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Toxoplasma gondii SAG2, SAG3 and GRA6 alleles and single nucleotide polymorphism in congenital infections with known parasite load and clinical outcome

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ABSTRACT

Amniotic fluid DNA samples were genotyped by multilocus-nested-PCR-RFLP, but only three of 11 markers amplified 113 of 122 (92.6%) samples, resulting in 12 untyped and 101 partial non-archetypal genotypes. The 101 typed samples were subdivided into four groups: G1 with 73 samples (5'and 3' SAG2 allele I + SAG3 allele III + GRA6 allele III), 53 had parasite load ≤ 10^2 parasites/mL (43 asymptomatic, 10 mild infections), 17 had load > 10^2 and ≤ 10^3 (one mild, 13 moderate and three severe), and three had load > 10^3 parasites/mL (three severe); G2 with 22 samples (5'and 3' SAG2 allele I + SAG3 allele III), all parasite load levels ≤ 10^2 parasites/mL (18 asymptomatic and four mild); G3 with five samples (5' and 3' SAG2 allele I + SAG3 allele II), parasite load ≤ 10^2 parasites/mL (three asymptomatic and two mild); G4 with one sample (5' and 3' SAG2 allele II + SAG3 allele II + GRA6 allele I), a parasite load < 10^2 parasites/mL in an asymptomatic infant. After DNA sequencing, restriction sites confirmed SAG2, SAG3 and GRA6 alleles in 98.7%, 100% and 100% of the cases, respectively, while single nucleotide polymorphisms confirmed 90% of 5'-SAG2 allele I; 98.7% of 3'-SAG2 allele I; 98% of SAG-3 allele III, but only 40% of GRA6 allele III results. For the moment, partial non-archetypal genotypes of parasites did not show any relationship with either parasite load in amniotic fluid samples or clinical outcome of infants at the age of 12 months.

KEYWORDS: Toxoplasmosis. Congenital toxoplasmosis. Genotyping. Single nucleotide polymorphism. Parasite load. Parasite burden.

INTRODUCTION

Toxoplasma gondii is a widely distributed parasite that usually causes mild infections in humans, but toxoplasmosis can be severe in immunocompromised patients, such as organ transplant recipients, patients undergoing chemotherapy for cancer treatment, people living with HIV and immunologically immature fetuses[1].

In congenital toxoplasmosis, the gestational age at which vertical transmission occurs is known as an independent risk factor[1]. The genetic predisposition of the host to the presence of certain HLA alleles may also confer susceptibility to toxoplasmosis[2]. Furthermore, the parasite load level in amniotic fluid samples at the time of diagnosis has been associated with the presence and the severity of symptoms at the end of the first year of life[3].

The parasite genotype has been considered a possible risk factor in congenital toxoplasmosis[4]. When *T. gondii* virulence is determined through infections in

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laboratory mice (biotest), the classic archetypal genotype I is associated with more virulent and lethal parasites, while archetypal genotypes II and III are associated with less virulent parasites and development of chronic infections[5].

A multilocus-nested-PCR-RFLP of 11 markers, namely SAG1, 5' and 3' SAG2, alt. SAG2, SAG3, bTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico was developed and used to test *T. gondii* strains from North America and Europe, with most strains and isolates falling into one of three archetypal genotypes (I, II and III) and human congenital infections mainly associated with parasites harboring the archetypal genotype II[6].

On the contrary, in South America, non-archetypal genotypes and several new genotypes have been described since the beginning, sometimes leading to potentially fatal infections as in the Amazonian toxoplasmosis[7,8]. More recently, a greater diversity of phenotypes attributed to archetypal genotype II parasites have been described in association with severe cases of congenital toxoplasmosis in the European continent[9,10].

In Brazil, 46 *T. gondii* isolates from cats living in 11 cities in Sao Paulo State were genotyped by the multilocus-nested-PCR-RFLP of 11 markers (SAG1, 5' and 3'-SAG2, SAG3, bTUB, GRA6, c22-8, c29-2, L358, PK1, Apico and CS3), revealing 20 genotypes, although the archetypal genotypes I, II and III were not found. Interestingly, there were two isolates likely harboring more than one parasite genotype at the same time. The parasite genotypes in this study were added to data from other reports resulting in a total of 125 isolates from humans and other animal species from different Brazilian States, resulting in 48 genotypes, 26 of which were represented only once and four genotypes were considered common Brazilian lineages, namely BrI, BrII, BrIII and BrIV, as they were detected in multiple isolates. The authors concluded for the greater genetic diversity of the *T. gondii* population in Brazil and pointed to the existence of four local lineages (BrI, BrII, BrIII and BrIV) that managed to expand in the Brazilian territory, being predominant and frequently found in both, human and animal infections to date[11], while in the North American and European parasite populations, the archetypal genotype II has been predominant, contrasting with the nonidentification of the archetypal genotype II in Brazil[11-13].

