Published in final edited form as:

Br J Ophthalmol. 2009 November ; 93(11): 1546–1550. doi:10.1136/bjo.2009.162412.

BILATERAL RETINOCHOROIDITIS CAUSED BY AN ATYPICAL STRAIN OF *TOXOPLASMA GONDII*

Juliana Bottós¹, Robin H. Miller³, Rubens N. Belfort^{1,4}, Ana Carolina Macedo¹, UNIFESP Toxoplasmosis Group², Rubens Belfort Jr^{1,*}, and Michael E. Grigg^{3,*}

¹Vision Institute, Department of Ophthalmology, Federal University of São Paulo, UNIFESP Brazil

²UNIFESP Toxoplasmosis Group (Cristina Muccioli MD, Maria Cristina Martins MD, Dr. Miguel N. Burnier Jr MD PhD, Cláudio Silveira MD, Luiz V. Rizzo MD)

³Molecular Parasitology Unit, Laboratory of Parasitic Diseases, NIAID, National Institutes of Health, Bethesda, MD, USA

⁴Henry C Witelson Ocular Pathology Laboratory, Department of Ophthalmology, McGill University, Montreal, Quebec, Canada

Abstract

Background—A 53-year-old man presented with an acute bilateral posterior uveitis with extensive necrotizing retinochoroiditis but without chorioretinal scarring. A thorough workup did not reveal any underlying disease. The possibilities of atypical ocular toxoplasmosis as well as herpetic retinal necrosis were considered and specific therapy instituted, with little improvement. The patient died within two months as result of an undifferentiated squamous cell carcinoma.

Methods—Histopathological examination, immunohistochemistry and multi-locus polymerase chain reaction confirmed *T. gondii* infection of the retina

Results—Macroscopic examination of enucleated globe showed extensive retinal necrosis and vitreous detachment. Histological examination of retinal tissue identified numerous round-to-elliptical toxoplasmic cysts within the retina, with retinal necrosis and minimal choroidal inflammation. Immunohistochemical analyses confirmed the cysts were due to *Toxoplasma gondii*. DNA extracted from formalin-fixed, paraffin-embedded tissue sections was subjected to multi-locus PCR analysis at the following typing loci: SAG1, SAG2, SAG3, SAG4, B1, NTS2, GRA6, and GRA7. DNA sequencing of positive PCR products at the NTS2, SAG1, and GRA7 loci confirmed the presence of a non-archetypal strain of *T. gondii* infecting the eye of the patient experiencing a severe, atypical ocular toxoplasmosis

Correspondence: Juliana Bottós, Rua Mirassol 236 ap 22, 04044-010, São Paulo – Brazil, Phone: 55 11 8186-6804, jubottos@gmail.com.

*Both authors contributed equally to this study

LICENCE FOR PUBLICATION

The Corresponding Author has the right to grant on behalf of all authors and does grant on behalf of all authors, an exclusive licence on a worldwide basis to the BMJ Publishing Group Ltd to permit this article to be published in BJO and any other BMJPL products and sublicences such use and exploit all subsidiary rights, as set out in our licence (<http://bjo.bmj.com/fora/licence.pdf>).

Competing Interest: None declared.

Conclusion—A highly divergent, non-archetypal strain of *Toxoplasma gondii* was responsible for causing a severe, atypical bilateral retinochoroiditis in a patient from Brazil.

