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Use of GRA6-Derived Synthetic Polymorphic Peptides in an Immunoenzymatic Assay To Serotype *Toxoplasma gondii* in Human Serum Samples Collected from Three Continents▽

Susana Sousa,[1,4] Daniel Ajzenberg,[4] Manuel Vilanova,[2] José Costa,[3] and Marie-Laure Dardé[4]*

Center of Parasite Immunology and Biology, INSA, Rua de S. Luis 16, 4000-509 Porto, Portugal[1]; Instituto de Ciências Biomédicas de Abel Salazar, Largo do Professor Abel Salazar 2, 4099-003, Porto, Portugal[2]; Laboratory for Veterinary Research, LNIV-INRB, I.P. Rua dos Lagidos, Lugar da Madalena, 4485-655 Vairão VCD, Portugal[3]; and Université de Limoges, EA 3174-NETEC, and National Reference Center and Biological resource Center for Toxoplasmosis, CHU Dupuytren, 2 Av. Martin Luther King, 87042 Limoges Cedex, France[4]

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Serotyping is a simple typing method that consists of an immunoenzymatic assay (enzyme-linked immunosorbent assay [ELISA]) using synthetic polymorphic peptides derived from *Toxoplasma gondii* antigens. We developed a new ELISA based on GRA6 C-terminal polymorphic peptides. Serum samples from 41 human infections due to 23 archetypal (type I, II, or III) and 18 nonarchetypal strains were selected in order to validate this approach. For 20 out of the 23 archetypal infections, there was a clear correlation between microsatellite genotype and GRA6 serotyping. All infections due to nonarchetypal strains were misclassified as archetypal strain infections. The GRA6 C-terminal peptides from these strains were analyzed to explain this misclassification. A second group of 455 patients with acute and chronic toxoplasmosis due to unknown genotypes from different European, African, and Latin American countries were included in this study, and the strain type predicted by this method. The results suggest that serotyping is a promising method for typing strains, although limitations exist for African and South American strains as a consequence of higher peptide polymorphism. Other peptides from different markers must be studied in order to discriminate archetypal from nonarchetypal strains.

Toxoplasmosis is a worldwide disease that causes particularly serious injuries in congenitally infected children and immunocompromised patients. *Toxoplasma gondii* was initially described as having a highly clonal structure. Types I, II, and III are the three archetypal lineages that predominate in Europe and North America (2, 7, 19). Nonarchetypal strains with atypical genotypes are more frequent in other geographical areas, such as Africa and South America (1, 23). There is no clear correlation between strain genotype and human disease. Without knowledge of strains infecting asymptomatic patients, it is unclear if strain distribution in human toxoplasmosis depends more on geographical origin than on clinical presentation. Congenital infections are commonly associated with type II strains in France and the United States (3, 18, 19), but they were found to be associated with atypical or type I genotypes in Colombia (16) and Brazil (15). Type I and unusual genotypes have been associated with acquired ocular toxoplasmosis (17, 21, 24, 28, 29). The role of the infecting strain seems more obvious in immunocompetent individuals infected with atypical genotypes, as shown by severe cases of toxoplasmosis, especially in remote regions of French Guiana (6) and Suriname (8).

T. gondii strains have been collected from patients with active infection. However, in order to fully understand the pathogenesis of toxoplasmosis, it will be important to know if isolates from chronic asymptomatic infections and from acute infections have the same genotype. A previously reported genotyping method based on a serological test using strain-specific peptides derived from dense granule antigens (GRA6 and GRA5) was shown to be capable of distinguishing type II from non-type II infections (22, 26, 27) and may be used to determine which strains are associated with symptomatic or asymptomatic infections.

In this work, the serological response of human serum samples to strain-specific peptides derived from GRA6 was evaluated. To validate this approach, strains belonging to the *Toxoplasma gondii* bank of strains from the Biological Resource Centre (BRC) ToxoBS group were selected and corresponding serum samples tested. In order to better understand the pathogenesis of toxoplasmosis, serum samples collected from patients with different *Toxoplasma*-associated pathologies were studied. Serum samples from different geographical origins were also included in this study to demonstrate the utility and limitations of these peptides in serotyping infections due to nonarchetypal strains.