When the Brazilian lineages were investigated according to mortality rates in laboratory mice (biotest), BrI showed a highly virulent behavior, BrIII was non-virulent, while BrII and BrIV showed intermediate virulence. These results were in agreement with the alleles found in the CS3 marker, suggesting that *T. gondii* has a particular population structure in Brazil and the four non-archetypal Brazilian lineages have different virulence when compared to the classic archetypal genotypes[11].

It is worth mentioning that these Brazilian non-archetypal genotypes present a combination of alleles that are also found in archetypal strains by multilocus-nested-PCR-RFLP, but they can also harbor unique alleles, suggesting that they may have an older origin[14]. The virulent BrI lineage is characterized by the presence of allele I in most multilocus-nested-PCR markers (SAG1, 5' and 3'-SAG2, SAG2 alt., bTUB c22-8, c29-2, PK1, Apico and CS3), with the exception of SAG3 allele III and GRA6 allele II. This allelic distribution suggests that the non-archetypal Brazilian lineages are distinct from the three classic archetypal genotypes I, II and III, classified in the *Toxoplasma gondii* (ToxoDB) data base[15] as ToxoDB#10 (archetypal genotype I) ToxoDB#1 (archetypal genotype II) and ToxoDB#2 (archetypal genotype III).

Lorenzi *et al.*[16] performed a comparative genomic investigation on the population diversity of *T. gondii* parasites and their relationships with closely related coccidian parasites (*H. hammondi*, *Sarcocystis neurona* and *N. caninum*). Whole genomes of related parasites, 62 *T. gondii* strains comprising the 16 existing haplogroups, in addition to prototype strains such as ME49, were obtained and showed an unusual population structure of *T. gondii* characterized by a clade-specific inheritance of large conserved haploblocks. This shared inheritance suggests an ancestry different from that raised by whole genome analysis, suggesting that the genome, virulence and transmission of *T. gondii* parasites may be somehow related, even if the mechanisms seem much more complex than previously thought.

The description of an increasing number of non-archetypal genotypes of *T. gondii* worldwide, even in the Northern Hemisphere, while South America has always stood out, since the beginning, as a hotspot of genetic diversity of *T. gondii*[8] may be explained by a natural increase in *T. gondii* biodiversity through the occurrence of genetic crosses between parasites over time, on all continents, in addition to a greater number of studies carried out with more representative samples and better resolution genotyping techniques[17].

The present study aimed to genotype *T. gondii* on DNA samples from amniotic fluid by multilocus-nested-PCR-RFLP, followed by sequencing of amplified alleles to analyze restriction enzyme sites and single nucleotide polymorphisms that should ensure the genotyping accuracy. Then, alleles were compared with parasite load levels in amniotic fluid samples and severity of congenital infections determined by a 12 months' clinical and laboratory follow-up. These two parameters were used as surrogate markers of the virulence of parasites.

MATERIALS AND METHODS

Ethic aspects

The study was approved by the Research Ethics Committee of Hospital das Clinicas, Sao Paulo, Brazil (CAAE 87530118.0.0000.0068).

Study design

This was a prospective laboratory study performed with DNA samples from amniotic fluid specimen belonging to a cohort of confirmed cases of congenital toxoplasmosis. These DNA samples were stored at –20 ºC from 2010 to 2020 and they had been submitted to two thawing-freezing cycles when previous studies were carried out[2,3].

Inclusion criteria

All the infants included in this study presented a positive *T. gondii* DNA detection on amniotic fluid samples by Real Time PCR, as previously described[3].

Molecular diagnosis of congenital toxoplasmosis

The gold standard for the diagnosis of congenital toxoplasmosis has been the Polymerase Chain Reaction (PCR) for more than two decades[18]. After ultrasound-guided amniocentesis to obtain amniotic fluid samples, they were submitted to DNA extraction with the QIamp DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Amniotic fluid DNA samples were tested by quantitative Real Time (qPCR) using primers from the 35-copy B1 gene of the parasite, according to a previously described protocol[3].

