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Original article

Serotyping of *Toxoplasma gondii*: striking homogeneous pattern between symptomatic and asymptomatic infections within Europe and South America

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Abstract

Field isolates of *Toxoplasma gondii* in Europe and North America have been grouped into three clonal lineages that display different virulence in mice. Whether the genetic structure of the parasite is related to clinical expression in humans has not yet been demonstrated. We developed an enzyme-linked immunosorbent assay which uses lineage-specific, polymorphic polypeptides derived from the dense granule antigens, GRA5 and GRA6. Our goal was to compare serotypical patterns observed in asymptomatic versus symptomatic (ocular disease and severe infection in human immunodeficiency virus (HIV)-positive patients) infections among patients from Europe and South America. Independent of the clinical presentation of the disease, serotypes differed according to geographical origin, with a homogeneous distribution of serotype II in Europe and of serotypes I and III in South America. We conclude that GRA5–GRA6 serotyping is an interesting tool to study serotype prevalence in populations but it is not an accurate marker of pathogenicity of *Toxoplasma* infection in humans.

Keywords: *Toxoplasma gondii* isolates; Serotyping; Type I, II, III; GRA5; GRA6

1. Introduction

Toxoplasma gondii is a ubiquitous intracellular protozoan parasite that infects all warm blooded organisms. In humans, the infection induces a wide range of clinical manifestations according to the immune status of the patient and the clinical setting. In immunocompetent children and adults, acquired infection is usually asymptomatic [1]. In some cases, chorioretinitis and even life-threatening visceral effects can be observed. Interestingly, such severe infections in immunocompetent patients seem to be more frequent in South America [2]. Antenatally infected newborns usually have a normal appearance, although severe neurological abnormalities may be observed [3]. Evolution of congenitally acquired toxoplasmosis

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is unpredictable and is marked by the risk of late-onset retinal lesion and relapses that can occur many years after birth [4]. In immunocompromised patients, toxoplasmosis, which is mainly due to reactivation of chronic infection, induces potentially lethal effects in multiple organs. Although severity of the disease is known to be linked to the immune status or age at maternal infection during pregnancy [1], factors that influence the severity of infection in humans are poorly known.

Genetic analyses have initially shown that the majority of strains isolated mainly in Europe and North America can be classified into three clonal lineages that exhibit different pathogenicity in mice [5,6]. Type I is reported to be highly virulent whereas both types II and III are relatively less virulent [6]. In humans, this correlation appears to be less clear. However, parasite genotyping is limited by the fact that, except in severe cases or for pregnant women with a positive amniocentesis, infective isolates cannot usually be obtained. Serological typing, using synthetic peptides derived from SAG2A, GRA3, GRA- and GRA7 toxoplasmic antigens, has been reported to predict accurately the strain responsible for infection in mice [7]. Such a test allows the investigation of an unlimited number of samples from human infections of various degrees of severity.

We previously investigated the serotype of strains that induce chronic sub-clinical infections in pregnant women in order to determine which serotype of strain is responsible for the majority of human infections that give rise to no patent disease. Our results using polymorphic polypeptides specific for the three clonal lineages and derived from two dense granule antigens (GRA5 and GRA6) clearly showed a homogeneous distribution of serotype II in European samples and of serotypes I and III in the Colombian population [8]. In this study, we investigated strain serotype in acutely ill patients from South America (Colombia and Brazil) and Europe (France and Switzerland) and compared the serological patterns with those obtained in asymptomatic patients. The aim of the study was to determine whether there is a relationship between clinical expression of the disease and strain serotype.

2. Materials and methods

2.1. Recombinant antigens

Type I, II and III variants of both the N-terminal hydrophilic portion of GRA5 [9] and the C-terminal hydrophilic region of GRA6 [10] were expressed as polypeptides fused to gluthathione *S*-transferase (GST) using the pGEX-3X vector 1. Plasmid constructions as well as production and purification of fusion proteins were reported previously [8].

2.2. Serum samples

2.2.1. Asymptomatic infections

A cohort of 269 sera from chronically infected pregnant women was constituted, including 212 from Europe (93 women from Lyon (France), 60 from Pavia (Italy), 59 from Aarhus (Denmark)) and 57 from South America (40 from Armenia (Colombia) and 17 from French Guyana). Eighteen adults from Colombia who had asymptomatic infections were also included in this cohort, leading to a total number of 287 sera (Table 1). The European cohort and the 40 sera from Colombia were already used in the previous study [8].