MATERIALS AND METHODS

Human serum samples. Three distinct geographical areas were selected for this study: Europe, Africa, and Latin America. Human serum samples from Europe were collected in France and Portugal; those from Africa were collected from patients originating from Ivory Cost, Congo, Angola, Cameroon, and Gabon; and those from Latin America were collected in French Guiana, Suriname, Colombia, and Mexico.

To demonstrate the validity of serotyping as a reliable typing method, human serum samples related to 41 strains from the *Toxoplasma* bank of the BRC

* Corresponding author. Mailing address: Laboratoire de Parasitologie-Mycologie, CHRU Dupuytren, 2 Av. Martin Luther King, 87042 Limoges Cedex, France. Phone: 33 555 056 160. Fax: 33 555 056 177. E-mail: darde@unilim.fr.

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TABLE 1. Amino acid sequences for *GRA6* marker[a]

Strain	Position(s) of variable amino acids																		
	7	12	17	47	50	92	94	105	180	185	186	198	204–208	213	219	223	224	227	230
RH	H	R	V	G	K	T	E	V	Q	D	G	G	GYGGR	A	P	E	R	V	Y
Beverley	Y	.	L	.	R	.	D	A	.	G	.	.	-----	-	.	G	S	E	F
NED	.	.	L	D	.	.	D	.	P	.	.	R	.....	G	A	.	.	.	.
GUY-2003-BAS	.	.	L	D	.	A	D	.	P	.	D	.	..R..	G	A	G	S	.	.
GUY-2004-TER	.	.	L	D	.	A	D	.	P	.	D	.	..R..	G	A	G	S	.	.
GUY-2002-MAT	.	H	L	.	.	.	D	.	P	.	D	.	..R..	G	A	.	.	.	.
GUY-2002-KOE	.	.	L	.	.	.	D	.	P	.	D	.	..R..	G	A	.	.	.	.
GUY-2004-AKO	.	.	L	.	.	.	D	.	P	.	D	.	..R..	G	A	.	H	.	.

[a] RH, Beverley, and NED are, respectively, type I, II, and III reference strains. Periods (.) indicate amino acids identical to the sequence of RH strain and dashes (—) indicate deletion.

ToxoBS group were selected (see Tables 2 and 3). The strains, previously typed by the analysis of five microsatellite markers (MS) (*TUB2*, *TgM-A*, *W35*, *B17*, and *B18*) as described by Ajzenberg et al. (4), had the following genotypes: (i) 1 type I strain (lymphadenopathy following a laboratory accidental infection with RH strain); (ii) 19 type II strains, 18 from congenital infections and 1 from disseminated infection in a transplanted patient; (iii) 3 type III strains from congenital infections; (iv) 1 type I/III strain associated with a case of ocular toxoplasmosis; and (v) 6 atypical strains associated with 17 cases (12 of them caused by a single strain isolated during a *Toxoplasma* outbreak in Suriname) (8).

Human serum samples from 455 patients infected with *Toxoplasma* strains with unknown genotypes were grouped according to geographical origin and subgrouped according to *Toxoplasma*-associated pathology.

(i) The serum samples from Europe were from 211 patients of whom (a) 44 had congenital infections; (b) 3 were HIV patients of whom 2 had cerebral and 1 had pulmonary toxoplasmosis; (c) 3 had ocular toxoplasmosis; (d) 31 were immunocompromised patients with asymptomatic infections; and (e) 130 were immunocompetent patients with asymptomatic chronic infections.

(ii) The serum samples from Africa were from 89 patients of whom (a) 1 had a congenital infection; (b) 8 were immunocompromised patients with asymptomatic infections; and (c) 80 were immunocompetent patients with asymptomatic chronic infections.

(iii) The serum samples from Latin America were from 155 patients of whom (a) 1 had a congenital infection; (b) 1 had ocular toxoplasmosis; (c) 1 was an immunocompetent patient with acute toxoplasmosis; (d) 4 were immunocompetent patients with multivisceral toxoplasmosis; and (e) 148 had asymptomatic chronic infections.

To establish cutoff values, 116 *T. gondii*-negative sera were selected from the following countries: France (29 sera), Portugal (17 sera), Mexico (22 sera), Suriname (21 sera), French Guiana (5 sera), and Ivory Coast (22 sera). Different cutoff values were defined for Europe, Africa, and Latin America.