Definition of a confirmed case of congenital toxoplasmosis

All recruited neonates were followed up to 12 months of age, during which time specific treatment was prescribed even to asymptomatic infants. The treatment comprised sulfadiazine and pyrimethamine and supplementation with folinic acid, and this schedule was maintained as continuously as possible during the first year of life, being temporarily replaced by spiramycin or suspended in the presence of adverse effects[19]. Serology for toxoplasmosis was repeated (IgM and IgG titers) every three months and cranial ultrasound or tomography to investigate central nervous system lesions were performed at the beginning and at the end of the follow-up. In addition, the eye fundus was evaluated every three months for the investigation of chorioretinitis. A congenital case was considered positive when qPCR was positive on amniotic fluid samples, as the finding of the parasite DNA in placenta is not an unequivocal proof of infection transmission. Moreover, most cases were confirmed at birth through neonatal serological evaluation or during the 12-month follow-up due to the presence of IgM and/or IgG titers significantly higher than those of the mother[3].

Classification of congenital toxoplasmosis according to severity

Congenital toxoplasmosis cases were considered asymptomatic or symptomatic, and the symptomatic cases were further divided into three categories: mild cases were those that presented a sign or symptom that resolved quickly; moderate cases were those with hepatosplenomegaly, jaundice, fever, petechiae, among others, requiring hospitalization, but resolved favorably; severe cases were those with at least one of the Sabin's triad of symptoms: hydrocephalus, cerebral calcifications and chorioretinitis[3].

T. gondii genotyping by multilocus-nested-PCR

All 122 DNA samples from confirmed cases of congenital toxoplasmosis were tested by 11-marker multilocus-nested-PCR (SAG1, 5' and 3'-SAG2, SAG3, GRA6, bTUB, c22-8, c29-2, L358, PK1, apico and CS3), according to a previously described protocol[12,13]. In the first round, 200 µM of dNTPs (Thermo Fisher Scientific, Waltham, MA, USA), 0.25 µM of the 11 external primers (IDT, USA), 2X QIAGEN Multiplex PCR Master Mix (QIAGEN, Hilden, Germany), Q-solution, 10 X primer mix and RNase-free H_2O to complete 35 µL, plus 100 ng of DNA from amniotic fluid samples or 10 ng of DNA from positive controls representing the prototypes for genotypes I, II and III (RH strain, ME49 and VEG, respectively), in a final volume of 50 µL. The initial denaturation step at 95 °C for 15 min was followed by 40 cycles at 95 °C for 30 s, 57 °C for 90 s and 72 °C for 90 s, ending with a final extension of 10 min at 72 °C. In the second round of amplification, each of the 11 markers was analyzed in separate microtubes containing 1X buffer (Thermo Fisher Scientific), 200 µM dNTPs (Thermo Fisher Scientific), 0.2 µM of the 11 internal primers (IDT), 2.5 U of Taq DNA Polymerase (Thermo Fisher Scientific), 2 µL of the first round amplification product and sterile water to a final volume of 50 µL. After an initial denaturation step at 95 °C for 5 min, 40 cycles were performed at 95 °C for 30 s, 55 °C for 90 s and 72 °C for 60 s were performed, ending with a final extension of 5 min at 72 °C. Amplification products were visualized on 2% agarose gels (Invitrogen, Thermo Fisher

Scientific) prepared in TAE buffer (Tris-acetate-EDTA 1M) and stained with 0.5 mg/mL ethidium bromide (PlusOne, Thermo Fisher Scientific). The gels were subjected to horizontal electrophoresis (HE-58, GE Healthcare, Thermo Fisher Scientific) at 80 volts for 30 min.

RFLP

After testing the 122 DNA samples from amniotic fluid, we noticed that only three of the 11 multilocus-nested-PCR markers generated amplification products, namely, 5' and 3'-SAG2, SAG3 and GRA6, and none of the markers amplified all amniotic fluid samples. This is the reason why restriction enzyme digestions were performed only for these three markers, following the manufacturer's instructions. The restriction enzyme HhaI (New England Biolabs, USA) was used to cleave the 3'-SAG2 amplification product; Sau3AI to cleave the 5'-SAG2 amplification product; NciII to cleave the SAG3 amplification product and MseI to cleave the GRA6 amplification product. Briefly, 10 µL of volume containing 3-10 U of the specific restriction enzyme and its specific buffer, plus 10 µL of the amplification product, were incubated at 37 °C for 3 h. Total digestion volume (20 µL) was visualized on agarose/NuSieve 2:1 gels (Invitrogen) prepared in TAE buffer (Tris-acetate-EDTA 1M) and subjected to horizontal electrophoresis (80 volts for 30 min).