2.2.2. Symptomatic infections

A cohort of 302 sera from symptomatic infections (Table 1) comprised 236 sera from immunocompetent individuals, including: (1) 223 sera from individuals who had active toxoplasmic chorioretinitis (123 from South America: 57 from Brazil and 66 from Columbia) and 100 from Europe (Switzerland); and (2) 13 sera from immunocompetent patients from French Guyana who had been hospitalized for severe primary toxoplasmic infection, with clinical manifestations that consisted mainly of pulmonary involvement and altered general status. Such an unusual severe primary infection has already been reported and seems to be related to atypical strains of *T. gondii* [11]. Sixty six human immunodeficiency virus (HIV)-infected patients from Paris (France) who suffered from acute infection with visceral

Table 1
Geographical origin and clinical status of patients included in the study

Asymptomatic infections		Symptomatic infections	
Number of patients	Country	Number of patients	Country
Pregnant women		**HIV-negative chorioretinitis**	
Europe		*Europe*	
93	France	100	Switzerland
60	Italy		
59	Denmark		
South America		*South America*	
40	Colombia	66	Colombia
17	French Guyana	57	Brazil
Non- pregnant women		**HIV-negative severe toxoplasmosis**	
South America		*South America*	
18	Colombia	13	French Guyanna
		HIV-positive severe toxoplasmosis	
		Europe	
		66	France

involvement, encephalitis or pulmonary manifestations were also included in the cohort.

2.3. Enzyme-linked immunosorbent assay (ELISA) using recombinant antigens

An ELISA test was set up for each type of antigen as described previously [8]. Briefly, flat-bottomed microtiter plates were coated with the optimal concentration of recombinant antigen and matched quantities of GST. Each test was run in triplicate. The final optical density (OD) for each serum was expressed as the result of the subtraction of the mean OD obtained with control GST from the mean OD obtained with recombinant GRA5 or recombinant GRA6.

2.4. Statistical analysis

Statistical analysis and graphs were performed under the R program environment (Ishaka J Comp Graph Stat 1996, R Development Coreteam 2005). In order to make an effective qualitative analysis, a mosaic plot was used to describe the serotyping data. The mosaic plot is a graphical representation of a contingency table, and the surface of each rectangle is proportional to the number of individuals. Under the null hypothesis of independence, all the rectangles are white (Fig. 1). The color code in the legend represents the standardized residuals and shows whether the number of observed subjects is greater than that theoretically expected (blue and shaded blue) or smaller (red and shaded red). It contributes to the rejection of independence for Fisher's chi-squared (χ^2) test.

To increase the exactitude, the Monte Carlo test was used: the observed χ^2 value was compared with its distribution estimated from 20 000 replications of random uniform sampling within contingency tables with the same column and row margins.

In order to allow the reader to reproduce the results and to change the cut-off, all data sets are available at http//pbil.univ-lyon1;fr/members/lobry/repro/toxo2. For this study, we used the same specificity thresholds as those determined in a previous paper (i.e. high threshold values for GRA6 I and GRA6 III) [8].

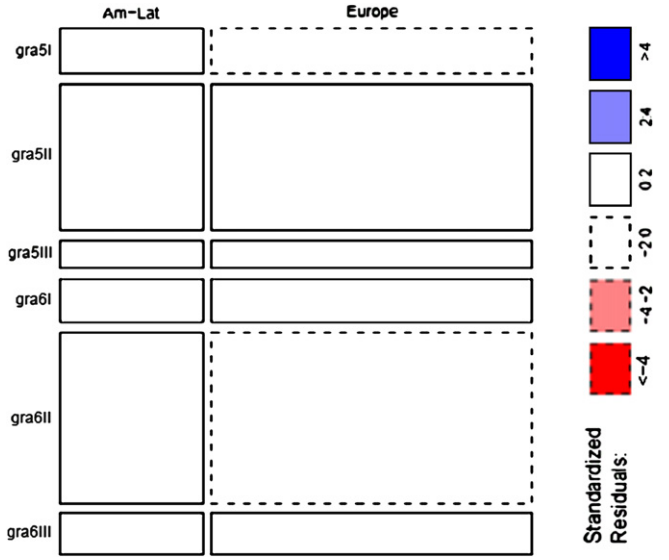

Fig. 1. Expected mosaic plot representation of serotyping results were independent of the origin of the sera. Under the null hypothesis of independence, all the rectangles are white. The size of each rectangle is proportional to the number of subjects. A rectangle with a solid border indicates that the value is higher than that expected under the null hypothesis of independence between serotyping results and the origin of sera. Conversely, a rectangle with a dashed border indicates that the number of patients is smaller than that expected. The color code is used to outline the most salient deviations. It shows whether the number of theoretical subjects is greater than that observed (blue or shaded blue) or smaller (red or shaded red). It contributes to the rejection of independence for Fisher's χ^2 test.

