JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2011, p. 1513–1517 Vol. 49, No. 4
0095-1137/11/$12.00 doi:10.1128/JCM.02196-10

Direct Genotyping of *Toxoplasma gondii* in Ocular Fluid Samples from 20 Patients with Ocular Toxoplasmosis: Predominance of Type II in France[▽]

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Received 12 November 2010/Accepted 7 January 2011

We report the direct genotyping analysis of *Toxoplasma gondii* in ocular samples collected from 20 patients, as well as associated clinical and epidemiological data. This work was aimed at better understanding the impact of genotypes of *Toxoplasma gondii* strains on toxoplasmic retinochoroiditis. For this purpose, we studied the aqueous humor (AH) or vitreous humor (VH) of 20 patients presenting with ocular toxoplasmosis (OT) in 2 hospitals in France. Genetic characterization was obtained with microsatellite markers in a multiplex PCR assay. In contrast to the results of previous studies, we found no association between atypical *Toxoplasma gondii* genotypes and the occurrence of OT. Considering the local epidemiological data, our OT patients seemed to be infected more frequently by ordinary type II strains found in the environment. In conclusion, direct genotyping of *Toxoplasma gondii* strains from aqueous or vitreous humor showed a predominance of the type II genotype in ocular toxoplasmosis; this may be due to a high exposure rate of this genotype in humans.

Ocular toxoplasmosis (OT) is the main cause of posterior uveitis worldwide. OT is a consequence of infection by the protozoan parasite *Toxoplasma gondii*, which can occur in virtually all warm-blooded animals.

Different factors, such as exposure rate, geographic location, host genetic background or immune status, and time of infection (congenital versus postnatal contamination), may explain variations in the occurrence, clinical presentation, relapse rate, or severity of ocular lesions. However, knowledge of the roles of these factors would benefit from further study.

For example, beyond the high prevalences of eye disease observed in southern Brazil (16, 19, 29), Colombia (7), and Africa (15), little is known about the effect of geography on OT in humans. Additionally, the high frequencies of OT in these areas, together with variations in clinical presentation, severity of lesions, and frequency of recurrence, could be due to many factors, including the genetic makeup of the parasite. Thus, genetic variability among isolates involved in the disease must also be assessed. Although the population structure of *T. gondii* in Europe is well defined, with >95% of strains fitting into one of three major clonal lineages (types I, II, and III), a different pattern of *T. gondii* strains has been found in other regions (1, 21). Using strains isolated mainly from Brazil and the Guianas, several recent publications reported a higher-than-expected genetic variability in these areas (1, 8, 23). These atypical South American strains, initially called "exotic," belong to several haplogroups which are endemic to South America (22, 24).

Moreover, variations in virulence between the different strains have been well established for mice, in which type I is particularly virulent. This concept is less clearly demonstrated for humans, although circumstantial evidence suggests that certain genetic types of *T. gondii* may be associated with clinical toxoplasmosis. Nevertheless, several reports have demonstrated an association of atypical strains with severe forms of the disease, including pulmonary or multivisceral toxoplasmosis in immunocompetent patients (6, 8, 9) and disseminated congenital toxoplasmosis (2). Among patients with AIDS, the *T. gondii* strain genotype is strongly associated with geographical origin (18).

The influence of genotypes on ocular toxoplasmosis has been studied mainly in patients from Brazil (23, 31), the United States (17), and Poland (30). In spite of differences in the number and design of genetic markers and in the origin of samples (ocular fluids or tissues versus peripheral blood), these studies found mainly a clear predominance of type I and atypical genotypes in ocular disease, regardless of the geographical location of patients. While these results were not surprising for areas such as Brazil, where many different atypical genotypes have been described, they were highly unexpected for North America, where type II predominates (21), and for Europe, where type II strains are considered to be largely predominant in animals and humans, regardless of the clinical presentation. For example, type II strains are involved in >96% of consecutive cases of congenital toxoplasmosis in France (2), in 85% of immunocompromised patients who acquired *Toxoplasma*

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▽ Published ahead of print on 19 January 2011.

infection in Europe (4), and in up to 100% of animals from several European countries (from 73% to 100%) (10, 13, 18). Furthermore, serotyping analysis confirmed the predominance of type II serotypes not only in chronically infected persons but also in patients with ocular disease in Europe (26, 28).

The aim of the present study was to determine the distribution of genotypes in retrospective cases of ocular toxoplasmosis in France.

(Some of the data in this study were presented at Toxo100: Toxoplasma Centennial Congress, September 2008, Buzios, Brazil.)