DNA sequencing

All amplification products of the markers 5'-SAG2, 3'-SAG2, SAG3 and GRA6 generated by multilocus-nested-PCR were sequenced to ensure the reliability of enzymatic digestions (RFLP) and to identify the presence of single nucleotide polymorphisms along the sequence of markers. Prior to DNA sequencing, total amplification products were purified using the Wizard SV Gel kit and PCR Clean-Up system (Promega Biotechnology, Madison, WI, USA). The concentration of PCR products was estimated using the Low DNA Mass Ladder reagent (Invitrogen, Thermo Fisher Scientific). Sequencing reactions were prepared using the BigDye® Terminator Cycle Sequencing kit (Applied Biosystems, Thermo Fisher Scientific). Briefly, for 10 µL of reaction mixture containing BigDye 1:16; 1.5 µL of 5 X sequencing buffer, 5 µM of each primer was added. Then, the reactions were carried out in a thermocycler under the following conditions: 25 cycles at 95 °C for 10 s, 50 °C for 5 s, 60 °C for 2 min. To remove unincorporated products, DNA was precipitated using the BigDye® X Terminator Purification kit (Applied Biosystems, Thermo Fisher Scientific), following the manufacturer's instructions. After sequencing, the electropherograms were manually edited by BioEdit Sequence Alignment and then analyzed by Codon Code Aligner. After editing, the AF sequences, as well as the prototype controls (RH, ME49 and VEG) were compared using the CLUSTALW tool to determine the degree of similarity between sequences.

RESULTS

One hundred and twenty-two DNA samples from amniotic fluid belonging to confirmed cases of congenital toxoplasmosis were investigated. Data from pregnant women and the 12-month clinical and laboratory follow-up of infants were described elsewhere[2,3]. Briefly, maternal infections occurred in the first trimester of pregnancy in 18 of 122 (14.7%) cases and in 104 of 122 (85.2%) cases, vertical transmission occurred in the second trimester. At birth, IgM was present in 107 of 122 (87.7%) neonates and infections were symptomatic in 36 of 122 neonates (29.5%), thus 50% of infections were acquired in the first trimester. Six of 36 symptomatic infections were severe, corresponding to 16.7% of symptomatic neonates and 4.9% of the total cases, and these infections were acquired in the first trimester of pregnancy[2,3].

Genotyping by multilocus-nested-PCR-RFLP

In the total of 122 DNA samples, partial amplifications were obtained in 113 (113/122 or 92.6%) samples, and the amplified markers were 5'and 3'-SAG2, SAG3 and GRA6. Nine of the 122 samples (7.4%) did not amplify any of the 11 markers. Table 1 shows the distribution of the 122 DNA samples according to the alleles found in the amplified markers and the number of unamplified samples for each marker. Briefly, SAG2 amplified 113 (92.6%) samples that after RFLP resulted in 100 (100/113 or 88.5%) samples with allele I, 11 (9.7%) samples with allele II, and two

Table 1 - Distribution of the 122 DNA samples from amniotic fluid according to SAG2, SAG3 and GRA6 alleles, the multilocus-nested-PCR-RFLP marker amplified and the number of non-amplified samples for each marker.

DNA samples	5' and 3'-SAG2	SAG3	GRA6
Allele I	100	0	1
Allele II	11	11	1
Allele III	2	95	73
Amplified samples	113 (92.6 %)	106 (86.8 %)	75 (61.5 %)
Non amplified samples	9 (7.4 %)	16 (13.1 %)	47 (38.5 %)
Total	122	122	122

(1.8%) samples with allele III. SAG3 amplified 106 (86.9%) samples, that after RFLP resulted in 11 (10.4%) samples with allele II and 95 (89.6%) samples with allele III. GRA6 amplified 75 (61.5%) samples that after RFLP resulted in one sample with allele I (1.3%), one with allele II (1.3%) and 73 (97.4%) with GRA6 allele III.

SAG2, SAG3 and GRA6 allele typing

Although we could amplify at most three of the 11 multilocus-nested-PCR markers from a DNA sample, the allele type analysis was performed in most cases, and two distinct allele types were found in at least two different markers, suggesting that the parasites were non-archetypal in the majority of samples, i.e., they were different from the classic archetypal genotypes I, II and III considering this genotyping tool.

Table 2 summarizes the analysis of the 5' and 3'-SAG2, SAG3 and GRA6 alleles in 113 DNA samples after amplification by multilocus-nested-PCR-RFLP. While 101 (89.4%) DNA samples generated partial non-archetypal genotypes, in the remaining 12 samples (*), this analysis could not be performed because only one marker was amplified or more than one marker was amplified, but the results indicated the same allele type at distinct markers.