Peptides. Strain-specific synthetic peptides derived from GRA6 and a control peptide were used. These peptides were based on those described by Kong et al. (22) with the following modification: the GRA6 C-terminal region between residues 220 and 230 was repeated three times in a single synthetic peptide. Peptide 3.6II (LHPGSVNEFDFLHPGSVNEFDFLHPGSVNEFDF) is specific for GRA6 type II strains, peptide 3.6I/III (LHPERVNVFDYLHPERVNVFD YLHPERVNVFDY) is specific for GRA6 type I and type III strains, and peptide 3.ctrl (EVVHDYRLFNPEVVHDYRLFNPEVVHDYRLFNP) is the peptide control.

Enzyme-linked immunosorbent assay protocol. Immobilizer amino plates (Nunc, Denmark) were coated with each peptide diluted to 10 μg/ml or RH strain lysate diluted to 5 μg/ml in 0.05 M carbonate-bicarbonate buffer, pH 9.6, by incubation overnight at 4°C. The wells were blocked with phosphate-buffered saline (PBS)–bovine serum albumin (BSA) 3% for 1 h at 37°C in a moist atmosphere and then washed three times with PBS–Tween 0.3%. The sera were diluted to 1/50 in PBS–Tween 0.3%–BSA 3% and incubated for 2 h at 37°C in a moist atmosphere. The wells were washed three times with PBS–Tween 0.3%. Anti-human immunoglobulin G alkaline phosphatase conjugate (Pierce, United States) was diluted at 1/5,000 in PBS–BSA 3%–Tween 0.3% and incubated for 1 h at 37°C in a moist atmosphere. The wells were washed three times with PBS–Tween 0.3% and developed with *p*-nitrophenyl phosphate for 15 min at 37°C. Absorbance was measured at 415 nm.

Optical density (OD) indices were calculated by subtracting the OD of the peptide control from the OD of each peptide. Different cutoff values were calculated for the three geographical regions. Serum samples negative for *T. gondii* were used to calculate the cutoff. The cutoff was set on the mean absorbancy readings of negative sera plus 2 standard deviations.

***GRA6* sequencing.** The *GRA6* coding region from five atypical strains from French Guiana and Suriname (GenBank accession numbers EF512225 [GUY-2003-BAS]; EF512226 [GUY-2004-TER]; EF512231 [GUY-2002-MAT]; EF512233 [GUY-2002-KOE]; and EF512234 [GUY-2004-AKO]) was amplified by PCR using the following primers (14): forward primer, 5′-GTAGCGTGCTTGTTGGCGAC-3′, and reverse primer, 5′-TACAAGACATAGAGTGCCCC-3′. The PCR mix was composed by using a Qiagen PCR multiplex kit (Qiagen, Courtaboeuf, France) with 2× Qiagen multiplex PCR master mix at a final concentration of 1×, 1 μM of each primer, and 4 μl of DNA in a total volume of 25 μl. The DNA was amplified using the following conditions: one cycle of 15 min at 95°C for initial denaturation, 35 cycles of 94°C for 30 s, 65°C for 3 min, 72°C for 1 min, and a final extension step at 60°C for 30 min. Amplifications were carried out in a GeneAmp PCR system 2700 thermal cycler (Applied Biosystems, Courtaboeuf, France). The PCR products were visualized on a 2% agarose gel under UV light. The PCR products were purified by using a Concert rapid PCR purification system kit (Marligen Biosciences, Montrouge, France) and then directly sequenced in both directions by using a BigDye Terminator version 1.1 cycle sequencing kit (Applied Biosystems, Courtaboeuf, France) in a GeneAmp PCR system 2700 thermal cycler (Applied Biosystems, Courtaboeuf, France). The fragments were purified by isopropanol precipitation. The sequencing gel was run on an ABI Prism 3100 genetic analyzer (Applied Biosystems, Courtaboeuf, France), and sequences were evaluated by using Sequencing Analysis 3.7 software.

Sequence analysis. The sequences were aligned with type I (RH), type II (Beverley), and type III (NED) reference strains by using CLUSTALW software. The nucleotide translation was made with EXPASY software (ExPASy Proteomics Server).

Statistical analysis. Statistical analysis was performed using SPSS version 12.0 for Windows. The Chi-squared test was performed to assess the statistical significance of differences in the prevalence of the GRA6 serotype for different geographical regions and for different pathologies. *P* values of less than 0.05 were considered significant.