MATERIALS AND METHODS

Patients. Patient data are presented in Table 1. We studied 17 aqueous humor (AH) and 3 vitreous humor (VH) samples from 20 patients who presented with OT at the Departments of Ophthalmology in two French hospitals (Pitié-Salpêtrière Hospital, Paris, and Centre Hospitalier Universitaire Dupuytren, Limoges) between 2003 and 2008. Patients included 7 males and 13 females; the mean age was 53.3 years. The patients presented different kinds of ocular lesions suggestive of atypical *Toxoplasma gondii* retinochoroiditis. To establish the diagnosis and rule out other lesion etiologies, anterior chamber paracentesis and (in three cases) vitrectomy were performed. Sera were collected simultaneously. Among the 20 patients, 12 were considered immunocompetent and 8 were immunocompromised. Among the latter, there were three cases of HIV, two cases of lymphoma, one case of Waldenström disease, one case of bone marrow transplantation, and one case of Good's syndrome (thymoma associated with hypogammaglobulinemia).

For all patients, any history of congenital infection was unknown.

Laboratory tests. Biological diagnoses were established by detecting *Toxoplasma gondii* DNA and, in some cases, by determining the production of a local specific antibody in AH.

Diagnostic real-time PCR using TaqMan technology (Applied Biosystems) was performed with AH as previously described (14). The targets of the PCR assay were a portion of the repetitive B1 gene and a 529-bp repeat element (REP) that exists at 200 to 300 copies/genome (20, 25). The primers were 5′-AGAGACACCGGAATGCGATCT-3′ (sense) and 5′-TTCGTCCAAGCCT CCGACT-3′ (antisense) for the B1 gene and 5′-GAAAGCCATGAGGCACT CCA-3′ (sense) and 5′-TTCACCCGGACCGTTTAGC-3′ (antisense) for the REP sequence. The hybridization probes were 5′-TCGTGGTGATGGCGGAG AGAATTGA-3′ for the B1 gene and 5′-CGGGCGAGTAGCACCTGAGGAG ATACA-3′ for the REP sequence. Parasite DNA was extracted from 10 μl of AH by use of a QIAmp DNA blood minikit (Qiagen). A decontamination step with uracil DNA-glycosylase was used to prevent carryover contamination. For ocular samples, measurement of IgG was achieved by enzyme immunoassay (EIA). Levels of total IgG in sera and in ocular samples were determined by using an immunodiffusion method (NOR-Partigen IgG-HC and LC-Partigen IgG for serum and aqueous humor, respectively; Dade-Behring). The Goldmann-Witmer coefficient (GWC) was calculated as previously described, and immunoblotting analysis (Western blotting) was performed according to a well-established protocol (14).

The serum of patient 11 was positive for IgM antibody determination, but a negative immunosorbent agglutination assay (ISAgA A) result and calculation of an avidity index were both in agreement for diagnosing a past infection (>4 months). Other patients had serological evidence of chronic infection.

Genotyping analysis. Genetic analysis of *Toxoplasma* DNA extracts was performed using a multiplex PCR for 5 microsatellite markers as described elsewhere (3). Briefly, five pairs of primers were used for a multiplex PCR assay. In each pair, one primer was 5′ end labeled with fluorescein to allow sizing of PCR products with an automatic sequencer. After amplification, the length polymorphism of microsatellite regions was assessed with an automatic sequencer.

RESULTS

Genotyping analysis. Genotyping results for the 20 ocular fluids are reported in Table 2. The genotype of *T. gondii* DNA is listed as not determined (ND) for samples supported by fewer than 3 successfully amplified markers, as genetic analysis based solely on 1 or 2 markers may lead to a misleading genotype designation because of insufficient genetic data. There were 7 samples with incomplete genotyping. Among the 13 complete genotyping results, we observed the type II genotype for 10 samples, the Africa 1 genotype for 2 samples, and a mixed type II/III genotype for 1 sample.

Correlation between genotype and toxoplasmosis. Among the six genotypes successfully identified in immunocompromised patients, all were type II, whereas among the seven successfully identified genotypes in immunocompetent patients, three were different from type II.

Symptom onsets were not related to any particular genotype. Also, recurrent disease was observed three times, twice with type II. The genotype of the third strain was incomplete and comprised a type II allele.