Sequencing analysis of amplification products to analyze restriction enzymes sites and single nucleotide polymorphisms in 5' and 3'-SAG2, SAG3 and GRA6 alleles

After sequencing (Sanger) the three markers found in the DNA samples from amniotic fluid and the prototype strains (I -RH; II-ME49 and III-VEG), quality readings were analyzed in two ways: at the restriction site of the specific enzyme used in RFLP and at single nucleotide polymorphisms along the sequences of the three markers. This approach has already been applied to assess parasite genotypes when only one or a few markers are amplified by multilocus-nested-PCR-RFLP[20-22].

Among the 113 sequences amplified by 5' and 3'-SAG2 primers, 79 (69.9%) underwent the restriction site analysis, and allele I was confirmed in 78 of 79 (98.7%) samples. Regarding SAG3, only 51 of the 106 (48.1%) sequences were analyzed at the restriction site and allele III was confirmed in all 51 samples (100%), and in GRA6, only 40 (53.3%) of the 75 sequences were analyzed at restriction sites and allele III was confirmed in all samples (100%).

In 5'-SAG2, 101/113 (89.4%) sequences were analyzed at positions 76 and 116 and the combination 76 (T) and 116 (T) corresponding to allele I, was found in 87 (86.1%) samples of the total, with four additional samples that could also harbor allele I (or allele III), nine samples with allele III and one sample with allele II (Table 3). Therefore, 91/101 (90%) samples had 5'-SAG2 alleles confirmed by this analysis.

In 3'-SAG2, 79/113 (69.9%) sequences were analyzed at positions 1211 and 1231, and the combination 1211 (C) and 1231 (T) corresponding to allele I (RH) or allele III (VEG) predominated in 78/79 (98.7%) of the samples, and only one sample harbored allele II, although it could also be allele I or allele III (Table 3). Therefore, 78/79 (98.7%) samples had their 3'-SAG2 alleles confirmed by this analysis.

In SAG3, nine polymorphisms were analyzed at positions 981, 1001, 1005, 1037, 1044, 1046, 1053, 1061 and 1076. Among the 106 quality readings, only 51

Table 2 - Detection of 5' and 3'-SAG2, SAG3 and GRA6 alleles by multilocus-nested-PCR-RFLP. Considering the total number of tested DNA samples (n=122), 113 (92.6%) samples amplified at least one marker, among which 101 (89.4%) amplified more than one marker and there two distinct allele types were identified in at least two different markers, resulting in non-archetypal parasites, while the remaining 12 samples were called untyped (*), as there was only one amplified marker, or more than one marker was amplified, but the alleles were of the same type.

Nº DNA samples	5' and 3'-SAG2	SAG3	GRA6	Genotype
73	I	III	III	Non-archetypal
1	II	II	I	Non-archetypal
1*	II	II	II	Untyped
22	I	III	NA	Non-archetypal
5	I	II	NA	Non-archetypal
4*	II	II	NA	Untyped
5*	II	NA	NA	Untyped
2*	III	NA	NA	Untyped
Total 113	**113**	**106**	**75**	**101 non-archetypal, 12 untyped**

*these 12 DNA samples from amniotic fluid were considered untyped; NA = non-amplified DNA sample.

Table 3 - Single nucleotide polymorphism analysis of the 5'- and the 3'SAG2- marker. Two polymorphisms were evaluated in each of the SAG2 ends, in each of the three archetypal prototypes (RH for type I, ME49 for type II and VEG for type III) and in the studied DNA samples from amniotic fluid of confirmed cases of congenital toxoplasmosis.

Samples	5'- SAG2		Allele	Number (%)
	76	116		
RH (type I prototype)	T	T	I	4
ME49 (type II prototype)	T	**G**	II	4
VEG (type III prototype)	**C**	T	III	4
76 (T), 116 (T)	I or II	I or III	I	87 (86.1)
76 (C or T), 116 (T)	I or II or II	I or III	I or III	4 (4.0)
76 (C), 116 (T)	III	I or III	III	9 (8.9)
76 (T), 116 (G)	I or II	II	II	1 (1.0)
Total				**101 (100)**
	3'- SAG2			
	1211	1231		
RH (type I prototype)	C	T	I	4
ME49 (type II prototype)	**G**	**G**	II	4
VEG (type III prototype)	C	T	III	4
1211 (C), 1231 (T)	I or III	I or III	I or III	71 (89.9)
1211 (C), 1231 (T or G)	I or III	I or II or III	I or III	5 (6.3)
1211 (C), 1231 (G or T)	I or III	I or II or III	I or III	2 (2.5)
1211 (C or G), 1231 (T or G)	I or II or III	I or II or III	I or II or III	1 (1.3)
Total				**79**

The addition of 87 cases of allele I and 4 cases of allele I (or III) results in 91 cases or 90% of the total.