3. Results

3.1. Serological profiles within Europe

Fig. 2 represents a mosaic plot of the serological results from the European population: 212 chronic, asymptomatic cases (from Denmark, France and Italy) and 166 patients (from France and Switzerland) who developed typical symptoms of toxoplasmosis. The tendencies for each serotype (which is described by white rectangles) were identical in both groups, with a clear over-representation of type II strains, as determined by GRA5 and GRA6 serotyping.

The non-significant p-value ($p = 0.46$) of the Monte Carlo test used on the corresponding contingency table confirmed this result. The two groups of acute infection (immunocompetent Swiss patients and immunocompromised French patients) displayed the same homogeneous GRA5–GRA6 serotype II pattern ($p = 0.32$, data not shown).

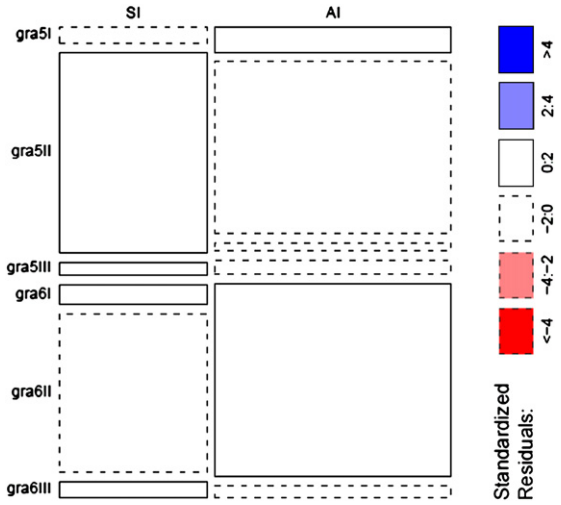

Fig. 2. Serotyping results among European patients with symptomatic (SI) and asymptomatic (AI) infections (OD > specificity thresholds). Mosaic plot representation of serotyping results for acute (166 cases) and chronic (212 cases) infections in European patients. The white color of all the rectangles indicates a homogeneous distribution of type II serotypes (see the size of GRA5 and GRA6 type II rectangles).

3.2. Serological profiles within South America

The same test described in Section 3.1 was carried out with the 211 South American sera. New samples from asymptomatic infections (17 from Guyanese pregnant women and 18 from non-pregnant Colombian individuals) were added to the 40 samples from chronically infected pregnant Colombian women previously used [8], leading to a total number of 75 sera from chronic South American cases. This group was compared with 136 sera from immunocompetent Brazilian, Colombian and Guyanese patients with severe infections. The mosaic plot showed homogeneous patterns between acute and chronic infections (Fig. 3), with an over-representation of GRA5–GRA6 serotypes I and III and the simulated χ^2 test was not significant ($p = 0.49$). Although the Guyanese patients displayed a higher OD (data not shown), they fitted within this serological pattern.

3.3. Distribution of serotypes between Europe and South America

Fig. 4 shows the GRA5–GRA6 serotyping data for the pooled populations with respect to their geographical origin (Europe versus South America) regardless of their clinical status. The color code used in this comparison allowed us to demonstrate the over-representation of serotypes I and III and under-representation of type II strains in South America, with GRA5 I, GRA5 III, GRA6 I and GRA6 III appearing in blue while both GRA5 II appeared in red and GRA6 II in shaded red. In contrast, among European samples, a converse color pattern was observed, with GRA5 I, GRA5 III, GRA6 I and GRA6 III appearing in shaded red while GRA5 II appeared in blue and GRA6 II in shaded blue. These results demonstrated a predominant type II serotype in Europe.

