*HLA-A**01 allele: a risk factor for dengue haemorrhagic fever in Brazil's population

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*Severe forms of dengue, such as dengue haemorrhagic fever (DHF) and dengue shock syndrome, are examples of a complex pathogenic mechanism in which the virus, environment and host immune response interact. The influence of the host's genetic predisposition to susceptibility or resistance to infectious diseases has been evidenced in several studies. The association of the human leukocyte antigen gene (*HLA*) class I alleles with DHF susceptibility or resistance has been reported in ethnically and geographically distinct populations. Due to these ethnic and viral strain differences, associations occur in each population, independently with a specific allele, which most likely explains the associations of several alleles with DHF. As the potential role of* HLA *alleles in the progression of DHF in Brazilian patients remains unknown, we then identified* HLA-A *alleles in 67 patients with dengue fever and 42 with DHF from Rio de Janeiro, Brazil, selected from 2002-2008 by the sequence-based typing technique. Statistical analysis revealed an association between the* HLA-A**01 allele and DHF [odds ratio (OR) = 2.7, p = 0.01], while analysis of the* HLA-A**31 allele (OR = 0.5, p = 0.11) suggested a potential protective role in DHF that should be further investigated. This study provides evidence that* HLA *class I alleles might be important risk factors for DHF in Brazilian patients.*

Key words: dengue fever - dengue haemorrhagic fever - *HLA-A* - *HLA* typing - Brazil

Dengue fever (DF) is caused by four distinct serotypes (1-4) related to the dengue virus (DENV), a mosquito-borne Flavivirus (Henchal & Putnak 1990, Westaway & Block 1997) that is a frequent cause of illness and death in the tropics and subtropics. DENV has been reported in more than 100 countries and can expand to new areas where the ecology has been changed by mosquitoes and where human demographics have been altered by activities that include urban expansion, augmented vector abundance, amplified trade, population growth and travel (Gubler 2002, 2006).

The spectrum of the illness ranges from unapparent mild disease to a severe and occasionally deadly haemorrhagic clinical form. Most dengue infections are symptomless and may present with classical DF, a mild illness, or with a potentially lethal complication, the dengue haemorrhagic fever (DHF) or dengue shock syndrome (DSS) (WHO 1997, 2009). Dengue infection affects infants, young children and adults, with an estimated 50-100 million cases annually and approximately 250,000-500,000 DHF annually and 22,000 deaths due to DHF, mainly among children (WHO 2009). In the summer of 2001 and in the summer of 2002, the worst Brazilian dengue epidemics took place, starting at the state of Rio de Janeiro (RJ) and soon reaching the other 11 states (Nogueira et al. 2001, Barbosa da Silva Jr et al. 2002). A total of 1,090,058 cases in 2001/2002 with 3,433 DHF and DSS cases infections and 198 deaths were notified (MS 2011). Thereafter, there was a decline in the number of cases, but a new outbreak emerged in 2007/2008 with an even greater number of the cases of DHF and DSS and deaths. Brazilian health authorities have reported a national total of 1,060,234 cases of dengue, including 5,337 DHF/DSS cases with 786 deaths (MS 2011). In the same period, RJ reported 255,116 cases of dengue, including 1,871 DHF/DSS cases with 279 deaths (MS 2011).

DENV-3 has been the predominant circulating serotype in RJ since the major epidemic in 2002. In the 2007/2008 outbreak, the state experienced the renewed circulation of DENV-2 (Nogueira et al. 2007, Teixeira et al. 2009).

In August 2011, Brazilian health authorities had already recorded 616,593 cases of dengue, including 2,753 DHF cases and 398 deaths. In RJ, 143,668 cases of dengue, including 868 DHF/DSS cases and 111 deaths were recorded (MS 2011).