Five patients presented with retinal necrosis, of whom three were immunocompromised (2 with HIV infection and one with Good's syndrome) and two were immunocompetent. Interestingly, complete genotypes (type II) were obtained for two of the immunocompromised patients with retinal necrosis. Incomplete genotypes were found for the three other patients with retinal necrosis. For the latter patients, evidence of a type II allele was found in one immunocompetent and one HIV-infected patient, and evidence of a type III allele was found in the remaining immunocompetent patient.

DISCUSSION

For the present study, we performed direct genotyping of *Toxoplasma gondii* strains from AH or VH samples from 20 patients presenting with OT. Genotyping was incomplete for seven patients. Considering only the 13 complete genotypes, type II isolates represented 77% ($n = 10$) of cases and non-type II isolates represented 23% ($n = 3$) of cases. This predominance of type II strains is in agreement with French data on other clinical forms of human toxoplasmosis or environmental samples (13). Therefore, it is coherent to detect more type II strains, be they in environmental specimens or in human samples such as AH.

In our study, recurrent disease was observed three times, twice with type II strains. However, considering the predominance of type II strains in France, we cannot state that there is a relationship.

The origins of the included patients were very diverse. Among the patients born overseas, most were from Africa ($n = 6$). Some of the patients had been living in France for years, while others lived in their native country but traveled regularly in France. Since all of the patients had evidence of chronic infection, it was largely impossible for us to determine the areas where the patients became infected and, consequently, the origins of the toxoplasmic strains. Even if we assumed that the patients were infected in their native countries, we could not determine if the infecting strain was common or scarce, as the genotypes that predominate in these countries are not well known. Among the 7 immunocompetent patients with complete genotypes, 4 were infected with type II strains and 3 were infected by atypical strains (one type II/III strain and 2 Africa 1 strains). The type II/III strain was seen in an immunocompetent French patient who traveled in China, India, and Iceland a few years ago. The Africa 1 genotype was recently reported for cases of toxoplasmosis (cerebral, pulmonary, or

TABLE 1. Epidemiological and clinical data[a]

Patient no.	Isolate	Yr of isolation	Age (yr)/sex of patient	Country of birth/travel	Immune status/medical history	Clinical findings	Onset of symptoms	Sample type
Immunocompetent patients								
1	PSP015-2005-ADO	2005	26/F	Ghana (living in France for 9 years)	IC	Unilateral granulomatous uveitis	NR	AH
2	PSP014-2005-ALL	2005	21/F	Ivory Coast	IC	Unilateral granulomatous uveitis	7 days	AH
3	PSP016-2005-BRO	2005	66/F	France/Martinique Island, Turkey	IC	Unilateral panuveitis	A few days	AH
4	PSP019-2005-DAV	2005	45/M	France/China, India, Iceland	IC	Unilateral posterior uveitis, vascularitis	5 days	AH
5	PSP023-2005-JAN	2005	62/F	France/Spain, Italy, Switzerland, England	IC	Unilateral panuveitis, vascularitis, and hyalitis lasting over 2 months	2 months	AH
6	PSP028-2005-TAA	2005	41/M	Tunisia	IC/NIDD	Unilateral posterior uveitis lasting over 2 months	2 months	AH
7	LGE024-2007-YAC	2007	29/F	Cameroon	IC	Unilateral panuveitis, hyalitis cataract	NR	AH
8	PSP069-2006-DAB	2006	39/F	NR	IC	Bilateral retinal necrosis	NR	AH
9	PSP032-2006-ROB	2006	35/M	NR	IC	Panuveitis	NR	AH
10	PSP036-2007-BOU	2007	75/M	NR	IC/diabetes mellitus over last 18 years	Unilateral posterior uveitis	NR	AH
11	PSP038-2007-DOM	2007	54/F	Portugal	IC	Retinal necrosis	NR	AH
12	PSP057-2008-DUR	2008	60/F	NR	IC	Unilateral, recurrent granulomatous panuveitis, hyalite	4 months	AH
Immunocompromised patients								
13	PSP022-2005-GUE	2005	64/M	Algeria (living in France for 43 years)	AIDS	Unilateral posterior uveitis lasting over 2 months	>2 months	AH
14	PSP056-2008-CVE	2008	45/M	NR	AIDS	Retinal necrosis	3 days	AH
15	PSP065-2008-VIO	2008	75/F	France/frequent travel in Europe, Africa, Canada, India	Good's syndrome	Retinal necrosis	8 months	VH
16	LGE005-2003-JAU	2003	65/F	France	WD	Unilateral, recurrent posterior uveitis, hyalitis	NR	VH
17	LGE017-2008-DEB	2008	70/F	France	Lymphoma (rituximab)	Bilateral, recurrent panuveitis, hyalitis	NR	AH
18	LGE028-2008-LAC	2008	86/F	France	Lymphoma (rituximab)	Unilateral posterior uveitis, hyalitis, vascularitis	NR	VH
19	PSP040-2007-KHA	2007	41/F	Algeria	AIDS (8 $CD4^+$ cells/mm^3)/HCV infection	Unilateral retinal necrosis, other eye lost (acute retinal necrosis)	NR	AH
20	LGE026-2008-HAZ	2008	67/M	NR	BMT	NR	NR	AH

