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HLA-DQA1/B1 alleles as putative susceptibility markers in congenital toxoplasmosis

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ABSTRACT

Host and parasite genotypes are among the factors associated with congenital toxoplasmosis pathogenesis. As HLA class II molecules play a key role in the immune system regulation, the aim of this study was to investigate whether HLA-DQA1/B1 alleles are associated with susceptibility or protection to congenital toxoplasmosis. One hundred and twenty-two fetuses with and 103 without toxoplasmosis were studied. The two study groups were comparable according to a number of socio-demographic and genetic variables. HLA alleles were typed by PCR-SSP. In the HLA-DQA1 region, the allele frequencies showed that *01:03 and *03:02 alleles could confer susceptibility (OR= 3.06, p = 0.0002 and OR= 9.60, p= 0.0001, respectively) as they were more frequent among infected fetuses. Regarding the HLA-DQB1 region, the *05:04 allele could confer susceptibility (OR = 6.95, p < 0.0001). Of the 122 infected fetuses, 10 presented susceptibility haplotypes contrasting with only one in the non-infected group. This difference was not statistically significant after correction for multiple comparison (OR = 9.37, p=0.011). In the casuistic, there were two severely damaged fetuses with high parasite loads determined in amniotic fluid samples and HLA-DQA1 susceptibility alleles. In the present study, a discriminatory potential of HLA-DQA1/B1 alleles to identify susceptibility to congenital toxoplasmosis and the most severe cases has been shown.

Introduction

Toxoplasma gondii is a widely distributed apicomplexan parasite, and human infections can be severe in immune compromised patients and in fetuses/newborns.[1-3]

In the congenital toxoplasmosis model, the severity of infection appears to depend mainly on the gestational age at which the transmission took place, but also on the parasite load determined in amniotic fluid samples, and possibly on the parasite genotype and the genetic predisposition of the host.[3-7]

All human diseases have genetic components, which can be decisive for the outcome of the disease, or be combined with environmental factors. It is already known that, in a given population, there are subjects that are more vulnerable (susceptible) to certain diseases, while others are resistant (protected).[8,9]

HLA class II molecules are responsible for the presentation of processed peptide antigens to CD4 T cells and thus play a key role in the immune system regulation.[9-12] In the present study, the choice for these regions (DQA1 and DQB1) was due to their crucial role in encoding the

HLA class II molecules. In fact, these regions, especially the exons 2 and 3 which encode the $\alpha 1$ and $\beta 1$ chains of the HLA-DQ subunits A1 and B1, are the regions that form the antigen cleft, where most of the genetic polymorphisms are concentrated.[8,9]

It is likely that the severity of congenital toxoplasmosis might be influenced by the presence or absence of one HLA allele or more alleles forming haplotypes. This is the reason why the present study sought to investigate the association between HLA-DQA1/DQB1 alleles and haplotypes with susceptibility or protection to congenital toxoplasmosis.

Patients and methods

Group of fetuses with toxoplasmosis

Pregnant women who received prenatal care in public hospitals of the city of Sao Paulo in the southern region of Brazil and who had a screening serology result showing seroconversion to toxoplasmosis during pregnancy evaluated by commercial enzyme

immunoassays, immune chemiluminescent or immunofluorometric tests, were enrolled in the present study. These serological results were confirmed by indirect immunofluorescence tests or by IgM capture enzyme immunoassay techniques, depending on each reference hospital's expertise. The second parameter that was required to meet the inclusion criteria of the present study was a positive detection of *T. gondii* DNA in amniotic fluid samples by conventional nested PCR and also by a Real Time PCR according to previously described protocols.[13,14] Finally, a case of congenital toxoplasmosis must have been confirmed at birth or at some point during the follow-up of the infants, which were performed until the age of 12 months, coinciding with the end of the toxoplasmosis treatment in infants. The confirmation of the newborn/infant diagnosis was based on the presence of anti-*T. gondii* IgM antibodies and/or high IgG titers (significantly higher than the maternal titers). In the absence of positive IgM antibodies, high IgG titers should necessarily be associated with signs and symptoms suggestive of toxoplasmosis, and/or cranial ultrasound and/or eye fundus abnormalities. These complementary tests and the serology were repeated every three months during the 12 months follow-up.

Amniocentesis was performed under ultrasound guidance during the second trimester (15-25 weeks of gestation), with a seroconversion occurring approximately four weeks earlier. At the time that amniocentesis was performed, all of the pregnant women had been treated with spiramycin for a minimum of one week to a maximum of four weeks before the procedure.

The exclusion criteria for this study included inadequate amniotic fluid samples due to the presence of blood, meconium, or sample insufficiency (less than 3 ml of amniotic fluid). All patients were informed of the amniocentesis risks and the nucleic acid procedures and consented with all subsequent laboratory testing.

Group of fetuses without toxoplasmosis (control group)

A group of women who had also received prenatal care in the same public hospitals was recruited. Only pregnant women with no abnormalities detected in the prenatal care and presenting serological results indicating a previous contact with *Toxoplasma gondii* (positive and low titers of IgG and negative IgM), therefore presenting a minimum risk of toxoplasmosis transmission to the fetus were included. In this control group, at birth, in the delivery room, blood samples were collected directly from the placentas, and transferred onto sterile EDTA tubes.