(48.1%) could be analyzed and in 50 (98%) samples, the combination 981 (C), 1001 (G), 1005 (A), 1037 (C), 1044 (G), 1046 (A), 1053 (C), 1061 (C), 1076 (A) corresponding to allele III was found, as well as in VEG strain (allele III prototype). Only one sample showed the allele II (Table 4). Therefore, 50/51 (98.0%) samples had SAG3 alleles confirmed by this analysis.

In GRA6, 40/75 (53.3%) sequences were analyzed at five polymorphic positions 41, 71, 106, 162 and 171. Only one sample showed a perfect match for allele II (ME49 strain), while 16 (40%) samples had 41 (C), 106 (T), 162 (A), 171 (A) matching allele III (VEG strain), and, more importantly, the predominant group of 23 (57.5%) samples had 41 (C), 71 (T), 106 (C), 162 (A), 171 (A), that does not match any of the prototype strains, resulting in two possible GRA6 alleles, I and III together or I and

Table 4 - Single nucleotide polymorphism analysis of the SAG3 marker. A total of nine polymorphisms were evaluated in each of the three archetypal prototypes (RH for type I, ME49 for type II and VEG for type III) and in the studied DNA samples from amniotic fluid of confirmed cases of congenital toxoplasmosis.

Samples	SAG3 nucleotide position									Genotype	Number
	981	1001	1005	1037	1044	1046	1053	1061	1076		
RH (type I)	C	G	A	T	G	G	**A**	**G**	A	I	4
ME49 (type II)	**T**	**A**	**G**	**T**	**A**	**G**	**C**	**C**	**G**	II	4
VEG (type III)	C	G	A	**C**	G	**A**	C	C	A	III	4
981 (C), 1001 (G), 1005 (A), 1037 (C), 1044 (G), 1046 (A), 1053 (C), 1061 (C), 1076 (A)	I or III	I or III	I or III	III	I or III	III	II or III	II or III	I or III	III or I/III or III/II	50
981 (T), 1001 (A), 1005 (G), 1037 (T), 1044 (A), 1046 (G), 1053 (C), 1061 (C), 1076 (G)	II	II	II	I or II	II	I or II	II or III	II or III	II	II or II/I or II/III	1
Total											**51**

II and III together, increasing the chances of infections caused by more than one parasite genotype (Table 5). It is noteworthy that only 16/40 (40%) samples had the GRA6 allele confirmed by this analysis.

SAG2, SAG3 and GRA6 alleles with respect to parasite load in amniotic fluid samples and the outcome of congenital toxoplasmosis

Table 6 summarizes the findings of 101 partially genotyped DNA samples. Briefly, 81 samples had a parasite load $\leq 10^2$ parasites/mL; 17 samples had parasite load $>10^2$ and $\leq 10^3$; three samples had parasite load $> 10^3$ parasites/mL. Regarding the severity of infections, there were 65 (64.4%) asymptomatic cases and 36 symptomatic (35.6%) ones, classified as mild (n=17 or 47.2%), moderate (n=13 or 36.1%) or severe (n=6 or 16.7%) cases of congenital toxoplasmosis[3].

In the first group of 73 samples with 5' and 3'-SAG2 allele I + SAG3 allele III and GRA6 allele III parasites, there were 53 infants with parasite load $\leq 10^2$ parasites/mL (43 asymptomatic and 10 mild infections), 17 infants with parasite load $> 10^2$ and $\leq 10^3$ (one mild, 13 moderate and three severe infections), and three infants with parasite load $> 10^3$ parasites/mL (three severe congenital infections). In the second group of 22 samples with 5' and 3'-SAG2 allele I + SAG3 allele III, all infections had parasite load $\leq 10^2$ parasites/mL (18 asymptomatic and

Table 5 - Single nucleotide polymorphism analysis of the GRA6 marker. A total of five polymorphisms were evaluated in each of the three archetypal prototypes (RH for type I, ME49 for type II and VEG for type III) and in the studied DNA samples from amniotic fluid of confirmed cases of congenital toxoplasmosis.

	GRA6 nucleotide position					Genotype	Samples
	41	71	106	162	171		N
RH (prototype I)	C	**G**	C	G	A	I	4
ME49 (prototype II)	**T**	T	C	G	**G**	II	4
VEG (prototype III)	C	T	**T**	**A**	A	III	4
41 (C); 106 (T); 162 (A); 171 (A)	I or III	II or III	III	III	I or III	III or I/III or I/II/III	16
41 (T); 71 (T); 106 (C); 162 (G); 171 (G)	II	II or III	I or II	I or II	II	II or II/III or II/I	1
41 (C); 71 (T); 106 (C); 162 (A); 171 (A)	I or III	II or III	I or II	III	I or III	I/II/III or I/III or II/III	23
Total							**40**

Table 6 - Distribution of the 101 partially non-archetypal genotypes according to 5' and 3'-SAG2, SAG3 and GRA6 alleles detected by multilocus-nested-PCR-RFLP, parasite load levels in DNA samples from amniotic fluid and severity of congenital infections determined after 12 months of clinical and laboratory follow-up.