[a] F, female; M, male; NR, not reported; IC, immunocompetent; WD, Waldenström disease; HCV, hepatitis C virus; BMT, bone marrow transplantation; AH, aqueous humor; NIDD, non-insulin-dependant diabetes; VH, vitreous humor; ND, not determined.

TABLE 2. Genotype allelic combinations from 20 ocular samples from patients with ocular toxoplasmosis

Patient no.	Isolate	Centre National de Référence code	Genotype (no. of positive alleles/total no. of alleles)	Allele at indicated locus[a]				
				TUB2	*W35*	*Tg-MA*	*B18*	*B17*
Immunocompetent patients								
1	PSP015-2005-ADO	TgH 29015A	II (4/5)	2.3	2.3	2	2	NA
2	PSP014-2005-ALL	TgH 29014A	II	2.3	2.3	2	2	2.3
3	PSP016-2005-BRO	TgH 29016A	II	2.3	2.3	2	2	2.3
4	PSP019-2005-DAV	TgH 29019A	Mixed II/III	2.3	2.3	3	2	2.3
5	PSP023-2005-JAN	TgH 29023A	Africa 1 (3/5)	NA	1	3	1.3	NA
6	PSP028-2005-TAA	TgH 29028A	II (4/5)	NA	2.3	2	2	2.3
7	LGE024-2007-YAC	TgH 21024A	Africa 1	1	1	3	1.3	1
8	PSP069-2006-DAB	TgH 29069A	ND (1/5)	NA	NA	2	NA	NA
9	PSP032-2006-ROB	TgH 29032A	ND (1/5)	NA	2.3	NA	NA	NA
10	PSP036-2007-BOU	TgH 29036A	ND (1/5)	2.3	NA	NA	NA	NA
11	PSP038-2007-DOM	TgH 29038A	ND (2/5)	NA	NA	3	1.3	NA
12	PSP057-2008-DUR	TgH 29057A	ND (1/5)	NA	NA	2	NA	NA
Immunocompromised patients								
13	PSP022-2005-GUE	TgH 29022A	II	2.3	2.3	2	2	2.3
14	PSP056-2008-CVE	TgH 29056A	II (4/5)	2.3	2.3	2	2	NA
15	PSP065-2008-VIO	TgH 29065A	II	2.3	2.3	2	2	2.3
16	LGE005-2003-JAU	TgH 21005A	II	2.3	2.3	2	2	2.3
17	LGE017-2008-DEB	TgH 21017B	II (4/5)	2.3	2.3	NA	2	2.3
18	LGE028-2008-LAC	TgH 21028A	II	2.3	2.3	2	2	2.3
19	PSP040-2007-KHA	TgH 29040A	ND (1/5)	NA	NA	2	NA	NA
20	LGE026-2008-HAZ	TgH 21026A	ND (2/5)	NA	NA	NA	2	2.3

[a] Allelic polymorphisms of the *TUB2*, *W35*, *TgM-A*, *B18*, and *B17* markers are expressed as numbers relative to classical typing of *T. gondii*, as follows: alleles 1, 2, and 3 are reserved for clonal lineages I, II, and III; allele 1.3 means that types I and III share the allele; allele 2.3 means that types II and III share the allele; and allele 4 and above are used for atypical alleles. NA, not amplified; ND, not determined.

disseminated) in immunocompromised patients who acquired the infection in sub-Saharan Africa (4). In the present study, the Cameroonian origin of patient 7 (who lived there until 2004) and the corresponding infection with an Africa 1 genotype strongly suggested an origin in sub-Saharan Africa. However, the second Africa 1 type was found in patient 5, who was born in France and who traveled only in Europe (Spain, Italy, Switzerland, and England) in the 1960s and 1970s. Immunocompromised patients were infected exclusively with type II strains. Vallochi et al. suggested that type I strains are responsible for ocular infections in immunocompetent patients in Brazil (31). However, they studied only one of the genes that are used to determine genetic background (*SAG2*). Moreover, there is almost no type II *T. gondii* in Brazil (11, 12). Furthermore, although OT is more frequent and severe in Erechim than in São Paulo, they did not find different genotypes between these two cities, suggesting that genotype is not reliably associated with clinical severity. For Erechim, Khan et al. used multilocus nested PCR analysis of 4 different loci combined with direct sequencing of a polymorphic intron to study strain isolates from blood in the setting of ocular toxoplasmosis (23). Their findings indicated that most Brazilian strains collected in this study were genetically distinct from the clonal lineages seen in North America or Europe.