DNA extraction and DNA concentration assessment

Amniotic fluid samples (study group) or blood samples (control group) were submitted to DNA extraction using a commercial kit (QIAamp DNA Mini Kit, QIAGEN) according to the manufacturer's instructions. DNA concentration was estimated by UV spectrophotometry at 260 nm (NanoDrop 1000, Thermo Scientific).

Analysis of the HLA-DQA1 and –DQB1 alleles in fetuses with and without toxoplamosis

The genotyping of the HLA-DQA1 and -DQB1 alleles was performed as previously described,[15,16] using sequence-specific primers amplifications (PCR-SSP). In the DQA1 region, 10 alleles have been identified, while in the DQB1 region, which is much more polymorphic than the DQA1 region, 19 alleles have been identified. Briefly, the master mix of reagents for all of the alleles of the DQA1 and the DQB1 region was prepared with 50 ng of genomic DNA, 0.4 uM of each primer; 200 uM of dNTPs; 1.5 mM of $MgCl_2$; 1.25 U of Taq DNA polymerase (Promega Corp.) for reactions with a final volume of 25 μL. The amplification conditions used for the DQA1 alleles were: an initial denaturation step at 95°C for 3 min.; 30 cycles of 95°C for 30 sec., 65°C for 60 sec. and 72°C for 60 sec.; ending with a final extension at 72°C for 3 min. For the DQB1 alleles the conditions were: an initial denaturation step at 95°C for 3 min.; 40 cycles of 95°C for 30 sec., 65°C for 60 sec. and 72°C for 60 sec.; ending with a final extension at 72°C for 3 min. The amplified products were applied to agarose gels 2:1 (agarose: NuSieve) (Invitrogen™ and Cambrex BioScience) prepared in TAE buffer 1X (Tris - Glacial Acetic Acid - 1M EDTA) and subjected to horizontal electrophoresis (HE- 58, GE Healthcare) at 80 V for 30 minutes, and subsequently stained with ethidium bromide at 0.5 ug/ml (PlusOne Ethidium Bromide, GE Healthcare). The amplification results were visualized in a transilluminator apparatus with ultraviolet light (BioDoc-It 210, UVP).

Statistical analysis

A data bank of HLA-DQA1 and -DQB1 processing results was created in Microsoft Excel 2007. Data were expressed as allele frequency, calculated as the ratio of the number of times that different alleles appeared in the sample to the total number of alleles. The p-value was calculated using a Chi-square (χ^2) test or the Fisher's

exact. Odds ratios (OR) at a 95% confidence interval (CI) were calculated. The Bonferroni correction was performed by multiplying the p-value by the number of alleles detected for each locus. Thus, regarding the DQA1 region, where 10 alleles were amplified, significant pc-values were those < 0.005, while in the DQB1 region, due to a high ambiguity at the *06 locus, the frequencies of the *06 alleles were not discriminated, and the results of this locus were expressed as the total number of alleles at *06 locus. Therefore, in the DQB1 region p-values were divided by 12 alleles so that significant pc-values were those < 0.0041. To compare the frequency of DQA1 and DQB1 haplotypes in the two groups, nine possible combinations of alleles forming haplotypes were considered and significant pc-values were those < 0.005. All statistical analyzes were performed using the Sigma Stat 3.5 program (Advisory Statistics for Scientists) and the Prism software (Graph Pad 5.0).

To determine if the allele frequencies were in Hardy–Weinberg equilibrium (HW) we used the equation available at http://www.oege.org/software/hwe-mr-calc.shtml.

Ethical approval

This research was approved by the Institutional Ethics Committee (CAPPesq protocol number 0347/2011) and was performed in São Paulo, which is the most populous state in Brazil, located in the southeastern region of the country.

Results

We analyzed 122 fetuses in the group with toxoplasmosis and 103 fetuses in the control group. This was a convenience sample consisting of all positive cases that could be recruited in the four participating services in the three

Table 1. Distribution of the two groups (with toxoplasmosis and without toxoplasmosis - control) according to the ethnicity, demographic, social and economic characteristics and other parameters that are of interest in the context of toxoplasmosis.