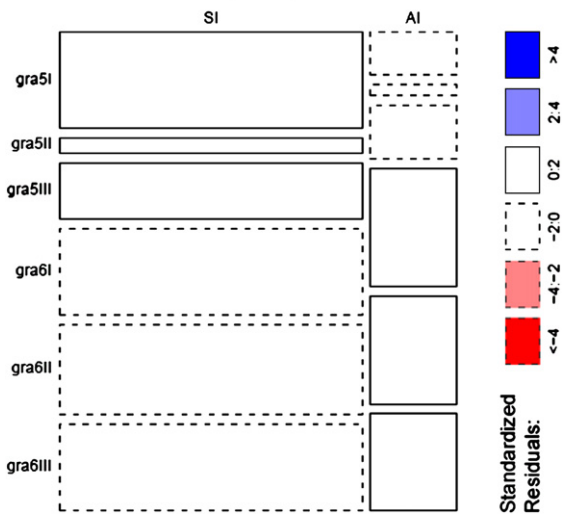

Fig. 3. Serotyping results among South American patients with symptomatic (SI) and asymptomatic (AI) infections (OD > specificity thresholds). Mosaic plot representation of serotyping results for acute (136 cases) and chronic (75 cases) infections in South American patients. The white color of all the rectangles indicates a homogeneous distribution of type I and III serotypes (see the size of GRA5 and GRA6 type I and III rectangles).

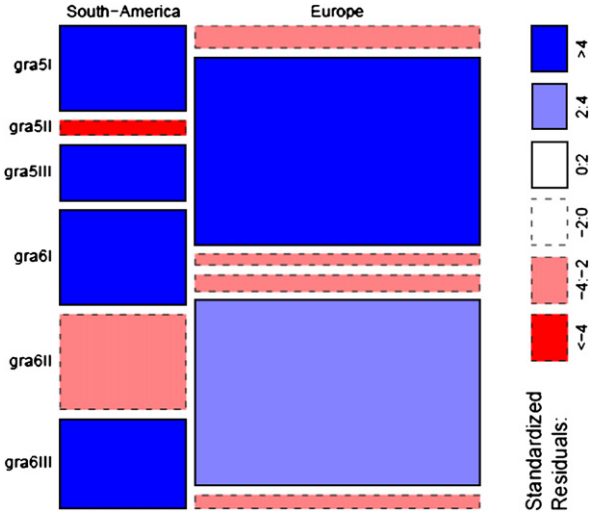

Fig. 4. Global serotyping results from South America and Europe (OD > specificity thresholds). Mosaic plot representation of serotyping results as a function of geographical origin without considering clinical status. The color code shows a clear separation between South America and Europe with an over-representation of type I and III and type II, respectively.

Our previous results had demonstrated the predominance of serotype II in European and of serotypes I and III in South American chronically, asymptomatic pregnant women [8]. Our current results highly support the notion that the geographical origin of the patients and not their clinical status has a major impact on serotype. Indeed, when focusing on the population of symptomatic patients from both Europe and South America, the same pattern as in the whole population was observed, with significant over-representation of GRA5–GRA6 serotypes I and III in patients from South America and of type II in European patients (Fig. 5).

4. Discussion

The pathogenicity of *T. gondii* infection in humans remains poorly understood. Although it is usually asymptomatic, the disease can be severe and even fatal. In mice, it has been reported that the three archetypal lineages correlate with parasitic virulence [12–14]. In addition, the geographical origin of isolates appears to be related to strain genotype [15]. In this study, we investigated the relationship between strain serotype and different clinical patterns of toxoplasmic infection in patients from South America and Europe. Using the mosaic plot presentation and the Monte Carlo test, we demonstrated that serotype II is over-represented in patent and asymptomatic infections in Europe (Fig. 2) and that serotypes I and III predominate in South America (Fig. 3), independently of clinical status. We demonstrated that geographical origin strongly correlates with the strain serotype (Fig. 4). Even among patients with acute infection and different immunological status, geographical origin remains strongly linked to serotype (Fig. 5). In Europe, immunocompetent patients with ocular disease

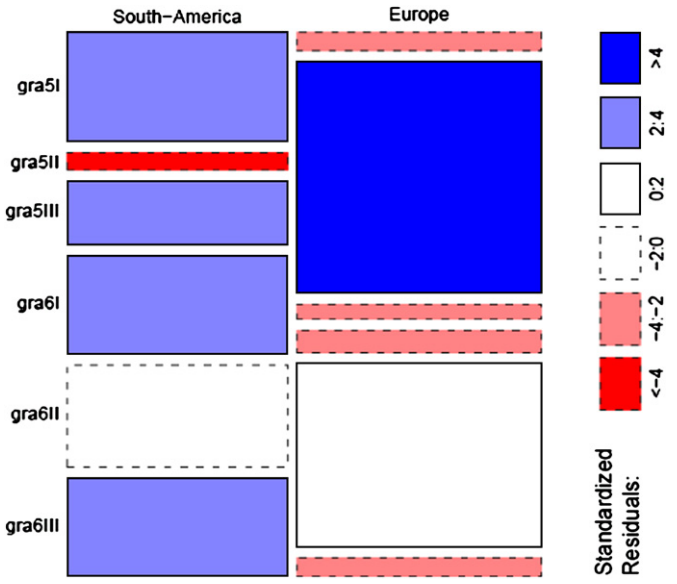

Fig. 5. Serotyping results as function of geographical origin in acute infections (OD > specificity thresholds). Only serotyping data from acute infections with respect to their geographical origin are represented. The color code and rectangle size clearly show over-representation of type I and III in South America and a predominance of type II in Europe, which confirms the results observed in Fig. 4.

and HIV patients with life-threatening disease displayed the same serotype II pattern.