Several risk factors have been proposed for the DHF/DSS evolution in infected dengue individuals. These include the increased possibility of DHF in secondary infections, known as antibody-dependent enhancement (Halstead 1970, 1980, 1988), cell-mediated pathogenesis (Guzman & Kouri 2008), viral strain differences (Diamond et al. 2000), the levels of virus circulating in individuals during the acute phase (Gubler et al. 1981, Vaughn et al. 2000), the nutritional status of the infected individuals (Thisyakorn & Nimmannitya 1993) and the individual's genetic background (de la C Sierra et al. 2007). However, the potential factors contributing to a higher risk of DHF progression are not yet clear.

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Several studies suggest associations between host genetic factors and DHF (Guzman & Kouri 2002, Fink et al. 2006, Coffey et al. 2009). The human leukocyte antigen (*HLA*) gene, which mainly encodes the major histocompatibility complex (MHC) class I and II molecules, has been described as a marker of dengue disease outcome in some countries (Chiewsilp et al. 1981, Paradoa Perez et al. 1987, Loke et al. 2001, LaFleur et al. 2002, Stephens et al. 2002, Polizel et al. 2004, Sierra et al. 2007, Nguyen et al. 2008, Falcon-Lezama et al. 2009, Appanna et al. 2010).

The high polymorphism of the *HLA* gene leads to differences in the nature and magnitude of the immune response (Chaturvedi et al. 2006, Fink et al. 2006). The T cell recognises antigen as a peptide bound by a particular allelic variant of an MHC molecule and will not recognise the same peptide bound to other MHC molecules (Shiina et al. 2009). In fact, the associations with a specific *HLA* allele have not been consistently reproduced as independent studies reported different *HLA* alleles class I and II in associations with both DHF progression and protection (Chiewsilp et al. 1981, Paradoa Perez et al. 1987, Loke et al. 2001, Stephens et al. 2002).

The potential role of *HLA* alleles in the progression of DHF in Brazilian patients remains unknown. Genetic marker identification in dengue prognosis may help contribute to an understanding of DHF immunopathogenesis, allowing therapeutic intervention, information for vaccine design and DHF/DSS prediction in clinical practice. The purpose of this study was to identify the *HLA-A* alleles as risk factors associated with dengue severity in the Brazilian population.

PATIENTS, MATERIALS AND METHODS

Study design and settings - A case-control study of DENV infection was conducted at the Evandro Chagas Clinical Research Institute (IPEC)/Oswaldo Cruz Foundation (Fiocruz). This institute is a reference centre for infectious diseases, including dengue that provides care on an inpatient and outpatient basis. It is one of the hospitals participating in the Brazilian Public Health System (BPHS) in RJ. The Research Ethical Committee of IPEC approved and reviewed this study on September 23 2004 and it is registered at National System of Ethics in Research (portal.saude.gov.br/sisnep/) (0019.1.009.000-04).

Subjects and data collection - The study population was selected during 2002-2008 and was formed by outpatients and inpatients with dengue infection at IPEC/FIOCRUZ, as well as inpatients at the hospital of BPHS. All clinical, serological and demographic data were retrospectively reviewed. The patients' data were extracted from the hospital's clinical protocols. The signs, symptoms and other clinical characteristics of DF were classified as being present, if they were registered on the medical records, or absent, if they were registered as absent or "not registered" on the records.

The patients of control group (DF) and case group (DHF) were selected according to clinical diagnosis following World Health Organization criteria (WHO 1997) and the diagnosis of dengue infection was confirmed by positive serology for anti-dengue IgM.

DF was deemed as an acute febrile illness (less than 7 days of fever) with two or more of the following symptoms: headache, retro-orbital pain, muscle or joint pain, rash or leukopenia. Typical cases of DHF were characterized by DF, in addition to any haemorrhagic manifestation, thrombocytopenia (i.e., platelet count < 100.000/mm^3) and evidence of plasma leakage revealed by more than a 20% rise in haematocrit over baseline. DSS is defined as DHF with signs of circulatory failure, including rapid and weak pulse, narrow pulse pressure (< or equal to 20 mmHg), hypotension relative to individual's age, cold and clammy skin and restlessness. To avoid any misclassification bias, individuals who had DF accompanied by haemorrhagic manifestation were excluded.