RESULTS

Sequencing. The alignment of the five atypical strains and the three reference strains showed 23 polymorphic positions at the amino acid level. Four different amino acid sequences were found for the atypical strains (Table 1). Strains GUY-2003-BAS and GUY-2004-TER shared the same sequence. Strains GUY-2002-MAT and GUY-2002-KOE shared with strains RH (type I) and NED (type III) the same polymorphisms in the C-terminal region (amino acids 220 to 230). GUY-2003-BAS and GUY-2004-TER shared two polymorphic amino acids (positions 223 and 224) with strain Beverley (type II strain) and two polymorphic amino acids (positions 227 and 230) with strains RH (type I strain) and NED (type III). GUY-2004-AKO shared with strain RH (type I) the same polymorphisms at the C-terminal region, except at position 224, where the arginine residue was replaced by a histidine.

TABLE 2. Serotyping results for samples from patients with infections due to archetypal strains (type I, II, or III)[a]

Case no.	Pathology	Geographical origin	OD index for:[b]		GRA6 serotype[c]	GRA6 C-terminal polymorphism[d]	Genotype
			GRA6 II	GRA6 I/III			
1	Congenital	France	0.054	−0.029	ND	GSEF	II
2	Congenital	France	0.269	−0.019	II	GSEF	II
3	Congenital	France	0.213	0.011	II	GSEF	II
4	Congenital	France	0.302	−0.025	II	GSEF	II
5	Congenital	France	0.071	0.023	ND	GSEF	II
6	Congenital	France	0.096	−0.011	II	GSEF	II
7	Congenital	France	0.432	−0.044	II	GSEF	II
8	Congenital	France	0.330	0.023	II	GSEF	II
9	Congenital	France	2.088	−0.004	II	GSEF	II
10	Congenital	France	0.217	−0.018	II	GSEF	II
11	Congenital	France	0.110	0.002	II	GSEF	II
12	Congenital	France	0.298	−0.032	II	GSEF	II
13	Congenital	France	0.279	−0.029	II	GSEF	II
14	Congenital	France	0.195	−0.025	II	GSEF	II
15	Congenital	France	0.183	−0.034	II	GSEF	II
16	Congenital	France	0.150	−0.007	II	GSEF	II
17	Congenital	France	0.218	0	II	GSEF	II
18	Congenital	France	0.159	−0.007	II	GSEF	II
19	Congenital	France	−0.031	0.123	I/III	ERVY	III
20	Congenital	France	0.011	0.118	I/III	ERVY	III
21	Congenital	France	0.008	0.312	I/III	ERVY	III
22	Lymphadenopathy	France	0.081	1.149	I/III	ERVY	I
23	Disseminated	France	0.005	0.020	ND	GSEF	II

[a] For congenital infections, the OD indices presented were obtained for serum samples from newborns, except for cases number 6, 8, and 10 obtained from mothers.
[b] OD indices were calculated by subtracting the OD of the peptide control from the OD of each peptide. The cutoff values for European samples were 0.088 for GRA6 II and 0.076 for GRA6 I/III.
[c] ND, not determined because the serum sample had an OD index that was below the cutoff value.
[d] The amino acid polymorphisms from the C-terminal region of GRA6 are at positions 223, 224, 227, and 230.

Validation of serotyping test. Forty-one typed infections were studied. The serotyping results were in agreement with the MS genotyping results for 20 out of 23 cases associated with strains belonging to the three main lineages (Table 2). Serotyping was successful in serum samples from mothers and newborns for cases 3, 12, and 14 to 21. Serotyping was successful only in sera from newborns for cases 2, 4, 7, 9, 11, and 13. For cases 6, 8, and 10, only sera from the mothers were serotyped. The remaining three cases presented OD indices that were under the cutoff established for both peptides (Table 2).

The serotyping results for samples from 18 patients infected by nonarchetypal strains are reported in Table 3. Nonarchetypal strains isolated from cases 40 (GUY-2002-KOE) and 41 (GUY-2002-MAT) share for the C-terminal region the same polymorphisms as type I and III strains. As a consequence, serum samples from these patients reacted as serotype I/III. The nonarchetypal strain isolated from case 39 (GUY-2004-AKO) shares for the C-terminal region the same polymorphisms as type I and III strains except for position 224 (H instead of R). Serum samples from this patient reacted as type I/III strains. Cases 26 to 37 include patients from an outbreak of toxoplasmosis in Suriname associated with the same strain (8). This strain shares with type II strains two polymorphic residues at the C-terminal region (G at position 223 and S at position 224) and with type I and III strains two polymorphic residues (V at position 227 and Y at position 230). Different responses were obtained for the 12 patients infected with this same strain: serotype II in one case, serotype I/III in two cases, reaction with both peptides in five cases, and no recognition of the peptides in four cases. These results demonstrate that serotyping based only on these two peptides is not a reliable method for typing infections due to nonarchetypal strains.