Partial non-archetypal genotypes	Parasite load and severity of CT		
	$\leq 10^2$	$>10^2$ to $\leq 10^3$	$>10^3$
73 5'and 3' SAG2 allele I + SAG3 allele III + GRA6 allele III	**53** 43 asymptomatic 10 mild	**17** 1 mild 13 moderate 3 severe	**3** severe
22 5'and 3' SAG2 allele I + SAG3 allele III	**22** 18 asymptomatic 4 mild	None	None
5 5' and 3' SAG2 allele I + SAG3 allele II	**5** 3 asymptomatic 2 mild	None	None
1 5' and 3' SAG2 allele II + SAG3 allele II + GRA6 allele I	**1** 1 asymptomatic	None	None
Total 101	**81**	**17**	**3**

Parasite load in amniotic fluid samples were expressed in parasites/mL; CT = congenital toxoplasmosis. Severity of congenital toxoplasmosis was classified as asymptomatic or symptomatic, and the latter group was further subdivided into mild, moderate or severe cases according to the follow-up of 12 months (see M&M).

four mild infections). In the third group of five samples with 5' and 3'-SAG2 allele I + SAG3 allele II, all samples had parasite load ≤ 10^2 parasites/mL (three asymptomatic and two mild infections). The fourth group of samples comprised only one non-archetypal genotype with 5' and 3'-SAG2 allele II + SAG3 allele II + GRA6 allele I, a parasite load ≤ 10^2 parasites/mL in an asymptomatic infant.

DISCUSSION

In our laboratory, the molecular diagnosis of toxoplasmosis is performed by Real Time PCR targeting the parasite's repetitive B1 gene, which generates more sensitive amplifications[3] than the multilocus-nested-PCR which is based on single-copy genes[8,14]. Thus, it is not surprising that only part of the DNA samples from amniotic fluid with positive Real-Time PCR could be successfully genotyped by multilocus-nested-PCR. This lack of the multilocus amplification has already been described by others dealing with human and animal samples[20-22].

A recent Brazilian study in which 148 samples (35 dogs, 105 wild life animals and eight amniotic fluid samples) were subjected to isolation of parasites in mice and genotyping by multilocus-nested-PCR-RFLP obtained only 22 (14.9%) isolates (nine dogs, one cat, 10 wild animals two pregnant women) among which there were 11 genotypes (nine previously described, two new genotypes and one potentially harboring two types of parasite at the same time). Considering the nine genotypes already known, the archetypal type III (ToxoDB#2), two Brazilian lineages (the highly virulent BrI and the non-virulent BrIII), in addition to ToxoDB#14, ToxoDB#41, ToxoDB#108, ToxoDB#140, ToxoDB#166 and ToxoDB#190 have been described[23]. Although several studies have succeeded in isolating parasites from amniotic fluid samples of treated pregnant women, which were thereafter genotyped by genotyping systems[24,25], in the mentioned study, the two isolates from congenital infections did not amplify any of the multilocus markers, the same situation found in our study[23].

As we were unable to amplify the 11 multilocus-nested-PCR markers, including the CS3 marker whose alleles seem to correlate with virulence in mice, we called the genotypes partial and non-archetypal, as they do not harbor the same allele type in at least two distinct markers, and this was the behavior found in most samples in our study (Table 2), as well as in previous reports[20-22]. These authors have further explored their partial genotypes by analyzing the presence and combination of single nucleotide polymorphisms in the sequences, as we did.

In our investigation, the low number of parasites in amniotic fluid samples is the most likely cause for the multilocus-nested-PCR-RFLP genotyping failure. The requirement of a minimum number of parasites for a successful genotyping has already been recognized[17] and only 20 of 122 (16.4%) samples in this investigation had parasite load levels > 10^2 parasites/mL. Another factor that could potentially have impaired the amplifications was the use of the same 122 DNA samples in two previous studies[2,3], submitting DNA samples to thawing-freezing cycles.