Serological tests - The serological tests were performed with PanBio Dengue IgM/IgG Capture ELISA Kit according to the manufacturers' instructions. The primary infection was defined by the presence of dengue-specific IgM in the serum sample from the fifth-seventh day of illness and the absence of IgG antibodies until the fifth day of illness. The secondary infection was defined by the presence of both antibodies (IgM and IgG) in the serum sample from the fifth until the seventh day of illness. All eligible patients were contacted by phone or post and invited to participate in this study and 5 mL blood were collected after obtaining written informed consent.

Genetic molecular analyses - Blood samples containing EDTA were stored at -20ºC until DNA extraction. Genomic DNA was extracted from whole blood samples using the QIAmp DNA Blood Mini Kit (Qiagen, Santa Clarita, CA), according to the manufacturer's instructions. In this study, a molecular technique of high resolution *HLA* sequence-based typing technique (SBT) was used (Santamaria et al. 1993, Bettinotti et al. 1997, Gerlach 2002).

Fragments 462 bp and 571 bp were amplified by polymerase chain reaction (PCR) using primers designed for exon 2; 5'GCCTCTGCGGGGAGAAGCAA3' and 5'GGATCTCGGACCCGGAGACTG3', exon 3; 5'GGTCCGAGATCCACCCCGAA3' and 5'GATTCCTCTCCCTCAGGACCA3', corresponding to the forward and reverse primers, respectively. The 50 µL reaction mixtures were comprised of 90 ng genomic DNA, 0.4 µM each of exon *HLA-A* specific primers, 0.2 mM deoxynucleotide triphosphates (dNTPs), 1.5 mM MgCl2 and 2.5 units of Platinum Taq DNA polymerase (Invitrogen by Life Technology, Carlsbad, CA, USA).

Temperature cycling was performed in an Eppendorf-Master Cycling-PCR machine with the following conditions: denaturation at 95ºC for 3 min, followed by 35 cycles at 95ºC for 50 s, 63ºC for 60 s, 72ºC for 50 s and extension at 72ºC for 8 min. The resulting amplicons were analyzed by agarose gel 1% electrophoresis and visualized by ethidium bromide staining. The DNA image was digitalized using a transiluminator with a system of image capture L-PIX-ST and L-PIX IMAGE 7.1 M Pixel. Images were captured with the software L-PIX IMAGE 1.0.1 (Loccus Biotecnologia, São Paulo, SP, Brazil).

The amplified DNA bands were excised from the gel and purified using the Wizard SV Gel and PCR Clean-Up System Kit (Promega, USA), according to the manufacturer's instructions. Purified PCR products were then sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Inc, USA). Cycle sequencing was prepared as described in the manufacturer's protocol. Each template was sequenced in forward and reverse sequence orientation for exon 2 and exon 3. The excess primers, dNTPs, genomic DNA and dye terminators were removed from the sequencing products using Dye EX 2.0 Spin (Qiagen, Germany), according to the manufacturer's instructions. The reaction products were reconstituted with 15 µL of Hi-Di™ Formamide (PE Applied Biosystems/Perkin-Elmer, Foster City, CA, USA) and analyzed on the ABI Prism® 3730 DNA Analyser at Plataforma Genômica - Sequenciamento de DNA-RPT01A-PDTIS/FIOCRUZ (Otto et al. 2008).

The sequences were edited and assigned using BioEdit software (Hall 1999). *HLA-A* alleles groups were determined by comparing their assembly sequences with a library containing all known *HLA-A* alleles in an IMGT/HLA database (ebi.ac.uk/imgt/hla).

Statistical analysis - The proportion of individuals carrying each *HLA-A* allele was computed for cases and controls separately. Differences between cases and controls were expressed as odds ratios (ORs). Crude ORs were obtained with their 95% confidence limits intervals by the exact method, taking the alleles as exposures and DHF as outcome. There were no missing data in this analysis.

Proportions (%) for categorical data (gender, skin colour etc.), means and standard deviations (SDs) were used to describe characteristics in the study population. Data analysis involved comparisons of proportions or means.