Prediction of *T. gondii* GRA6 serotype. Four hundred fifty-five patients with untyped infections were serotyped, and the results analyzed according to geographical origin and related pathology.

Geographical distribution of *T. gondii* GRA6 serotypes. The GRA6 type II profile was significantly more frequent ($P < 0.001$) in serum samples from European infections with unknown genotype, being found in 50.7% of the patients studied (76.4% if we consider only sera for which serotyping was successful) (Table 4). Interestingly, it was noted that in Portugal, the I/III profile was more common than in France ($P < 0.001$). This profile was found in 15% of the Portuguese patients and in only 2% of the patients from France. GRA6 type I/III and a mixed GRA6 profile (reaction with both peptides) were more frequent in serum samples from Africa and Latin America. In those regions, the frequency of the GRA6 type I/III profile was significantly higher than in Europe ($P < 0.001$), being found in 31.5% and 45.8%, respectively, of African and Latin American samples but in only 9.5% of the patients in Europe. The mixed GRA6 profile was obtained in 18.1% of Latin America patients, 14.6% of African patients, and 6.2% of European patients. The number of serum samples that did not recognize these peptides was more than 30% in the three continents. In Europe and Latin America, respectively, 33.6% and 31.6% of the cases were not serotyped, while in Africa this number grew to 42.7%. No relation was established between this lack of response, antibody titer, and time of infection.

TABLE 3. Serotyping results for samples from patients with infections due to nonarchetypal strains

Case no.	Strain	Pathology	Geographical origin[a]	OD index for:[b]		GRA6 serotype[c]	GRA6 C-terminal polymorphism[d]	Genotype
				GRA6 II	GRA6 I/III			
24		Ocular	Cameroon	0.067	0.029	ND		I/III
25		Asymptomatic	France	0.007	0.002	ND		Atypical
26	GUY-2004-TER	Congenital	Suriname	0.372	0.471	Mixed	GSVY	Atypical
27	GUY-2004-TER	Congenital	Suriname	0.371	0.588	Mixed	GSVY	Atypical
28	GUY-2004-TER	Multivisceral	Suriname	0.019	0.333	I/III	GSVY	Atypical
29	GUY-2004-TER	Multivisceral	Suriname	0.484	−0.022	II	GSVY	Atypical
30	GUY-2004-TER	Multivisceral	Suriname	0.052	−0.009	ND	GSVY	Atypical
31	GUY-2004-TER	Multivisceral	Suriname	0.034	0.045	ND	GSVY	Atypical
32	GUY-2004-TER	Multivisceral	Suriname	0.139	0.586	Mixed	GSVY	Atypical
33	GUY-2004-TER	Multivisceral	Suriname	−0.073	0.144	I/III	GSVY	Atypical
34	GUY-2004-TER	Multivisceral	Suriname	0.148	0.145	Mixed	GSVY	Atypical
35	GUY-2004-TER	Asymptomatic	Suriname	0.019	0.009	ND	GSVY	Atypical
36	GUY-2004-TER	Asymptomatic	Suriname	0.943	2.346	Mixed	GSVY	Atypical
37	GUY-2004-TER	Asymptomatic	Suriname	0.042	0.024	ND	GSVY	Atypical
38	GUY-2003-BAS	Multivisceral	French Guiana	0.121	0.030	II	GSVY	Atypical
39	GUY-2004-AKO	Multivisceral	French Guiana	0.054	0.838	I/III	EHVY	Atypical
40	GUY-2002-KOE	Multivisceral	French Guiana	0.022	0.476	I/III	ERVY	Atypical
41	GUY-2002-MAT	Multivisceral	French Guiana	0.076	1.241	I/III	ERVY	Atypical

[a] Cases 26 to 37 are from an outbreak of toxoplasmosis associated with the same strain (GUY-2004-TER).
[b] OD indices were calculated by subtracting the OD of the peptide control from the OD of each peptide. Cutoff values for African samples were 0.111 for GRA6 II and 0.079 for GRA6 I/III, for European samples were 0.088 for GRA6 II and 0.076 for GRA6 I/III, and for Latin American samples were 0.089 for GRA6 II and 0.067 for GRA6 I/III.
[c] ND, not determined because the serum sample had an OD index that was below the cutoff value; Mixed, serum sample reacted with both peptides.
[d] The amino acid polymorphisms from the C-terminal region of GRA6 are at positions 223, 224, 227, and 230.