Variable		Toxoplasmosis N=122 (%)	Control group N=103 (%)	p value*
Maternal age (years)	< 20	23 (18.8)	21 (20.4)	0.965
	20-29	65 (53.3)	56 (54.4)	
	30-34	20 (16.4)	16 (15.5)	
	≥ 35	14 (11.5)	10 (9.7)	
Mother's skin color	White	56 (45.9)	46 (44.6)	0.937
	Mixed-ethnicity[a]	47 (38.5)	42 (40.8)	
	Black	19 (15.6)	15 (14.6)	
Newborn's skin color	White	55 (45.1)	46 (44.6)	0.966
	Mixed-ethnicity[a]	48 (39.3)	42 (40.8)	
	Black	19 (15.6)	15 (14.6)	
Maternal education (years)	0-4	56 (46)	43 (41.8)	0.325
	5-8	64 (52.4)	60 (58.2)	
	9-11	2 (1.6)	0 (0)	
Family income (quartiles)[b]	≤ 1 (lowest)	74 (60.6)	65 (63.1)	0.865
	2	46 (37.7)	37 (35.9)	
	3	2 (1.7)	1 (1)	
Parity	None	65 (53.3)	53 (51.5)	0.823
	1	49 (40.2)	41 (39.8)	
	2	8 (6.5)	9 (8.7)	
Prenatal care[c]	Yes	98 (80.3)	103 (100)	< 0.0001
	No	24 (19.7)	0 (0)	
Delivery	Vaginal	78 (64)	60 (58.2)	0.383
	Cesarean	44 (36)	43 (41.8)	
Gestational age[d]	Preterm	22 (18)	0 (0)	< 0.0001
	Full term	100 (82)	103 (100)	
Birth weight (Kg)	>3	50 (40.1)	75 (72.8)	< 0.0001
	2.5 – 3	58 (47.5)	26 (25.2)	
	< 2.5	14 (11.5)	2 (1.9)	
Household contact with animals[e]	Yes	55 (45.1)	50 (48.5)	0.604
	No	67 (54.9)	53 (51.5)	
Consumption of meat per week	1	35 (28.7)	23 (22.3)	0.493
	2	49 (40.2)	48 (46.6)	
	3	38 (31.1)	32 (31.1)	
Ingestion of raw or undercook meat	Yes	18 (14.7)	17 (16.5)	0.718
	No	104 (85.3)	86 (83.5)	

[a] Mixed ethnicity refers to a mixture of black and white;
[b] The family income was divided into quartiles according to the Brazilian minimum wage (1 minimum wage = U$240 American dollars);
[c] Prenatal care: yes refers to > 3 prenatal care appointments; No refers to no prenatal care at all or < 3 appointments;
[d] Gestational age: preterm < 37 weeks and full term 37- 40 weeks (gestational ages were estimated at birth by the last menstrual period, the ultrasound performed at 21– 28 weeks, the ultrasound at 29 weeks and the method of Capurro, even in preterm infants;[17]
[e] Household contact with animals refers mainly to cats and dogs;
* p value was calculated using (χ^2) test or the Fisher's exact test was used when the cell counts were < 5.

years period in which the survey was conducted. Data related to the demographic, social and economic characteristics, as well as other parameters that are relevant in the context of toxoplasmosis are shown in Table 1. A statistical significant difference between the groups with and without toxoplasmosis was observed for the following variables: prenatal care, gestational age and birth weight (p<0 .0001, Chi-square test or Fisher's exact test).

When the allele frequencies of the group with toxoplasmosis was compared with the ones without toxoplasmosis, it was observed that the alleles *01:03 and *03:02 were more frequent in the fetuses with toxoplasmosis (20.5 and 8.6%, respectively) than in the control group (7.8 and 1.0%, respectively). These differences (OR= 3.06, p = 0.0002 and OR= 9.60, p= 0.0001, respectively) are statistically significant and suggest that the alleles *01:03 and *03:02 could confer susceptibility to toxoplasmosis. The findings of the HLA-DQA1 alleles in the two groups (with and without toxoplasmosis) are presented in Table 2.

Regarding the DQB1 region, the allele *05:04 could confer susceptibility as it was more frequent in fetuses with toxoplasmosis (14.7%, OR= 6.95 and p < 0.0001) (Table 3).

In this study, considering the susceptibility to toxoplasmosis, three alleles were identified: DQA1 *01:03 and *03:02, and DQB1 *05:04.

The comparison of the haplotype frequencies in the two groups was performed by the direct counting of the number of cases presenting two or three DQA1 and DQB1 susceptibility alleles, forming haplotypes. In the group of 122 infected fetuses, 10 presented susceptibility haplotypes contrasting with only one in the non-infected group. The Fisher's exact test showed the following results (OR = 9.37, p = 0.011), i.e., the difference between the two groups was not significant (Table 4).

The 10 infected fetuses presented the following haplotypes:

1. four fetuses were DQA1*01:03/DQB1*05:04 - heterozygous for both alleles;
2. two fetuses were DQA1*01:03/*03:02/DQB1*05:04 – heterozygous for the three alleles;
3. one fetus was DQA1*01:03/*01:03/DQB1*05:04 – homozygous for DQA1*01:03 and heterozygous for DQB1*05:04;
4. one fetus was DQA1*03:02/*03:02/DQB1*05:04 – homozygous for DQA1*03:02 and heterozygous for *05:04;
5. one fetus was DQA1*01:03/DQB1*05:04/*0504 – heterozygous for DQA1*01:03 and homozygous for DQB1*0504;
6. one fetus was DQA1*03:02/DQB1*05:04 - heterozygous for both alleles.

The only case found in the control group presented the haplotype DQA1*01:03/*01:03/DQB1*05:04 i.e., was homozygous for DQA1*01:03 and heterozygous for DQB1*05:04.

The distribution of the allele proportions in either the DQA1 region or the DQB1 region was in Hardy–Weinberg equilibrium (HW) in both groups.

Discussion

The aim of the present study was to investigate the association between HLA-DQA1/DQB1 alleles and haplotypes with susceptibility or protection to congenital toxoplasmosis.