Tests for proportions and the Student's *t* test (means) were used to determine the two-tailed statistical significance of the associations. Probability values less than 0.05 were considered significant. Probability values were corrected after multiplying by the number of comparisons made (Bonferroni correction) and were considered significant when $p < 0.05$.

RESULTS

Participants - The 473 outpatients at IPEC/Fiocruz were examined and had at least one blood sample collected for a serological test during their illness. Among these patients, 97 (20.5%) showed dengue-IgM antibodies: 81 (83.5%) were diagnosed with DF and 16 (16.5%) inpatients were diagnosed with DHF. Among the patients classified as DF, 14 individuals that had a haemorrhagic manifestation were excluded.

One hundred twenty-six patients diagnosed as DHF inpatients in BPHS hospitals were invited to participate in this study. Among these, 26 responded, attending the IPEC for the signature of informed consent and the collection of blood samples. Thus, 67 patients were included in the DF (control group) and 42 patients in the DHF (case group).

Patients' characteristics - The mean age of the subjects included in this study was 32.72 years old (range = 3-87 years). The DHF patients' ages ranged from 12-66 years old, with a mean age of 35.02 years old (SD = 14.8), whereas the DF patients' ages ranged from three-87 years old, with a mean age of 31.28 years old (SD = 13.7). The mean age among the groups (DF and DHF) were similar ($p = 0.19$).

Cases of DHF were more frequent in females (54.8%) than in males (45.2%); however, this was not significant ($p = 0.39$).

Of the 42 DHF cases, 78.6% were white individuals, 19% mulatto and 2.4% black. In the same way, there was also a predominance of whites in the DF patient group (73.1%) in relation to mulatto (20.9%) and black (6%). Therefore, our data did not show that ethnic origin might have an important role in dengue prognosis.

Concerning the serological response in the control group (DF), 12% were classified as having primary infection and 88% were classified as secondary infection. Among the 16 DHF inpatients at IPEC, only 19% reported previous dengue infection, which was confirmed serologically. On the other hand, in 26 DHF patients, it was not possible to determine the infection type because serology for dengue-specific-IgG was not part of the BPHS routine at the time. However, none of these patients reported a previous dengue infection history during the inclusion interview.

Regarding clinical information, the symptoms more frequent in the DF (control) group were prostration ($p = 0.004$), asthenia ($p < 0.01$), anorexia ($p = 0.029$) and back pain ($p = 0.026$). In the DHF (case) group, the symptoms that showed statistical significance were abdominal pain ($p < 0.01$) and diarrhoea ($p < 0.01$). The most commonly reported symptoms in general were fever, headache, myalgia, rash, vomiting, retro-orbital pain, nausea, arthralgia and hypotension (Table I). The least reported symptoms were chills, ascites, elevated liver transaminases and central nervous system alteration.

In DHF patients, the majority of symptoms related to the spontaneous bleeding phenomenon included gingival bleeding, followed by epistaxis, haematemesis, melena, metrorrhagia, hyphema and haematuria. The platelet count ranged from 12.000-99.000/mm^3, with a mean count of 50.000/mm^3. The platelet count in 57% of patients were below 50.000/mm^3. None of the cases selected were classified as DSS.

HLA-A typing - Specific amplification of the *HLA-A* gene was successfully achieved by PCR with designed primers and HLA-SBT was reliable in defining the *HLA-A* alleles groups. There were 12 different *HLA-A* specificities in the control group (DF): *HLA-A** 24 (21.6%), *HLA-A*02 (18.6%), *HLA-A*31 (14.2%), *HLA-A*01 (9%), *HLA-A*03 (8.2%), *HLA-A*30 (7.5%) and *HLA-A*11 (6.7%). The *HLA-A*29, *HLA-A*23, *HLA-A*26, *HLA-A*32 and *HLA-A*33 allele frequencies were under 5%. The case group (DHF) presented 13 different *HLA-A* specificities: *HLA-A*24 (23.8%), *HLA-A*01 (21.4%), *HLA-A*02 (14.3%), *HLA-A*29 (7.1%), *HLA-A*31 (7.1%) and *HLA-A*11 (5.9%). The alleles with frequencies under 5% were *HLA-A*03, *HLA-A*32, *HLA-A*30, *HLA-A*23, *HLA-A*33, *HLA-A*26 and *HLA-A*34.