Keywords

Microbiology; Pathology; Genetics; Infection; Diagnostic tests/Investigation

INTRODUCTION

In Brazil, 50–80% of the adult population is infected with *Toxoplasma gondii* [1]. Multiple routes of exposure have been implicated including waterborne transmission and the ingestion of undercooked meat [2, 3]. Infection is associated with high levels of ocular disease, especially in the Southern States where incidence can be as high as 18%, and recurrent disease is both serious and common [4]. This contrasts with Europe and the United States where the incidence of ocular toxoplasmosis is much lower, ranging from 2–5% [5]. Epidemiologic and population genetic data indicate that many Brazilian patients experiencing ocular disease acquire infection post-natally [4, 6], and that they are infected with strains genetically distinct from clonal lines circulating in Europe and United States [1, 7, 8, 9, 10]. Strain type is thought to be an important factor determining the severity of ocular disease, and recent molecular studies in Brazil have identified a rich diversity of strains infecting people and their companion animals. PCR-based typing at a single *SAG2* locus has previously identified a Type I lineage allele among outbreak patients experiencing high levels of ocular disease associated with atypical, necrotizing lesions [3, 11]. However, typing at a single locus fails to identify the true genotype of recombinant and/or atypical strains that bear combinations of archetypal and non-archetypal alleles, as are common in Brazil [1]. Typical features of ocular toxoplasmosis include focal acute necrotizing granulomatous retinochoroiditis that is often associated with a preexisting pigmented retinochoroidal scar, called satellite lesions. Atypical presentations include multiple large foci of bilateral acute necrosis, retinal hemorrhages and vasculitis. Such atypical lesions often present a challenge in establishing the diagnosis [12, 13].

Herein, we report a case of a Brazilian patient with bilateral retinal necrosis. Histological examination revealed numerous *Toxoplasma* cysts and multi-locus PCR-DNA sequencing analyses from formalin-fixed, enucleated eye tissue sections confirmed this patient was infected with a non-archetypal strain of *Toxoplasma gondii*. This report highlights the necessity of performing multi-locus genotyping analyses and describes new primers that amplify from formalin-fixed tissue sections making retrospective analyses possible.

METHODS

Clinical Information

A healthy, 53 year old man appeared complaining of an acute loss of vision and pain in both eyes. His past ocular and systemic histories were unremarkable. His best-corrected visual acuity was 20/50 in the right eye and 20/200 in the left eye.

Slit-lamp biomicroscopy showed granulomatous keratic precipitates and inflammatory cells in the anterior chamber and vitreous in both eyes. The left eye showed corneal edema, fibrin in the anterior chamber and posterior synechiae (Figure 1) Fundoscopy of the right eye revealed extensive and poorly-defined retinochoroiditis involving the inferior retina, associated with 2+ vitreitis, intraretinal hemorrhages, marked periphlebitis and periarteritis, and absence of chorioretinal scars (Figure 2). In the left eye the fundus exam was not possible due to 4+ vitreitis. Fluorescein angiography of the right eye disclosed hypofluorescence of the lesion during the early phases of the study, followed by progressive hyperfluorescence secondary to vascular leakage and occlusive vasculitis (Figure 2).

Multiple etiologic agents were considered in the differential diagnosis of bilateral acute uveitis with occlusive vasculitis and retinal necrosis. A work up for sarcoidosis, syphilis, tuberculosis, toxoplasmosis, cytomegalovirus, HIV, herpes, masquerade syndrome by intraocular lymphoma and Behçet's disease were negative, except for positive circulating IgG antibodies for toxoplasma and herpes simplex. A thorough systemic workup did not reveal any underlying disease.

Possible acute herpetic retinal necrosis was considered and the patient treated with intravenous and orally acyclovir and oral prednisone (1mg/kg/day). One month later, his best-corrected visual acuity was improved. However, after two months, his visual acuity decreased with worsening of the vitreous charges and increasing areas of retina necrosis in both eyes. An atypical presentation of ocular toxoplasmosis was considered and systemically trimethoprim/sulmethoxazole was added.

The following month, his systemic condition progressively deteriorated. He presented with loss of weight, a painful left shoulder, hoarseness and cervical lymphadenopathy. A full physical examination, complete laboratory assessment and radiologic work-up as well as endoscopic procedures, as indicated, were performed. Lymph node biopsy established the diagnosis of squamous cell carcinoma from an unknown primary site (CUP). Cervical lymph nodes, bone and liver metastasis were found. The patient died two months later and his eyes were donated for research.