Serological typing of *T. gondii* isolates, unlike genotyping, is performed using a non-invasive sampling method and allows an unrestricted number of patients to be tested, especially those who are asymptomatic and are generally not typeable by polymerase chain reaction methods. We have already demonstrated the feasibility of serotyping using the type I, II and III allelic variants that are found within both the GRA5 and GRA6 amino acid sequences. GRA5 and GRA6 are members of the dense granule proteins, an antigen family that has been reported to be highly immunogenic in humans [16]. Our assay clearly discriminates type II from serotypes I and III. For these two latter serotypes, cross-reactivity was frequently observed especially with sera that scored high ODs. This could be explained by the fact that allelic variation between type I and III polypeptides was found to be weaker than that between serotypes I–III and type II [8]. The choice of antigens can be a limitation, serotyping, as well as genotyping based on 2 loci, preclude the possibility of identifying recombinant strains. With regard to geographical distribution, our data are in agreement with previous studies that reported on genotyping in animals and humans. In Europe, a majority of isolates are from genotype II [13,17], whereas in Brazil and Colombia, isolates from chickens genotyped at SAG2 locus were reported to be from genotypes I and III but not from genotype II [18,19]. Of 15 isolates from Colombian cats genotyped at five different loci, only one was found to be genotype II and 11 displayed a combined type I and III genotype [20]. The rarity of genotype II isolates in both Colombia and Brazil favors a different *Toxoplasma* population structure in South America than in Europe, as reported by Lehmann et al. [15]. A Brazilian study using restriction fragment length polymorphism at eight different loci revealed a complex distribution of the parasite population [21]. Lehman et al. [15] analyzed seven polymorphic loci in isolates from domestic chickens collected around the world and found that genotypes from Europe, Africa and North America presented small genetic differences but were notably different from populations from South America. Whether this complex genotype is related to the severity of the disease frequently observed in this continent remains to be confirmed.

We can conclude that differential serological reactivity towards GRA5 and GRA6 polymorphic peptides does not correlate with the clinical expression of disease in patients on the same continent. Although acquired toxoplasmosis seems to be more severe in Latin America than in Europe [11,22], we showed that the combined GRA5–GRA6 serotype I and III is not an accurate marker of higher pathogenicity, since it was also observed at the same frequency in asymptomatic infections. Furthermore, type II strains, reported to be avirulent in mice [5], can induce severe encephalitis [23]. In humans, severe congenital toxoplasmosis [24] or life-threatening disease in HIV-infected patients due to reactivation of a chronic infection [5] have been reported with this genotype. It should be kept in mind that while ocular lesions can occur and relapse throughout life [4], their impact on eyesight is related to their localization in the retina. Similarly, depending on their localization in the brain, calcifications can be sub-clinical or induce hydrocephalus. Therefore, even if isolates of the same *Toxoplasma* genotype were to be found in different clinical settings, it is probable that the severity of infection would be linked to host and environmental factors. Genetic host factors were reported to be major determinants of the outcome of toxoplasmosis in animal models [25–27]. In humans, factors of susceptibility such as human leukocyte antigens, have been identified [28]. It is tempting to hypothesis that Latin American populations, which are genetically different from European populations, are more susceptible to toxoplasmic infection. Yet, the city of Erechin (Brazil), where the prevalence of ocular toxoplasmosis is 17% (the highest reported in the world), was settled by European immigrants in the twentieth century [29] and its genetic background is thus still likely similar to the European genetic background.

Taken together, our results support the notion that the outcome of toxoplasmosis in humans is far from being strictly related to the genetic origin of the strains but is rather the result of a subtle, yet not fully understood, interaction between parasitic infectivity and host resistance. GRA5–GRA6 serotyping is not a good proxy for the investigation of these two traits of infection, but appears to be a useful tool for population studies by enabling a larger number of subjects to be tested, especially those with sub-clinical infection.

5. Conflict of interest

None reported.

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