The OR of *HLA-A**01 was 2.75, indicating that there is an increased risk of DHF progression in subjects carrying this allele.

The OR of *HLA-A**31 allele in the DF group was twice as high as that in the DHF group (OR = 0.5). Although not significant, the OR < 1 value suggests that *HLA-A**31 may offer protection (Table II). After Bonferroni correction, the p value was not significant.

TABLE I

Clinical profile and potential predictors of dengue haemorrhagic fever (DHF) outcomes

Symptoms[a]	DHF (n = 42) n (%)	DF (n = 67) n (%)	p value[b]	Total (n =109) n (%)
Fever	32 (76.1)	59 (88)	0.174	91 (83.4)
Headache	30 (71.4)	55 (82.1)	0.285	85 (77.9)
Myalgia	29 (69)	56 (83.6)	0.122	85 (77.9)
Prostration	13 (30.9)	41 (61.1)	0.004	54 (49.5)
Asthenia	6 (14.2)	44 (65.6)	0.000	50 (45.8)
Rash	15 (35.7)	29 (43.3)	0.560	44 (40.3)
Vomiting	22 (52.3)	22 (32.8)	0.068	44 (40.3)
Retro-orbital pain	14 (33.3)	30 (44.7)	0.325	44 (40.3)
Anorexia	11 (26.1)	33 (49.2)	0.029	44 (40.3)
Nausea	15 (35.7)	26 (38.8)	0.904	41 (37.6)
Arthralgia	12 (28.8)	23 (34.3)	0.678	35 (32.1)
Back pain	7 (16.6)	26 (38.8)	0.026	33 (30.2)
Hypotension	9 (21.4)	11 (16.4)	0.687	20 (18.3)
Abdominal pain	10 (23.8)	0	0.000	10 (9.2)
Diarrhoea	8 (19)	0	0.000	8 (7.3)

a: the most commonly reported symptoms; *b*: value from proportion comparison hypothesis test; DF: dengue fever.

TABLE II

Frequency distribution and association of human leukocyte antigen (*HLA*) gene in dengue haemorrhagic fever (DHF) patients and controls

*HLA-A**	DHF AF n (%)	DF AF n (%)	Odds ratio (95% CI)	p[a]
01	18 (21.4)	12 (9)	2.75 (1.2 < OR < 6.2)	0.012
02	12 (14.3)	25 (18.6)	0.73 (0.3 < OR < 1.5)	0.412
03	4 (4.8)	11 (8.2)	0.57 (0.1 < OR < 1.8)	0.346
11	5 (5.9)	9 (6.7)	0.89 (0.2 < OR < 2.7)	0.843
23	2 (2.4)	4 (3)	0.63 (0.2 < OR < 4.2)	1
24	20 (23.8)	29 (21.6)	1.1 (0.6 < OR < 2.2)	0.708
26	1 (1.2)	4 (3)	0.3 (0.1 < OR < 3.3)	0.651
29	6 (7.1)	6 (4.5)	1.4 (0.5 < OR < 5.0)	0.543
30	3 (3.6)	10 (7.5)	0.5 (0.1 < OR < 1.6)	0.254
31	6 (7.1)	19 (14.2)	0.5 (0.2 < OR < 1.2)	0.115
32	4 (4.8)	3 (2.2)	1.6 (0.5 < OR < 8.7)	0.433
33	2 (2.4)	2 (1.5)	1.0 (0.3 < OR < 9.4)	0.640
34	1 (1.2)	0	1.6 (0.2 < OR < 12.0)	0.385

a: derived from chi-square test; AF: allele frequency; CI: confidence interval; DF: dengue fever; *HLA-A**: *HLA* gene *locus* A specificities.