To allow the comparison of the group of fetuses, with and without congenital toxoplasmosis, a number of socio-demographic and genetic factors such as the maternal age, the mothers and newborns ethnicity, maternal education, family income, mother's parity and

Table 2. Description of the 10 alleles investigated in the HLA-DQA1 region, the number of alleles and the allele frequency in the 122 fetuses with congenital toxoplasmosis (study group) and in the 103 fetuses of the control group.

DQA1 Alleles	N° of alleles		Allele frequency		OR (95% CI)	Bonferroni p < 0.005
	Control group	Study group	Control group	Study group		
*01:01	12	17	5.8	7.0	1.21 (0.56 – 2.59)	0.702
*01:02	37	22	18.0	9.0	0.45 (0.25 – 0.79)	0.0073
01:03	**16**	**50**	**7.8**	**20.5**	**3.06 (1.68 – 5.56)**	**0.0002**
*01:04	19	9	9.2	3.7	0.37 (0.16 – 0.85)	0.018
*02:01	25	12	12.1	4.9	0.37 (0.18 – 0.76)	0.005
*03:01	29	24	14.1	9.8	0.66 (0.37 – 1.18)	0.187
03:02	**2**	**21**	**1.0**	**8.6**	**9.60 (2.22 – 41.49)**	**0.0001**
*04:01	16	28	7.8	11.5	1.53 (0.80-2.93)	0.205
*05:01	48	53	23.3	21.7	0.91 (0.58 – 1.42)	0.734
*06:01	2	8	1.0	3.3	3.45 (0.72 – 16.47)	0.118
Total	206	244	100	100	–	–

OR: odds ratio; CI: confidence interval; Bonferroni correction was calculated considering significant
p value 0.05/10 combinations of DQA1 alleles. The alleles presented in **bold** could confer susceptibility to congenital toxoplasmosis.

Table 3. Description of the five loci and 12 alleles investigated in the HLA-DQB1 region, the number of alleles in each group and the allele frequency in the 122 fetuses with congenital toxoplasmosis (study group) and in the 103 fetuses of the control group.

DQB1 Alleles	N° of alleles		Allele frequency		OR (95% CI)	Bonferroni p < 0.0041
	Control group	Study group	Control group	Study group		
*02:01	22	20	10.7	8.2	0.74 (0.39-1.41)	0.417
*03:01	33	23	16.0	9.4	0.54 (0.30-0.96)	0.044
*03:02	5	8	2.4	3.3	1.36 (0.43-4.23)	0.779
*03:03	6	5	2.9	2.1	0.69 (0.20-2.32)	0.559
*03:04	3	7	1.4	2.9	1.99 (0.51-7.83)	0.356
*04:01	2	2	0.9	0.8	0.84 (0.11-6.04)	1.000
*04:02	13	12	6.3	4.9	0.76 (0.34-1.72)	0.542
*05:01	26	22	12.6	9.0	0.68 (0.37-1.25)	0.224
*05:02	2	7	0.9	2.9	3.01 (0.61-14.67)	0.189
*05:03	22	14	10.7	5.7	0.50 (0.25-1.02)	0.057
*05:04	**5**	**36**	**2.4**	**14.7**	**6.95 (2.67-18.09)**	**<0.0001**
*06[§]	67	88	32.5	36.1	1.17 (0.79-1.73)	0.485
Total	206	244	100	100	–	–

Notes. Due to a high ambiguity, the frequencies of the *06 alleles were not discriminated, and the results were expressed as the total number of alleles at the *06 locus. OR: odds ratio; CI: confidence interval; Bonferroni correction was calculated considering significant *p* values of 0.05/12 combinations of DQB1 alleles. The allele presented in **bold** could confer susceptibility to toxoplasmosis
[§] Due to a high ambiguity, the frequencies of the

type of delivery, were found to be comparable in both groups. In contrast, the percentage of prenatal care, as well as the newborns gestational age and birth weight were different, as expected (Table 1).

One could criticize the choice of the control group in this study because it was composed of uninfected fetuses whose mothers were healthy, presented a previous *T. gondii* infection revealed by the presence of anti-*T. gondii* IgG antibodies. Instead of these pregnant women, we could have used others that had also seroconverted during pregnancy but did not transmit the infection to the fetuses, and therefore presented negative PCR results in amniotic fluid samples. However, this alternative control group could face other criticisms because the transmission of *T. gondii* to the fetus can take place after the completion of amniocentesis especially when amniocentesis is performed before 18 weeks of gestation.[18] Moreover, it is recognized that the positivity of PCR depends on the gestational age at the time of maternal infection. The earlier the amniocentesis is performed, the lower the PCR sensitivity.[19] Montoya and Remington recommended that amniocentesis should not be performed at less than 18 weeks' gestation, and should be offered no less than 4 weeks after suspected acute maternal infection to lower the occurrence of PCR false-negative results.[18]

Another problem that might jeopardize the control group is that PCR could be negative due to a lack of

sensitivity of the amplification method, generating a false-negative result. It is known that more recent techniques such as Real Time PCR tend to be more sensitive than conventional PCR, but even these quantitative techniques are not able to rule out the presence of infection[20] i.e., there will be always around 10% of the casuistic that will be infected and present negative serological results at birth.[14,19-23]