Serology, Histopathology and Immunohistochemistry

Serum was tested for IgG reactivity for *Toxoplasma gondii*. Enucleated eye tissue was fixed in 10% neutral buffered formalin, trimmed, paraffin-embedded and 5–10 µm sections cut and stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed to screen for reactivity to antibodies against *T. gondii* on formalin fixed, paraffin-embedded tissues.

Molecular Characterization

Genomic DNA was extracted from 10 µm cut formalin fixed, paraffin-embedded sections according to the manufacturer's instructions of the DNeasy Tissue kit (Qiagen). Paraffin was xylene-extracted from embedded tissue sections prior to DNA isolation. Genomic DNA preparations were screened for the presence of *T. gondii* DNA by nested Polymerase Chain Reaction (PCR)-amplification at the following diagnostic, strain-typing loci: *B1*, *NTS2*,

SAG1, *SAG2*, *SAG3*, *SAG4*, *GRA6* and *GRA7*. One water (DNA-free) negative control and one Type I strain (RH) positive control was tested with each assay. Both rounds utilized 5 µl of PCR Buffer (10X SigmaTaq PCR buffer containing 15mM MgCl₂), 0.1mM dNTP mix, 10pmol of each primer, and 1.5U of Taq DNA polymerase in a total reaction volume of 50 µl. Each of the 35 cycles consisted of 94°C for 40 seconds, 58°C for 40 seconds, and 72°C for 40 seconds. Samples were amplified in an automated thermocycler (Eppendorf Mastercycler). PCR products were visualized using GelRed staining of 1–2% agarose gels. Primer sequences for B1, *SAG1*, *SAG2*, *SAG3*, *SAG4*, and *GRA6* have been described before [14, 15, 16]. Primer sequences for NTS2 and *GRA7* were: ***NTS2***, F_{ext}: TGTGCTCGTGACTTGATGTG, F_{int}: ACCAGAAAGCCAGTGGAAATG, R_{ext}: GGAAAACAAGCGGTGAGATT, R_{int}: TTTGTTTTTCTCGCGTAGAGG.; and ***GRA7***, F_{ext}: CCAGCATGGATAAGGCATCT, F_{int}: GACGCTGAAGTGACTGACGA, R_{ext}: GACACTGTCTCGAGCTCCT, R_{int}: TTAGCCCCCATATCCTACTGG (Shobab et al, submitted).

RESULTS

Macroscopic examination of enucleated globe showed retinal necrosis with ghost retinal vessels and vitreous detachment. Histopathologic examination with H&E-stained tissue sections disclosed numerous round-to-elliptical toxoplasmic cysts within the retinal necrosis. Immunohistochemistry confirmed the diagnosis (Figure 3).

To determine the strain type that caused infection, DNA extracted from the paraffin-embedded, formalin-fixed tissue sections was subjected to nested PCR using 8 highly sensitive, genetic typing loci: *B1*, *NTS2*, *SAG1*, *SAG2*, *SAG3*, *SAG4*, *GRA6* and *GRA7*. The *B1* gene is widely used for the molecular diagnosis of *T. gondii* infection due to its high sensitivity; there are 35 copies of this gene in the *Toxoplasma* genome. *NTS2* (Non Transcribed Spacer 2) is another multi-copy gene locus that lies in the region between the 28S and 18S rRNA genes. The majority of the other markers developed for multi-locus genotyping are derived from highly polymorphic surface antigens (SAGs) or parasite proteins secreted from a unique set of Apicomplexan organelles collectively known as dense granules (GRAs). Given the difficulties of PCR amplification using DNA extracted from formalin-fixed specimens, we evaluated a wide panel of genotyping markers for efficacy.

PCR amplification for *T. gondii* was successful using the *NTS2*, *SAG1*, and *GRA7* loci only (Figure 4A). All genetic markers were tested at least twice on independent DNA extractions from two separate tissue sections. All markers were negative against eye tissue extracted from a patient not infected with *Toxoplasma* (data not shown). Positive PCR products were purified and the allele determined by DNA sequencing. A Type I allele was identified at *NTS2*, a Type II or III strain allele was identified at *SAG1*, and a highly divergent, atypical allele was identified at *GRA7* (Figure 4B). Multi-loci genotyping established that the Brazilian patient was infected with a non-archetypal strain that appears to be a recombinant strain possessing a mixture of non-archetypal and archetypal alleles.