DISCUSSION

There are few studies documenting the influence of genetic factors for the DENV infection, especially in Latin American countries. The present study provides evidence for the association of *HLA* class I, particularly *locus* A, with progression to DHF in Brazilians patients.

Several studies have been conducted to identify *HLA* class I and II alleles in association with infectious diseases, including dengue (Blackwell et al. 2009). The *HLA* polymorphism has a profound effect on antigen recognition by T cells and the combination of polymorphisms greatly extends the range of peptides that can be presented to T cells by each individual and each population mediating the risk of DENV infection (Fink et al. 2006).

Initially, the identification of different HLA types was obtained almost exclusively by serology. In 1981, for the first time, a positive association was found for *HLA-A*1, *HLA-A*2, *HLA-A*9 and *HLA-B* blank with the severity of DENV infection and HLA-B13 related to protection (Chiewsilp et al. 1981). Another study conducted in 1987 showed a significant frequency increase in *HLA-A*1, *HLA-B* blank and *HLA-CW*1 in DHF/DSS patients, whereas the *HLA-A*29 antigen seems to protect against DHF/DSS (Paradoa Perez et al. 1987). In Thailand, the authors demonstrated that the *HLA-A*1, A2, A9 serotypes were associated with DHF (Chiewsilp et al. 1981) and in Cuba, the *HLA-A*1 association was presented with DHF (Paradoa Perez et al. 1987), while in Brazil an association of DF with *HLA* class I antigens was not found (Polizel et al. 2004).

At the end of the 1980s and through the 1990s, there was a major breakthrough in the molecular technology for *HLA* gene polymorphism identification. The *HLA* allele typing technology is classified as either low or high resolution, according to its specificity. The SBT, a high-resolution molecular typing, allows specific allele identification by homology, according to the IMGT-HLA (ebi.ac.uk/imgt/hla). The first study that used molecular methods evidenced the association of *HLA-A**24 with DHF progression in Vietnamese patients (Loke et al. 2001). Another study conducted in Thai patients showed that *HLA-A**0207 associated with DHF (Stephens et al. 2002).

The present study is the first to identify *HLA* polymorphisms class I alleles in dengue-infected Brazilian individuals using molecular methods. Our results showed that patients with *HLA-A**01 allele groups had a nearly threefold risk of DHF progression (p = 0.012). Similar results were described in Thai children (Chiewsilp et al. 1981) and in Cuban patients (Paradoa Perez et al. 1987). In contrast, the *HLA-A**31 allele was twice more frequent in DF patients than in DHF patients (OR = 0.5, p = 0.115) on a statistical basis. It was not possible to conclude that *HLA-A**31 has any role in the progression of DF to DHF; however, its estimated OR point suggests that its role in DHF protection should be further investigated. On the other hand, this association with *A**31 was observed in Cuban patients with DHF (Sierra et al. 2007).

The lack of consistency in studies on *HLA* allele associations with DHF progression or protection in different populations may be due to ethnic differences. Brazil is a country with a large miscegenation of European

Caucasians, native Amerindians and African blacks. The majority of Caucasians came from Portugal, Spain, Italy and Germany. Most of the Africans brought to Brazil in the colonial period were from equatorial Africa (Moraes et al. 1993). Due to the large size of Brazil and the territorial diversity following its colonization, different regions have a higher or lower predominance of each population's subtypes (Alves-Silva et al. 2000). Because of the significant miscegenation, prospective clinical studies are required to identify a specific *HLA* allele-related to each ethnic group, which would contribute to a deeper understanding of DHF immunopathogenesis in Brazil.

Epidemiological and clinical studies have proposed that sequential infection with different DENV serotypes predisposes the patients toward DHF/DSS (Halstead 1970, 1980, 1988, 2007, Guzman & Kouri 2002). Nonetheless, our findings showed that the majority (88%) of DENV disease patients that had secondary infections confirmed by serological test did not develop DHF and a study conducted in Mexican patients corroborates these findings (LaFleur et al. 2002). Despite this finding, it is important to consider the usefulness of secondary DENV infection as a DHF predictor in clinical practice. Because most dengue infection is symptomless (Guzman & Kouri 2002), physicians would depend on the serological IgG anti-DENV test results up until the seventh day of illness to make an appropriate clinical decision. The diagnostic test is not always available in public hospitals in Brazil; thus, even if secondary infection is an effective DHF predictor, it may not be suitable for clinical practice in this setting.