A factor that is likely to decrease the sensitivity of PCR is that all of the pregnant women were under treatment at the time of amniocentesis because of the protocol to treat pregnant women that is recommended by the Brazilian Ministry of Health. Due to the treatment administered to women who have seroconverted during pregnancy with either spiramycin, or a combination of sulfadiazine and pyrimethamine, and also to the fact that the newborns of these women also receive, even if in an indirect way, the drug through the placenta, it is common that fetuses are asymptomatic at birth and during the first months of life and also have undetectable IgM antibodies accompanied by IgG levels that are similar to the maternal ones or even lower, especially if the mothers began the treatment early in pregnancy, and received it for prolonged periods. Thus, the absence of IgM antibodies do not absolutely rule out the possibility of infection in newborns whose mothers were treated during pregnancy, i.e., specific antibody production may be inhibited

Table 4. The number of cases in each group carrying the susceptibility haplotypes in the HLA-DQA1 and -DQB1 regions.

Susceptibility haplotypes	Study group	Control group	OR (CI - 95%)	Bonferroni p<0.005
Cases/total (%)	10/122 (8.20)	1/103 (0.97)	9.37 (1.17-74.54)	0.011

OR: odds ratio; CI: confidence interval; Bonferroni correction was calculated considering significant *p* value 0.05/9 combinations of DQA1and DQB1 haplotypes.

by the maternal treatment, and also by the treatment administered to the infants during the first year of life.[3,24]

These are some of the reasons that explain why we have decided to use a control group composed of women who had already been exposed to toxoplasmosis and presented an absolutely normal prenatal care, and therefore had a minimum probability to transmit the infection to their fetuses. In these mothers, congenital toxoplasmosis would only occur in extremely rare cases of reactivation or reinfection with another strain of *Toxoplasma gondii*.[25-29]

Another interesting topic regarding the newborn/infants treatment is that in some cases, the production of specific anti-*Toxoplasma gondii* antibodies by the unsuspected infected infant is delayed until some months after the interruption of the treatment.[30] Then, after variable periods of time, the infected child will begin to produce antibodies to *T. gondii*, and this phenomenon is called serological rebound.[31]

Therefore a negative serological pattern can be a transitory phenomenon in congenital toxoplasmosis as a consequence of maternal and neonatal treatment, particularly when the maternal infection has occurred during the first two trimesters of pregnancy.[32,33]

A supplementary point that merits attention is the fact that even with prolonged and repeatedly negative anti-*Toxoplasma* serology, a congenitally infected infant can develop chorioretinitis much later, e.g., at the age of 13 years.[34]

The present study design does not allow the association of maternal genetic factors represented by the maternal HLA-DQA1/DQB1 that could predispose to toxoplasmosis, mainly regarding the vertical transmission of toxoplasmosis. However, it is possible that genetic maternal factors are not predominant when the development and severity of manifestations in infected infants are considered. In the present study the main goal was to determine the fetal HLA-DQA1/DQB1 alleles and haplotypes that could influence somehow the severity of congenital toxoplasmosis.

Considering the outcome of infections after a 12 months' follow-up, in only two fetuses with toxoplasmosis of the 122 studied (1.64%) the congenital infection was considered severe. These two infants presented with the Sabin's triad of symptoms (hydrocephalus, cerebral calcifications and chorioretinitis) and high parasite loads determined by Real Time PCR[14] in amniotic fluid samples (10^3 and 10^4 parasites/ml) despite the mothers' treatment. These two fetuses carried susceptibility alleles, one of them was heterozygous for HLA-DQA1 *01:03 and the other was homozygous for HLA-DQA1 *01:03, respectively.

It is noteworthy that the frequency of the DQA1 *02:01 allele was higher in the group of fetuses with toxoplasmosis [OR 0.37 (0.18-0.76) and pc-value= 0.005], Table 2, but as we decided to accept as statistically significant only results with pc-values < 0.005, this allele was not highlighted in the present study, nevertheless should be re-analyzed in replication studies.

In the present report, all of the HLA typing experiments were repeated due to the high ambiguity found in the DQB1 region, more specifically at the *06 locus. After the repetition, it was concluded that the primers described by Olerup et al. and Mullighan et al.[15,16] can be moderately/highly discriminatory in other populations, but have failed to discriminate the alleles at the *06 locus in our series of Brazilian pregnant women belonging to a population with a high degree of racial miscegenation. Therefore, as the problem took place in both studied groups (with and without toxoplasmosis), we analyzed the DQB1 alleles results at the *06 locus as a whole (locus DQB1 *06), and no statistical difference was found between the two groups. Anyway, we succeeded in classifying patients as being homozygous or heterozygous at the *06 locus since all the other amplifications corresponding to alleles present at DQB1 loci (*02; *03, *04, *05) were highly discriminatory and generated unambiguous results.