DISCUSSION

Atypical toxoplasmic retinochoroiditis has been described in immunocompromised patients including those with AIDS as well as in immunocompetent patients receiving immunosuppressive drug therapy or transplant recipients and elderly individuals [5, 12]. Variation in the clinical presentation and severity of disease has been attributed to several factors, including the genetic heterogeneity of the host and the genotype of the parasite responsible for infection [12, 17]. This study identified a novel recombinant strain of *T. gondii* that caused an atypical ocular toxoplasmosis. A Type I allele was identified at *NTS2*, a Type II or III strain allele was identified at *SAG1*, and a highly divergent, atypical allele was identified at *GRA7*. Thus, the strain infecting this patient appeared to be a novel recombinant genotype possessing a mixture of non-archetypal and archetypal alleles.

This study highlights the development of several robust, new multi-locus genotyping markers that were both highly sensitive and capable of annealing to parasite DNA extracted from formalin-fixed clinical tissue specimens. Primers against eight genetic typing loci (*B1*, *NTS2*, *SAG1*, *SAG2*, *SAG3*, *SAG4*, *GRA6* and *GRA7*), were tested and three markers (*NTS2*, *SAG1*, and *GRA7*) consistently amplified parasite DNA extracted from different cut sections from the paraffin-embedded, formalin-fixed block of the patient's eye tissue. These new polymorphic markers now extend the arsenal of molecular typing tools to formalin-fixed tissues making it possible to retrospectively perform multi-locus genotyping on archival clinical specimens. It is important to note that the B1 primers, which are routinely used for the molecular diagnosis of *T. gondii* infection, did not work against DNA preparations from the formalin-fixed specimen. Nor did the SAG2 primers, which have previously been applied successfully against acetone-fixed tissue specimens [9]. These facts underscore the necessity of applying a battery of molecular markers to maximize the potential for assigning a multi-locus genotype against DNA preparations extracted from formalin-fixed specimens. Application of these molecular markers should enable researchers and clinicians to test whether specific genotypes of circulating *T. gondii* are associated with the development of ocular toxoplasmosis. Multi-locus typing is preferred because the exclusive use of only a single marker is not sufficiently resolved to accurately determine strain genotype in regions of the world (for example, Brazil) where strain diversity is greater and non-archetypal and recombinant strains are detected more frequently [1, 2, 3, 19]. And for those cases of ocular toxoplasmosis where *T. gondii* DNA is either not available, or cannot be amplified due to prolonged fixation in formalin, it is now possible to ascribe a serotype by reacting patient serum in an ELISA format against a panel of synthetic polymorphic peptides derived against several *T. gondii* antigens that pull out strain-specific antibodies circulating in infected patients [18]. These new tools should make it possible to ask whether strain "type" is a predictor for the development of ocular disease and may ultimately become an important diagnostic and prognostic tool for management of ocular disease.

Whether the strain infecting this patient is related to, or identical to, strains previously identified infecting ocular toxoplasmosis patients from Brazil is not possible to address because the majority of previous investigations simply investigated a single typing locus, *SAG2*. In a waterborne outbreak in Parana State, Brazil, a high incidence of associated ocular toxoplasmosis was diagnosed and genotyping performed on two epidemiologically-