Clinical aspects and GRA6 serotypes. The GRA6 type II profile was the most frequently found in serum samples from symptomatic and asymptomatic patients from Europe (Table 5). There were no marked differences in the distribution of GRA6 serotypes (II, I/III, and mixed) according to the different categories of patients (congenital versus asymptomatic and immunocompromised asymptomatic versus immunocompetent asymptomatic) in Europe ($P > 0.05$).

The reduced number of samples from symptomatic patients from Africa and Latin America did not allow us to make a reliable statistical analysis of the GRA6 serotype frequency for different pathologies. Except for one case of congenital toxoplasmosis, all serum samples from Africa belonged to asymptomatic patients. The GRA6 type I/III profile was found in 62.5% of the immunocompromised patients and in only 28.8% of the immunocompetent patients. However, a difference between the two samples (8 immunocompromised patients versus 80 immunocompetent patients) which does not allow us to do a reliable comparison of results should be noted. GRA6 type II was only found in immunocompetent asymptomatic patients (11.3%) and in the only case of congenital infection from Africa (Table 6). Almost half of the serum samples (46.3%) from immunocompetent asymptomatic patients did not recognize both peptides. For samples from Latin America, the GRA6 type I/III profile was the most frequently found for asymptomatic cases (45.9%) and for severe multivisceral cases (50%), and it was found for the only case of congenital toxoplasmosis there (Table 7). GRA6 type II was found for one case of severe multivisceral infection, for one case of ocular infection, and for five (3.4%) asymptomatic patients.

TABLE 4. Geographical distribution of *T. gondii* GRA6 serotypes

Geographical origin (no. of samples)	% (no.) of samples with indicated GRA6 serotype(s)			
	II	I/III	Mixed[a]	ND[b]
Europe (211)	50.7 (107)	9.5 (20)	6.2 (13)	33.6 (71)
Africa (89)	11.2 (10)	31.5 (28)	14.6 (13)	42.7 (38)
Latin America (155)	4.5 (7)	45.8 (71)	18.1 (28)	31.6 (49)

[a] Mixed, serum sample reacted with both peptides.
[b] ND, not determined because serum samples had OD indices that were below the cutoff values. Cutoff values for European samples were 0.088 for GRA6 II and 0.076 for GRA6 I/III, for African samples were 0.111 for GRA6 II and 0.079 for GRA6 I/III, and for Latin American samples were 0.089 for GRA6 II and 0.067 for GRA6 I/III.

DISCUSSION

Peptides were selected from the C-terminal region of the GRA6 antigen. This region comprises the sequence between amino acid positions 220 and 230, where four polymorphic amino acids are present in the archetypal strains at positions

TABLE 5. *T. gondii* GRA6 serotypes and association with clinical aspects in Europe

Pathology (no. of samples)	% (no.) of samples with indicated GRA6 serotype(s)			
	II	I/III	Mixed[b]	ND[c]
Congenital (44)	65.9 (29)	11.4 (5)	0 (0)	22.7 (10)
Other[a] (6)	66.7 (4)	0 (0)	0 (0)	33.3 (2)
Immunocompromised asymptomatic (31)	58.1 (18)	6.5 (2)	9.7 (3)	25.8 (8)
Immunocompetent asymptomatic (130)	43.1 (56)	10 (13)	7.7 (10)	39.2 (51)

[a] Two cases of cerebral and one of pulmonary toxoplasmosis in HIV patients and three cases of ocular toxoplasmosis.
[b] Mixed, serum sample reacted with both peptides.
[c] ND, not determined because serum samples had OD indices that were below the cutoff values. Cutoff values were 0.088 for GRA6 II and 0.076 for GRA6 I/III.