There are few studies on the variability of HLA alleles in cases of congenital toxoplasmosis. Meenken et al.[35] have conducted the HLA serotyping of the regions (HLA-A, HLA-B, HLA-C and HLA-D) in 52 newborns with congenital toxoplasmosis and Mack et al.[36] have serotyped the HLA-DQ alleles of 23 infants with toxoplasmosis and hydrocephalus, 45 infants with toxoplasmosis without hydrocephalus, and a control group of 232 healthy individuals concluding that the frequency of the DQ3 allele was significantly higher in infants with toxoplasmosis and hydrocephalus.

In our study, the alleles DQA1 *01:03 and *03:02 were associated with increased susceptibility to toxoplasmosis, but the simple comparison with the results of Meenken et al. and Mack et al. is a difficult issue due to methodological differences (serotyping versus genotyping).[35,36] Nevertheless, it is likely that the DQ3 identified by Mack et al.[36] as a susceptibility allele corresponds to the allele *03:02 evidenced in the present study also associated with susceptibility to congenital toxoplasmosis. Meenken et al.[35] have only found differences in the HLA-B region, and we did not investigate this HLA region.

In 2005, Habegger de Sorrentino et al.[37] have studied 220 individuals, 112 HIV-negative healthy subjects, and 108 HIV-positive patients (18 with AIDS and neuro-toxoplasmosis, 49 with AIDS without neuro-toxoplasmosis, and 41 asymptomatic). HLA-DR and -DQ genotyping was performed by molecular biology techniques. The HLA-DQB *04:02 (OR = 20.43) and DRB1 *08 (OR = 11) alleles were associated with a higher risk of developing neuro-toxoplasmosis. In the present study, no

differences were found in the frequencies of the DQB1 *04:02 allele and we did not perform the investigation of the DRB1 region. In addition, we studied congenital toxoplasmosis and Habegger de Sorrentino et al.[37] studied HIV-positive patients with or without neuro-toxoplasmosis.

Regarding the frequency of HLA-DQA1/DQB1 haplotypes in the group of infected fetuses with respect to the non-infected ones, the results of the present study did not show a higher frequency of these haplotypes in the group of infected fetuses (pc-value found 0.011 vs. significant pc-value < 0.005), however, it is possible that in future studies, the statistical significance could be reached if one analyzes a higher number of patients (Table 4).

In the present study, the results of the HLA-DQA1/DQB1 allele frequencies are promising and original. Furthermore, the number of cases reported here, however limited to some extent, are higher than those of studies on the same subject.[35-37] Nevertheless, replication studies with a higher number of patients and controls are needed to corroborate the results presented herewith, ideally accompanied by the simultaneous determination of the mothers' HLA. Another interesting perspective will be the concomitant study of the DRB1 region.

A discriminatory potential of HLA-DQA1 and -DQB1 alleles to identify susceptibility to congenital toxoplasmosis and the most severe cases in this casuistic has been shown. This study opens up interesting prospects for the future such as the proposal of a new therapeutic approach for congenital toxoplasmosis. Using the genetic profile of infected fetuses it would be possible to address more aggressive treatment regimens in cases in which the fetuses carry HLA-DQA1 or -DQB1 alleles that have been associated with increased susceptibility to infection, possibly contributing to reduce the morbidity and mortality attributable to congenital toxoplasmosis.

Abbreviations

AIDS   Acquired immunodeficiency syndrome
DNA   Desoxyribonucleic acid
HIV   Human immunodeficiency virus
HLA   Human leucocytes antigens
IgG   Immunoglobulin G
IgM   Immunoglobulin M
OR   Odds ratio
PCR   Polymerase chain reaction
SSP   Sequence-specific primers
UV   Ultraviolet light

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Author contributions

PTS and LST carried out the acquisition of the data and performed the experiments, LY, JCR, KAK carried out initial analysis and the interpretation of the data; TSO conceptualized and designed the study, coordinated and supervised data collection, and wrote the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

References

[1] Hill DE, Chirukandoth S, Dubey JP. Biology and epidemiology of Toxoplasma gondii in man and animals. Anim Health Res Rev 2005; 6:41-61; PMID:16164008; http://dx.doi.org/10.1079/AHR2005100

[2] Dubey JP, Lago EG, Gennari SM, Su C, Jones JL. Toxoplasmosis in humans and animals in Brazil: high prevalence, high burden of disease, and epidemiology. Parasitology 2012; 139:1375-424; PMID:22776427; http://dx.doi.org/10.1017/S0031182012000765

[3] Robert-Gangneux F. It is not only the cat that did it: How to prevent and treat congenital toxoplasmosis. J Infect 2014; 68:S125-33; PMID:24119928; http://dx.doi.org/10.1016/j.jinf.2013.09.023

[4] Howe DK, Sibley LD. Toxoplasma gondii comprises three clonal lineages: correlation of parasite genotype with human disease. J Infect Dis 1995; 172:1561-6; PMID:7594717; http://dx.doi.org/10.1093/infdis/172.6.1561

[5] Costa JM, Pautas C, Ernault P, Foulet F, Cordonnier C, Bretagne S. Real-time PCR for diagnosis and follow-up of Toxoplasma reactivation after allogeneic stem cell transplantation using fluorescence resonance energy transfer hybridization probes. J Clin Microbiol 2000; 38:2929-32; PMID:10921953