linked isolates from the outbreak identified only Type I lineage alleles at SAG2 [3]. A study of 9 immunocompetent patients from Brazil bearing a severe, atypical ocular toxoplasmosis also identified a Type I lineage allele at SAG2 [3, 11]. The authors of these two studies concluded that Type I strains were associated with ocular toxoplasmosis in Brazil, but in the absence of multi-locus typing using a wider set of sequence-based markers, there is no way of telling whether the strain(s) associated with ocular toxoplasmosis in these patients were truly Type I or non-archetypal, recombinant strains like the one found infecting the patient described herein. In this study, a Type I lineage allele was identified at *NTS2*, so typing using only this locus would have led to an erroneous conclusion. It is, however, possible to distinguish this strain from the one(s) that caused a severe or atypical toxoplasmic retinochoroiditis in 12 otherwise healthy adult patients in the US because it possessed a Type II or III lineage allele at SAG1, whereas the US strains all possessed a Type I lineage allele. These results make it clear that future work should incorporate multi-locus typing to identify the diversity and epidemiologic origins of the strain(s) associated with the development of ocular disease in people both regionally and worldwide. Future studies should retrospectively be applied against banked clinical material and prospectively against patient cohorts with ocular disease to address what strains pose the greatest risk for developing a severe form of toxoplasmic retinochoroiditis.

The case presented herein also showed a patient with bilateral atypical ocular toxoplasmosis as the first sign of CUP origin, which is perceived to be a very aggressive and ominous disease carrying a poor prognosis. Patients with immunodeficiency, including those with cancer or treated with immunosuppressive drugs have a higher rate of opportunistic infections. The compromised immune system of the host may have been an additional factor responsible for the atypical and severe presentation in this case, considering that the strain involved was also an atypical one.

The ability to identify parasite types, although not routinely performed, may now provide new insights into the pathogenesis of this disease and, ultimately, become an important diagnostic and prognostic tool.

Acknowledgments

The authors would like to thank Erick James and Spencer Magargal for technical assistance and for the grants from FADA-UNIFESP and CNPq (Rubens Belfort Jr.). This work was supported by the Intramural Research Program of the NIH and NIAID. MEG is a scholar of the Canadian Institute for Advanced Research (CIFAR) Program for Integrated Microbial Biodiversity.

References

1. Khan A, Jordan C, Muccioli C, et al. Genetic divergence of *Toxoplasma gondii* strains associated with ocular toxoplasmosis, Brazil. *Emerg Infect Dis.* 2006; 12(6):942–9. [PubMed: 16707050]
2. Belfort-Neto R, Nussenblatt V, Rizzo L, et al. High prevalence of unusual genotypes of *Toxoplasma gondii* infection in pork meat samples from Erechim, Southern Brazil. *An Acad Bras Cienc.* 2007; 79(1):111–4. [PubMed: 17401480]
3. de Moura L, Bahia-Oliveira LM, Wada MY, et al. Waterborne toxoplasmosis, Brazil, from field to gene. *Emerg Infect Dis.* 2006; 12(2):326–9. [PubMed: 16494765]
4. Silveira C, Belfort R Jr, Muccioli C, et al. A follow-up study of *Toxoplasma gondii* infection in southern Brazil. *Am J Ophthalmol.* 2001; 131(3):351–4. [PubMed: 11239868]

