chorioretinitis and 6 uveitis. The ocular samples of OT negative individuals included 25 AH and 5 VH. At real time PCR, 29 out of

30 ocular fluid samples were negative and 1 was false positive. The specificity of the real time PCR in ocular fluid samples was 96.67% and the false positive rate was 3.33%. The serology had 16.67% specificity and 83.33% false positive rate (Table 2). Overall, 56 out of 60 real time PCR results were concordant with the clinical diagnosis and the diagnostic accuracy was 93.33%, the positive predictive value was 96.43% and the negative predictive value was 90.63%.

Twelve (12) patients were tested by real time PCR and IgG-IgM IB (Table 3). Ten (10) of these were OT positive at clinical diagnosis and 2 were negative. Real time PCR and IB were concordant positive in 7 out of 12 cases with clinical diagnosis of OT. In 5 cases results were discordant: among the patients with OT one was PCR negative and IB positive and two were PCR positive and IB negative. Among the patients without OT one resulted negative in PCR and positive in IB, the other was positive in PCR and negative in IB. Hence, clinical diagnosis, PCR and IB were concordant in 7 out of 12 samples, clinical diagnosis and PCR only were concordant in 10 out of 12 cases while IB and clinical diagnosis were concordant in 9 out of 12 patients, of which the diagnosis of just one case was based on IB only.

Discussion

Clinical signs are currently the main diagnostic evidences in OT infections. PCR is generally accepted as an adjunctive

Table 1: Characteristic of symptomatic patients whose samples were retrospectively investigated by real time PCR for ocular toxoplasmosis.

Patients	N=60
Gender	
Female	31 (51.67%)
Male	29 (48.33%)
Mean age	43 ± 22
Age range	1 month to 88 years
Ocular Diagnosis	
Chorioretinitis	49 (81.67%)
Uveitis	9 (15.00%)
Panuveitis	1 (1.67%)
Vitritis	1 (1.67%)
Sample type	
Aqueous Humor	52 (86.67%)
Vitreous Humor	8 (13.33%)

Table 2: Results of serology and real time PCR in ocular fluid samples from patients with possible ocular Toxoplasmosis. Sensitivity = No. PCR positive/ total No. OT clinically positive. Specificity = No. PCR Negative/ total No. OT clinically Negative

Clinical Diagnosis	Serology nr/ total nr (%)	PCR nr/ total nr (%)
sensitivity	30 / 30 (100)	27 / 30 (90)
specificity	5 / 30 (16.67)	29 / 30 (96.67)
False negative	0 (0)	3 / 30 (10)
False positive	25 / 30 (83.33)	1 (3.33)
Diagnostic accuracy	35 / 60 (58.33)	56 / 60 (93.33)



Table 3: Results of real Time PCR and IB in larger volume AH samples.

Larger volume AH samples (N=12)	Serology		Real Time PCR		IgG/IgM IB	
	POS	NEG	POS	NEG	POS	NEG
Clinical diagnosis						
OT	10/12	0	9/10	1/10	8/10	2/10
No OT	2/12	0	1/2	1/2	1/2	1/2

laboratory test, but its suitability as first line diagnostic tool remains controversial [1,16]. The lack of standardisation has resulted in inadequate sensitivity of molecular tests and has eventually played against implementation in an effective OT diagnosis algorithm. On the other hand, result comparison across studies is partially biased by heterogeneity in sample choice, sampling time, DNA extraction protocols, PCR targets and by the variety of assay used, from inhouse PCR to commercial PCR assays adjusted according to the available laboratory equipment or procedures [12,16].

In this study, the agreement between clinical diagnosis and real time PCR alone on ocular fluids was over 93%. In addition, sensitivity was 90% and specificity was higher than 96%.

The observed improvements are possibly justified by the use of a commercial standardised method for detection of *T. gondii* DNA. Standardization of the entire analytic procedure has been advocated as crucial to implement molecular diagnostics of *T. gondii* infections and the match between DNA extraction and amplification methods has been considered crucial to achieve adequate diagnostic performance [15]. In this study, the molecular method fully met the recent recommendation on development of molecular test for *T. gondii* diagnosis [15]. The assay targets the RE region that is repeated more than 38-75 folds in the *T. gondii* genome [18,19]. Such region has been demonstrated to achieve higher sensitivity [9,15,20]. In addition, we run the assay according to well-defined protocols for both extraction and amplification, which were designed and validated by the manufacturer in order to guarantee suitability of extracted DNA for amplification. In our view such conditions have likely contributed to improve the sensitivity and specificity of the molecular diagnosis. However, the agreement with the clinical diagnosis was further improved combining real time PCR and IB in the analysis of larger volume samples. In agreement with previous reports [16], in this study we observed two patients with a discordant diagnosis between PCR and IB and in one of them diagnosis was based on IB only. Indeed, as reported in literature IgG and IgM IB can be useful in the diagnosis of ocular lesions [1]. In addition, compared to clinical diagnosis we reported two false positives at laboratory test, one of PCR and one for IB. However, because of the limited information on the patient history and follow up, we cannot discuss these data more accurately.

Ocular fluid sampling is an invasive procedure. Though AH is possibly easier to sample, it is questioned whether it is sufficiently informative for OT investigation. This study did not aim to investigate the appropriateness of a specific ocular fluid for molecular diagnosis. Nonetheless, 88% of the tested samples were on AH, and the results show that AH is *per se* an adequate and informative matrix for real time PCR investigation of OT and for IgG IgM IB.

Based on these observations we propose that real time PCR can be used as first line test in laboratory confirmation of OT in ocular fluid samples independently from the type of patient, age, sex and immunocompetence status. When larger volumes of ocular fluid are available IB can be performed in order to obtain the best diagnostic efficacy.

Acknowledgements

The authors are grateful ELITech Group for supporting the project and to dr. Cristina Olivo for contributing to the data analysis and to the drafting and editing of the paper.

Valeria Meroni is a member of the European Study Group for Clinical Parasitology of The European Society of Clinical Microbiology and Infectious diseases.

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