[6] Romand S, Chosson M, Franck J, Wallon M, Kieffer F, Kaiser K, Dumon H, Peyron F, Thulliez P, Picot S. Usefulness of quantitative polymerase chain reaction in amniotic fluid as early prognostic marker of fetal infection with Toxoplasma gondii. Am J Obstet Gynecol 2004; 190:797-802

[7] Tan TG, Mui E, Cong H, Witola WH, Montpetit A, Muench SP, Sidney J, Alexander J, Sette A, Grigg ME, et al. Identification of T. gondii epitopes, adjuvants, and host genetic factors that influence protection of mice and humans. Vaccine 2010; 28:3977-89; PMID:20347630; http://dx.doi.org/10.1016/j.vaccine.2010.03.028

[8] Kotb M, Norrby-Teglund A, McGeer A, El-Sherbini H, Dorak MT, Khurshid A, Green K, Peeples J, Wade J, Thomson G, et al. An immunogenetic and molecular basis for differences in outcomes of invasive group A streptococcal infections. Nat Med 2002; 8:1398-404; PMID:12436116; http://dx.doi.org/10.1038/nm1202-800

[9] Trowsdale J, Knight JC. Major histocompatibility complex genomics and human disease. Annu Rev Genomics

Human Genet 2013; 14:301-23; http://dx.doi.org/10.1146/annurev-genom-091212-153455

[10] Klein J, Sato A. The HLA system. First of two parts. N Engl J Med 2000; 343:702-9; PMID:10974135; http://dx.doi.org/10.1056/NEJM200009073431006

[11] Klein J, Sato A. The HLA system. Second of two parts. N Engl J Med 2000; 343:782-6; PMID:10984567; http://dx.doi.org/10.1056/NEJM200009143431106

[12] Thorsby E. A short history of HLA. Tissue Antigens 2009; 74:101-16; PMID:19523022; http://dx.doi.org/10.1111/j.1399-0039.2009.01291.x

[13] Okay TS, Yamamoto L, Oliveira LC, Manuli ER, de Andrade HF, Jr, Del Negro GM. Significant performance variation among PCR systems in diagnosing congenital toxoplasmosis in São Paulo, Brazil: analysis of 467 amniotic fluid samples. Clinics (Sao Paulo) 2009; 64:171-6; PMID:19330240; http://dx.doi.org/10.1590/S1807-59322009000300004

[14] Teixeira LE, Kanunfre KA, Shimokawa PT, Targa LS, Rodrigues JC, Domingues W, Yamamoto L, Okay TS. The performance of four molecular methods for the laboratory diagnosis of congenital toxoplasmosis in amniotic fluid samples. Rev Soc Bras Med Trop 2013; 46:584-8; PMID:24409481; http://dx.doi.org/10.1590/0037-8682-0095-2013

[15] Olerup O, Zetterquist H. HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. Tissue Antigens 1992; 39:225-35; PMID:1357775; http://dx.doi.org/10.1111/j.1399-0039.1992.tb01940.x

[16] Mullighan CG, Bunce M, Welsh KI. High-resolution HLA-DQB1 typing using the polymerase chain reaction and sequence-specific primers. Tissue Antigens 1997; 50:688-92; PMID:9458131; http://dx.doi.org/10.1111/j.1399-0039.1997.tb02936.x

[17] Pereira AP, Dias MA, Bastos MH, da Gama SG, Leal Mdo C. Determining gestational age for public health care users in Brazil: comparison of methods and algorithm creation. BMC Res Notes 2013; 6:60; PMID:23402277; http://dx.doi.org/10.1186/1756-0500-6-60

[18] Montoya JG, Remington JS. Management of Toxoplasma gondii infection during pregnancy. Clin Infect Dis 2008; 47:554-66; PMID:18624630; http://dx.doi.org/10.1086/590149

[19] Sterkers Y, Pratlong F, Albaba S, Loubersac J, Picot MC, Pretet V, Issert E, Boulot P, Bastien P. Novel interpretation of molecular diagnosis of congenital toxoplasmosis according to gestational age at the time of maternal infection. J Clin Microbiol 2012; 50:3944-51; PMID:23035201; http://dx.doi.org/10.1128/JCM.00918-12

[20] Wallon M, Franck J, Thulliez P, Huissoud C, Peyron F, Garcia-Meric P, Kieffer F. Accuracy of real-time polymerase chain reaction for Toxoplasma gondii in amniotic fluid. Obstet Gynecol 2010; 115:727-33; PMID:20308831; http://dx.doi.org/10.1097/AOG.0b013e3181d57b09

[21] Bessières MH, Berrebi A, Cassaing S, Fillaux J, Cambus JP, Berry A, Assouline C, Ayoubi JM, Magnaval JF. Diagnosis of congenital toxoplasmosis: prenatal and neonatal evaluation of methods used in Toulouse University Hospital and incidence of congenital toxoplasmosis. Mem Inst Oswaldo Cruz 2009; 104:389-92; http://dx.doi.org/10.1590/S0074-02762009000200038