5. Holland GN. Ocular toxoplasmosis: a global reassessment. Part I: epidemiology and course of disease. *Am J Ophthalmol.* 2003; 136(6):973–88. [PubMed: 14644206]
6. Silveira C, Belfort R Jr, Muccioli C, et al. The effect of long-term intermittent trimethoprim/sulfamethoxazole treatment on recurrences of toxoplasmic retinochoroiditis. *Am J Ophthalmol.* 2002; 134(1):41–6. [PubMed: 12095806]
7. Ajzenberg D, Cogne N, Paris L, et al. Genotype of 86 *Toxoplasma gondii* isolates associated with human congenital toxoplasmosis, and correlation with clinical findings. *J Infect Dis.* 2002; 186(5): 684–9. [PubMed: 12195356]
8. Darde ML, Bouteille B, Pestre-Alexandre M. Isoenzyme analysis of 35 *Toxoplasma gondii* isolates and the biological and epidemiological implications. *J Parasitol.* 1992; 78:786–94. [PubMed: 1403418]
9. Howe DK, Honore S, Derouin F, et al. Determination of genotypes of *Toxoplasma gondii* strains isolated from patients with toxoplasmosis. *J Clin Microbiol.* 1997; 35(6):1411–4. [PubMed: 9163454]
10. Howe DK, Sibley LD. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. *J Infect Dis.* 1995; 172(6):1561–6. [PubMed: 7594717]
11. Vallochi AL, Muccioli C, Martins MC, et al. The genotype of *Toxoplasma gondii* strains causing ocular toxoplasmosis in humans in Brazil. *Am J Ophthalmol.* 2005; 139(2):350–1. [PubMed: 15734002]
12. Holland GN. Ocular toxoplasmosis: a global reassessment. Part II: disease manifestations and management. *Am J Ophthalmol.* 2004; 137(1):1–17. [PubMed: 14700638]
13. Moshfeghi DM, Dodds EM, Couto CA, et al. Diagnostic approaches to severe, atypical toxoplasmosis mimicking acute retinal necrosis. *Ophthalmology.* 2004; 111(4):716–25. [PubMed: 15051204]
14. Grigg ME, Boothroyd JC. Rapid identification of virulent type I strains of the protozoan pathogen *Toxoplasma gondii* by PCR-restriction fragment length polymorphism analysis at the B1 gene. *J Clin Microbiol.* 2001; 39(1):398–400. [PubMed: 11136812]
15. Grigg ME, Ganatra J, Boothroyd JC, et al. Unusual abundance of atypical strains associated with human ocular toxoplasmosis. *J Infect Dis.* 2001; 184(5):633–9. [PubMed: 11474426]
16. Miller MA, Grigg ME, Kreuder C, et al. An unusual genotype of *Toxoplasma gondii* is common in California sea otters (*Enhydra lutris nereis*) and is a cause of mortality. *Int J Parasitol.* 2004; 34(3): 275–84. [PubMed: 15003489]
17. Sibley LD, Boothroyd JC. Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage. *Nature.* 1992; 359(6390):82–5. [PubMed: 1355855]
18. Kong JT, Grigg ME, Uyetake L, Parmley S, Boothroyd JC. Serotyping of *Toxoplasma gondii* infections in humans using synthetic peptides. *J Infect Dis.* 2003; 187:1484–95. [PubMed: 12717631]
19. Pena HF, Gennari SM, Dubey JP, et al. Population structure and mouse-virulence of *Toxoplasma gondii* in Brazil. *Int J Parasitol.* 2008; 38(5):561–9. [PubMed: 17963770]

Figure 1.

Slit-lamp biomicroscopy of the anterior segment. A and B - Right eye showing granulomatous keratic precipitates, 2+ inflammatory cells in the aqueous humor and pigment on the anterior lens capsule; C and D – Left eye showing corneal edema, severe anterior uveitis with fibrin in the anterior chamber and posterior synechiae.

Figure 2.

Funduscopy examination with extensive necrotizing retinochoroiditis associated with vitreous inflammatory reactions and marked periphlebitis, periarteritis and absence of ophthalmoscopically visible chorioretinal scarring. Fluorescein angiography with progressive hyperfluorescence in late phases secondary to vascular leakage (inset).

Figure 3.

Toxoplasma gondii tissue cysts from formalin fixed, paraffin-embedded eye tissue stained with hematoxylin and eosin (H&E). Protozoal tissue cysts are indicated by arrows. Within the cyst are hundreds of zoites, characterized by an outer rim of pale basophilic cytoplasm and a central round, deeply basophilic nucleus.

Figure 4. PCR detection and DNA sequence analysis at the NTS2, GRA7, SAG1 and SAG3 loci. A). Parasite DNA amplified using nested primers against designated typing loci was separated by electrophoresis in a 1% agarose gel and visualized using GelRed. B). Polymorphic sites that exist between archetypal Type I, II and III lineages were compared with DNA sequences obtained from PCR amplified parasite DNA from two separate, formalin-fixed sections of eye tissue. The consensus sequence was defined as the nucleotide common to at least two of the three archetypes. Periods (·) indicate identity with the consensus sequence. The numerical positions annotated refer to the numbered sites in the published GenBank sequences Y13863 for *GRA7* and X14080 for *SAG1*