TABLE 6. *T. gondii* GRA6 serotypes and association with clinical aspects in Africa

Pathology (no. of samples)	% (no.) of samples with indicated GRA6 serotype(s)			
	II	I/III	Mixed[a]	ND[b]
Congenital (1)	100 (1)	0 (0)	0 (0)	0 (0)
Immunocompromised asymptomatic (8)	0 (0)	62.5 (5)	25 (2)	12.5 (1)
Immunocompetent asymptomatic (80)	11.3 (9)	28.8 (23)	13.8 (11)	46.3 (37)

[a] Mixed, serum samples reacted with both peptides.
[b] ND, not determined because serum samples had OD indices that were below the cutoff values. Cutoff values were 0.111 for GRA6 II and 0.079 for GRA6 I/III.

223, 224, 227, and 230. Peptide GRA6 type II has polymorphisms characteristic of type II strains (GSEF). Peptide GRA6 type I/III has specific polymorphisms (ERVY) of type I and III strains, since these strains share the same polymorphisms at the GRA6 C-terminal region. These peptides have already been described by Kong et al. (22), but coupled with a carrier protein. Here, we chose to use a three-time repeat of synthetic peptides that were allowed to coat a peptide immobilizer present on the enzyme-linked immunosorbent assay plate. The threefold repetition of the sequence was shown in a preliminary assay to amplify the reaction.

Toxoplasma strains circulating in Europe belong to the clonal lineages named I, II, and III (2, 19). Type II predominates in France (3, 13). In Portugal, the three genotypes have already been described (5, 9, 12), type II being the most frequently found. In our study, the GRA6 type II peptide was recognized by most European serum samples from patients with severe and asymptomatic infections. Similar results were obtained with samples from humans with congenital toxoplasmosis in Poland (26) and from chronically infected pregnant women from France, Italy, and Denmark (27). However, in the present study, we found a higher prevalence of serotype I/III among Portuguese patients than among French patients, which could suggest a different epidemiological pattern of strains circulating in these two countries. Little information exists about circulating *Toxoplasma gondii* strains in Africa. *SAG2* type III has been the genotype most frequently described in isolates from chickens from several African countries (10, 11). A multilocus genotyping study of isolates from Ugandan chickens revealed either type II, III, or I (25). The few strains

TABLE 7. *T. gondii* GRA6 serotypes and association with clinical aspects in Latin America

Pathology (no. of samples)	% (no.) of samples with indicated GRA6 serotype(s)			
	II	I/III	Mixed[a]	ND[b]
Congenital (1)	0 (0)	100 (1)	0 (0)	0 (0)
Other[a] (2)	50 (1)	0 (0)	50 (1)	0 (0)
Multivisceral (4)	25 (1)	50 (2)	0 (0)	25 (1)
Asymptomatic (148)	3.4 (5)	45.9 (68)	18.2 (27)	32.4 (48)

[a] One case of ocular toxoplasmosis and one of acute toxoplasmosis in an immunocompetent patient.
[b] Mixed, serum sample(s) reacted with both peptides.
[c] ND, not determined because serum samples had OD indices that were below the cutoff values. Cutoff values were 0.089 for GRA6 II and 0.067 for GRA6 I/III.

TABLE 8. Polymorphic peptides selected from *GRA6* marker

C-terminal GRA6 polymorphic peptides[a]	Strain(s)[b]	Strain type	Reference
LHPERVNVFDY	RH	I	21
. . . GS . . E . . F	Beverley	II	21
. . . ER . . V . . Y	NED	III	21
. . . GS . . V . . Y	GUY-2003-BAS and GUY-2004-TER	Atypical	This study
. . . ER . . V . . Y	GUY-2002-MAT and GUY-2002-KOE	Atypical	This study
. . . EH . . V . . Y	GUY-2004-AKO	Atypical	This study

[a] Periods (.) indicate amino acids that are identical to the sequence of strain RH. Underlined amino acids represent polymorphic residues.
[b] RH, Beverley, and NED are, respectively, type I, II, and III reference strains.

isolated from patients from West and Central Africa exhibited a mixture of type I and III alleles when studied by a multilocus microsatellite analysis (4). In this study, peptide GRA6 type I/III was recognized by 31.5% (54.9% if we exclude the serum samples that did not react with the peptides) of the African serum samples. Peptide GRA6 type II was less prevalent, being recognized by 11.2% of African serum samples.