[22] Morelle C, Varlet-Marie E, Brenier-Pinchart MP, Cassaing S, Pelloux H, Bastien P, Sterkers Y. Comparative assessment of a commercial kit and two laboratory-developed PCR assays for molecular diagnosis of congenital toxoplasmosis. J Clin Microbiol 2012; 50:3977-82; PMID:23035184; http://dx.doi.org/10.1128/JCM.01959-12

[23] Prusa AR, Kasper DC, Pollak A, Olischar M, Gleiss A, Hayde M. Amniocentesis for the detection of congenital toxoplasmosis: results from the nationwide Austrian prenatal screening program. Clin Microbiol Infect 2015; 21:191.e1-8; PMID:25596783; http://dx.doi.org/10.1016/j.cmi.2014.09.018

[24] Sensini A. Toxoplasma gondii infection in pregnancy: opportunities and pitfalls of serological diagnosis. Clin Microbiol Infect 2006; 12:504-12; PMID:16700697; http://dx.doi.org/10.1111/j.1469-0691.2006.01444.x

[25] Ladas ID, Rallatos CL, Kanaki CS, Damanakis AG, Zafirakis PK, Rallatos G. Presumed congenital ocular toxoplasmosis in two successive siblings. Ophthalmologica 1999; 213:320-2; PMID:10516521; http://dx.doi.org/10.1159/000027446

[26] Silveira C, Ferreira R, Muccioli C, Nussenblatt R, Belfort R, Jr. Toxoplasmosis transmitted to a newborn from the mother infected 20 years earlier. Am J Ophthalmol 2003; 136:370-1; PMID:12888070; http://dx.doi.org/10.1016/S0002-9394(03)00191-0

[27] Kodjikian L, Hoigne I, Adam O, Jacquier P, Aebi-Ochsner C, Aebi C, Garweg JG. Vertical transmission of toxoplasmosis from a chronically infected immunocompetent woman. Pediatr Infect Dis J 2004; 23:272-4; PMID:15014310; http://dx.doi.org/10.1097/01.inf.0000115949.12206.69

[28] Gavinet MF, Robert F, Firtion G, Delouvrier E, Hennequin C, Maurin JR, Tourte-Schaefer C, Dupouy-Camet J. Congenital toxoplasmosis due to maternal reinfection during pregnancy. J Clin Microbiol 1997; 35:1276-7; PMID:9114425

[29] Valdès V, Legagneur H, Watrin V, Paris L, Hascoët JM. Congenital toxoplasmosis due to maternal reinfection during pregnancy. Arch Pediatr 2011; 18:761-3; http://dx.doi.org/10.1016/j.arcped.2011.04.011

[30] Lago EG, Oliveira AP, Bender AL. Presence and duration of anti-Toxoplasma gondii immunoglobulin M in infants with congenital toxoplasmosis. J Pediatr (Rio J) 2014; 90:363-9;PMID:24530469; http://dx.doi.org/10.1016/j.jped.2013.12.006

[31] Wallon M, Cozon G, Ecochard R, Lewin P, Peyron F. Serological rebound in congenital toxoplasmosis: long-term follow-up of 133 children. Eur J Pediatr 2001; 160:534-40; PMID:11585075; http://dx.doi.org/10.1007/s004310100805

[32] Villena I, Aubert D, Leroux B, Dupouy D, Talmud M, Chemla C, Trenque T, Schmit G, Quereux C, Guenounou M, et al. Pyrimethamine-sulfadoxine treatment of congenital toxoplasmosis: follow-up of 78 cases between 1980 and 1997. Reims Toxoplasmosis Group. Scand J Infect Dis 1998; 30:295-300; PMID:9790140; http://dx.doi.org/10.1080/00365549850160963

[33] Jaisson-Hot I, Wallon M, al Kurdi M, Thulliez P, Kahi S, Cozon G, Peyron F. Congenital toxoplasmosis.

Transitory negative serology. Presse Med 2001; 30:1001-4; PMID:11433684

[34] Bidgoli S, Koch P, Caspers L. Toxoplasmic chorioretinitis: positive PCR on vitreous with negative serology for Toxoplasma gondii. J Fr Ophtalmol 2011; 34:384.e1-5; PMID:21492958; http://dx.doi.org/10.1016/j.jfo.2011.01.015

[35] Meenken C, Rothova A, de Waal LP, van der Horst AR, Mesman BJ, Kijlstra A. HLA typing in congenital toxoplasmosis. Br J Ophthalmol 1995; 79:494-7; PMID:7612565; http://dx.doi.org/10.1136/bjo.79.5.494

[36] Mack DG, Johnson JJ, Roberts F, Roberts CW, Estes RG, David C, Grumet FC, McLeod R. HLA-class II genes modify outcome of Toxoplasma gondii infection. Int J Parasitol 1999; 29:1351-8; PMID:10579423; http://dx.doi.org/10.1016/S0020-7519(99)00152-6

[37] Habegger de Sorrentino A, López R, Motta P, Marinic K, Sorrentino A, Iliovich E, Rubio AE, Quarleri J, Salomón H. HLA class II involvement in HIV-associated toxoplasmic encephalitis development. Clin Immunol 2005; 115:133-7; PMID:15885635; http://dx.doi.org/10.1016/j.clim.2005.01.003