In their North American study, Grigg et al. observed a strong bias toward type I or mixed type I/III strains in immunocompetent patients, whereas different kinds of strains were found in six immunocompromised patients (one strain with a type I allele, three type II strains, one type III strain, and one recombinant type I/III strain). Thus, clonal type II or III strains were found exclusively in immunocompromised patients (17). These interesting results from Grigg et al. suggest that immunocompromised patients are infected by common strains (e.g., type II strains, which predominate in the United States), whereas those who are immunocompetent are infected by particularly virulent strains possessing type I alleles. It should be noted that type I strains are rarely isolated in the United States. However, their study did not give any information concerning the geographical origins of the patients; the immunocompetent patients may have been native to South America or infected during travel. Furthermore, they could not be sure that the type I strains detected in their study were not recombinant or atypical strains, as they studied only a limited number of loci. However, we experienced the same difficulties, and our work presents the same limits.

More recently, Switaj et al., using direct genotyping from peripheral blood, found exclusively type I strains in patients with OT (30) in Poland, where type II has been found to predominate in cases of congenital toxoplasmosis (27). However, their results will need further confirmation, notably to rule out the possibility of cross-contamination.

With the methodological issues discussed above, these different studies suggest that strains containing a type I allele may be more capable of inducing OT, possibly due to a genetic background that enhances the virulence of genotype I strains. In our series of 20 patients from whom 13 complete genotypes were established, only two strains comprised a type I allele, and they were both characterized by a mixture of type I and III alleles, previously described as the Africa 1 (I/III) genotype. A third strain, isolated from immunocompetent patient 11, possessed type I and/or type III alleles, but unfortunately this genotype was incomplete.

Among the 7 incomplete genotypes, 4 had one allele that belonged exclusively to type II, and 2 had alleles that belonged

either to type II or to type III, suggesting that a majority of the isolates with incomplete genotyping were type II strains. However, due to an insufficient number of allelic amplifications, we cannot make definitive conclusions.

Our results show some similarities with those published by Grigg et al., who found type II strains exclusively in immunocompromised patients (17). We found only type II strains in immunocompromised patients. We found non-type II isolates only in immunocompetent patients. The last result might seem to indicate an increased virulence of non-type II strains, but in contrast to Grigg et al., we found that immunocompetent patients could be infected by type II strains as well.

Recently, Bottos et al. reported bilateral OT-associating posterior uveitis and extensive necrotizing retinochoroiditis in an immunocompromised Brazilian male (5). Multilocus genotyping analysis established that the strain was characterized by a mixture of type I, II, or III alleles and an atypical allele. It was suggested that the severity of the lesions might be related to both the immunodepression and the atypical strain. However, we observed severe toxoplasmosis with retinal necrosis in both immunocompromised ($n = 3$) and immunocompetent ($n = 2$) patients. In these cases, we obtained two complete genotypes (type II) (one HIV-infected patient and one patient with Good's syndrome) and two incomplete genotypes that showed the presence of a type II allele (one immunocompetent and one HIV-infected patient). In the fifth case, the immunocompetent patient was infected with a strain that contained a type III allele. These results are different from those previously reported and suggest that severe ocular toxoplasmosis may not be related exclusively to strains that contain type I or atypical alleles.

In conclusion, we found no evident relationship between the genotype of *Toxoplasma gondii* strains and the occurrence of OT in France. Moreover, our results show that type II strains can be responsible for OT in both immunocompromised and immunocompetent patients, suggesting that the genotype is not the only factor affecting OT. Consequently, our study does not support previous studies arguing that type I alleles or atypical strains are involved more frequently in the occurrence and level of severity of OT. Local epidemiological and host factors must be subjected to more in-depth testing to determine if a particular genotype is involved more frequently in OT. It is thus becoming evident that to better understand OT, the scientific community must focus its efforts on collecting and assessing data not only on the parasite but also on its host.

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