In Brazil, a recent report[26] described the first case of severe congenital toxoplasmosis due to a non-archetypal genotype of *T. gondii* in a neonate from Alagoas State, Northeastern Brazil. The pregnant woman seroconverted to toxoplasmosis in the second trimester of pregnancy and an umbilical cord blood sample was used to isolate *T. gondii* in mice, resulting in a ToxoDB#162 genotype that is related to the archetypal genotype III (ToxoDB#2) and the Brazilian BrIII lineage (ToxoDB#8). The isolate was non-virulent in mice, in agreement with the previous non-virulence of the Brazilian BrIII lineage[11], though disagreeing with the severity of the congenital infection, once again drawing attention to the lack of association between the parasite genotype and the outcome of congenital infections.

In this study, after DNA sequencing, the 5' and 3'-SAG2, SAG3 and GRA6 alleles detected by multilocus-nested-PCR-RFLP were confirmed by the restriction site analysis and also by single nucleotide polymorphisms of 5' and 3'-SAG2 and SAG3, excepting for the GRA6 marker, for which there was a predominance of infections likely caused by more than one type of parasite harboring distinct GRA6 alleles (Table 5), reinforcing that, in the present study, the parasites are different from the three classic archetypal genotypes[5,6], although they may still be identical to the Brazilian BrII lineage (ToxoDB#2), which is characterized by the presence of the same 5' and 3'-SAG2 allele I, SAG3 allele III and GRA6 allele III[12,13] and is associated with moderately virulent parasites in mice, while the clinical phenotypes of infected infants in this study varied from asymptomatic infants to severely impaired ones with the Sabin's triad of symptoms.

Possible matches for this combination of the SAG2, SAG3 and GRA6 alleles are ToxoDB#65, which predominated in another study carried out in Sao Paulo State, although this ToxoDB#65 showed a highly severe phenotype in human patients[27]. Other possibilities are ToxoDB#17[28], ToxoDB#9 or ToxoDB#14[16], ToxoDB#36[12], ToxoDB#210, ToxoDB#211 or ToxoDB#212[12,13], ToxoDB#210, ToxoDB#211 or ToxoDB#212[12], ToxoDB#226[13], ToxoDB#52, ToxoDB#111 and ToxoDB#143[28]. It is noteworthy that all these ToxoDB# genotypes are non-archetypal and they result in heterogeneous clinical phenotypes, the same situation hypothesized for most samples in this study.

Our study has strengths and limitations. As strengths, we highlight the large number of confirmed cases of congenital toxoplasmosis (n=122), and the outpatient follow-up extended for an entire year, characterizing the infants according to clinical, neurological, ophthalmological and laboratory parameters, including imaging tests[2,3].

The limitations of the study were the lack of information on the virulence of parasites in mice and the failure to amplify all the multilocus-nested-PCR-RFLP markers. To circumvent these limitations, we used two surrogate markers of virulence: the parasite load levels estimated in amniotic fluid samples by Real-Time PCR at the time of the diagnosis of toxoplasmosis in pregnant women, bearing in mind that this parameter has already proven to be associated with the presence of symptoms and the severity of congenital toxoplasmosis[3]; we also analyzed data from the clinical and laboratory follow-up until the end of the first year of life, which allowed us to classify the severity of congenital infections based on true clinical phenotypes.

After comparing the SAG2, SAG3 and GRA6 alleles with the levels of parasite load in amniotic fluid samples and the severity of congenital toxoplasmosis, we did not find any relationship between the parameters, but and these results are corroborated by studies carried out in South America[8,26,29] and abroad[9,10,30].

In future analyses, the virulence in mice should always be attempted and it would be interesting to change the line of procedures, starting with the collection and storage of amniotic fluid samples in tubes containing substances specifically designed to preserve nucleic acid molecules, increasing the chances of performing a whole genome sequencing of parasites instead of the multilocus-nested-PCR followed by RFLP analysis to obtain the parasite genotype.

CONCLUSION

In conclusion, when SAG2, SAG3 alleles confirmed by restriction enzyme sites and single nucleotide polymorphisms, in addition to GRA6 alleles confirmed by restriction site analysis but only partially confirmed by single nucleotide polymorphisms were compared with the levels of parasites in amniotic fluid samples and the clinical outcome of infections after a follow-up of 12 months, we did not find any relationship between the parameters.

AUTHORS' CONTRIBUTION

LST performed the genotyping experiments by multilocus-nested-PCR; EHS performed all the RFLP experiments; LY performed and supervised the analysis of single nucleotide polymorphisms; GAB organized the database and entered all data into the system; KAR performed the DNA extractions; MCR performed the DNA extractions; KAK performed and supervised the analysis of single nucleotide polymorphisms, elaborated the tables and revised the manuscript; TSO conceived the study, was responsible for the grant to finance the experiments and wrote the manuscript.

CONFLICT OF INTERESTS

The authors declare that they do not have any type of conflict of interests.

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