In South America, atypical genotypes are mainly found (1, 15, 20). Eleven cases of toxoplasmosis were reported in a village from Suriname; at least five isolates corresponded to only one nonarchetypal strain (8). In French Guiana, most of the reported cases of disseminated toxoplasmosis in immunocompetent patients were also associated with nonarchetypal strains (1, 6). Considering this, it should be expected that sera from these cases would not react with GRA6 peptides, since nonarchetypal strains possess specific polymorphisms that distinguish them from type I, II, and III strains. We observed that GRA6 sequencing of those strains showed that at the C-terminal region, they differ from clonal strains by 1 or 2 amino acids. Three different peptides can be described for the five atypical strains included in this study (Table 8). One peptide is shared by type I and type III strains. This peptide induced a misclassification of the atypical strains (GUY-2002-MAT and GUY-2002-KOE) as type I/III. One peptide differed from the peptide characterizing type I and III by a single amino acid at position 224 (E*H*-V-Y instead of E*R*-V-Y). This single amino acid substitution was not enough to distinguish *Toxoplasma* infections with strains harboring these alleles and could explain why GUY-2004-AKO serum reacted as type I and III sera. Another peptide (GS-V-Y) is a mixture of amino acid sequences characterizing type II and type I and III. Strains from French Guiana (GUY-2003-BAS) and Suriname (GUY-2004-TER) with these GRA6 C-terminal polymorphisms displayed different reactivity profiles. The same atypical allele may induce a type II response, a type I/III response, or a double response against both peptides.

Of serum samples from other Latin American countries, 45.8% (67% of successfully serotyped sera) recognized peptide GRA6 type I/III. Peyron et al. (27), described similar results with serotyping of chronically infected pregnant women from Colombia, where a type I and III profile was found, but no type II. In our study, a GRA6 type II profile was found in 4.5% of patients from Latin America (French Guiana and Mexico). But it could not be excluded that these infections were due to

nonarchetypal strains, as demonstrated by serotyping of infections due to known nonarchetypal strains in Suriname.

Serotyping is a typing method based on the antibody recognition of strain-specific polymorphic peptides. Although this method appears very promising for typing *T. gondii* strains, it presents, at this point, some limitations. The peptides used were derived from the archetypal strains and only differentiate strains with a GRA6 type II genotype from strains with a GRA6 non-type II genotype. It is therefore not possible to distinguish type I from type III and from atypical genotypes. Like a single-locus genotyping, serotyping based on these two peptides does not distinguish nonarchetypal strains. Moreover, we were unable to serotype some strains. A considerable number did not recognize the two peptides studied. This was actually the case for immunocompetent asymptomatic patients from Europe and Africa, where 39.2% and 46.3%, respectively, of the serum samples studied were not serotyped (Tables 4 and 5). However, for congenital infections and other *T. gondii*-related pathologies from Europe, the number of nonserotyped infections was significantly lower (22.7% and 33.3%, respectively) ($P < 0.009$). Similar results were obtained for congenital infections from Poland (15.4%) by Nowakowska et al. (26) and for cerebral and ocular toxoplasmosis from North America (29.2%) by Kong et al. (22).

It seems pertinent to link the immunoglobulin G isotype kinetics (antibody profile) with the time of infection. The kinetics of the humoral response might explain the high rate of nonserotyped asymptomatic chronic infections. Determination of the specific isotype present at each infection stage may be important for the study of the nonserotyped infections. Our results suggest that strain type may induce pathology in a geographical-origin-dependent manner. The different geographical origins and limitations inherent in the serotyping method (limited number of peptides) are two important biases in the interpretation of the relationship between serotype and clinical disease. To better understand the hypothetical association between serotype and clinical disease, serum samples from patients with a specific pathology from different geographical regions must be studied using a large number of discriminative peptides.

Studies on serotyping have previously been performed but on a restricted number of infections (mainly congenital) from Europe and North America (22, 26). A single study was performed with congenital infections from Colombia (27). Our study involves a large number of patients with different *Toxoplasma*-associated pathologies and is the first serotyping study involving asymptomatic chronic infections. This is also the first serotyping study with patients from Africa, French Guiana, Suriname, Mexico, and Portugal.

In conclusion, this study highlights a strong agreement between GRA6 serotype and MS genotype for infections due to archetypal strains. However, the designed peptides used have a poor specificity for serotyping of infections due to nonarchetypal strains. In Europe the prevalent profile is GRA6 type II, while in Africa and Latin America, GRA6 type I/III prevails. New peptides from different markers must be found in order to differentiate type I, type III, and nonarchetypal strains. Studies are currently going on with type I- and type III-specific peptides with encouraging results.

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