Regarding the gender distribution of DHF, several studies demonstrated discrepant rates in males and females. A significant female predominance was reported in Asia (Pinheiro & Corber 1997); in Mexico and other Latin American countries, there was no predisposition of a particular gender (Narro-Robles & Gomez-Dantes 1995, Pinheiro & Corber 1997, LaFleur et al. 2002). A study recently conducted in Mexico showed a significant difference of DHF in males ($p < 0.001$) (Gunther et al. 2009). Our findings demonstrated a female predominance, but it was not statistically significant.

The age distribution of DHF cases has changed progressively. The distribution in the Americas is different than that observed in Asia (Guzman & Kouri 2002). Dengue in the Americas, including Brazil, has been an adult disease (Zagne et al. 1994), while in Southeast Asia, DHF/DSS occurs in infants and children (Nimmannitya 1987). DHF or DSS occurred in infants who acquire the maternal dengue antibody and then subsequently experienced a dengue infection (Kliks et al. 1989).

In Brazil, a change in age group predominance was observed during the last countrywide dengue epidemic. More than 53% of cases were in children under 15 years old, while in previous years, the predominance of DHF cases were in the 20-40-year-old age group (Teixeira et al. 2008). This change in the epidemiologic pattern of dengue cases may be attributed to the cyclical oscillation of the individual serotypes (DENV-1, DENV-2 and DENV-3) whereby the dominant type is sequentially replaced over time (Nisalak et al. 2003). Following the pattern observed in Southeast Asia countries, the adults become immune, whereas a relatively large proportion of children and adolescents are still susceptible to dengue infection (Halstead 2006). However, our findings demonstrated that the patients' mean ages with DHF and DF were similar (35 and 31.2 years old, respectively). This could be attributed to the age of the population treated in hospitals in the study.

Regarding race as a risk factor for DHF/DSS, a recent study confirmed that race is an important risk factor for DHF/DSS. The prevalence of Negroid characteristics was associated with a lower incidence of DHF, when compared with those of Caucasoid characteristics (de la C Sierra et al. 2007, Sierra et al. 2007). Other studies in Cuba and Haiti reported a reduced risk for DHF/DSS in patients with African ancestries compared to those with European ancestry (Halstead et al. 2001, Sierra et al. 2007). However, our results showed that patients of white skin colour were predominant in the population studied, independent of clinical outcome (DF/DHF).

The lack of statistical significance between patients' characteristics and loss of significance in the *HLA* class I frequency after Bonferroni correction could be due to the small sample size, a potential limitation of this study.

The correction of p values by Bonferroni's procedure aims at an interpretation of the statistical test applied to the data so that the likelihood of false positives is greatly reduced.

However, it also increases the likelihood of false negatives; therefore, the use of this procedure does not have a consensus among all authors. Other procedures have been proposed to enable a proper interpretation of multiple tests, but they require additional information external to the data being analyzed; therefore, the probabilities of false positive and false negative results are estimated in an interpretable form.

The clinical manifestations of cases (DHF) and controls (DF) were similar to the findings of other studies (Guzman & Kouri 2002). Highlighting the spontaneous bleeding phenomena and thrombocytopenia below $50.000/mm^3$ in nearly half of the DHF cases, abdominal pain and diarrhoea were also observed in DHF cases only, in accordance with other studies (Coffey et al. 2009).

Finally, it is essential to conduct larger studies from different Brazilian regions to confirm the *HLA-A**01 allele association with DHF, which was found in this study and to further analyze the *HLA-A**31 association with DHF protection. In addition, further studies using multivariate analysis could offer greater understanding of the risks associated with DHF progression and could identify a suitable combination of potential predictors.

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