Polymorphisms IL10-819 and TLR-2 are potentially associated with sepsis in Brazilian patients

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Genetic variation in immune response is probably involved in the progression of sepsis and mortality in septic patients. However, findings in the literature are sometimes conflicting or their significance is uncertain. Thus, we investigated the possible association between 12 polymorphisms located in the interleukin-6 (IL6), IL10, TLR-2, Toll-like receptor-4 (TLR-4), tumor necrosis factor-α and tumor necrosis factor-β (lymphotoxin α - LTA) genes and sepsis. Critically ill patients classified with sepsis, severe sepsis and septic shock and 207 healthy volunteers were analyzed and genotyped. Seven of the nine polymorphisms showed similar distributions in allele frequencies between patients and controls. Interestingly, our data suggest that the IL10-819 and TLR-2 polymorphisms may be potential predictors of sepsis.

Key words: cytokines - outcome - SNP - septicaemia - immune system

Sepsis has been defined as a combination of signs of systemic inflammation secondary to an infectious process, confirmed or suspected (King 2007, Silva & Velasco 2007). However, the concept of sepsis is continuously debated in consensus meetings (Bone et al. 1992, Levy et al. 2003). A multicenter observational cohort study reported that the incidence of sepsis is about 57 per 1,000 patient-days (Silva et al. 2004) and another study showed that around 17% of intensive care unit (ICU) beds are occupied by these patients (Sales-Júnior et al. 2006). This illness is probably the leading cause of mortality in general and surgical ICU. The importance of studying genetic factors that may be associated with susceptibility to infections is demonstrated by incongruity observed in the predisposition or resistance to them in different individuals (Texereau et al. 2005).

The equilibrium of the immune system as a critical factor in sepsis has been a matter of great discussion because genetic variations that may influence the susceptibility and outcome of sepsis are correlated with several diseases (Henckaerts et al. 2009). Genes involved in both coagulation cascades and inflammation and their polymorphic variants are the main targets of these investigations. Usually, the polymorphisms chosen are single nucleotide polymorphisms (SNP) that occur within the gene promoter or within genic or intergenic regions, some of which can modify the protein expression or function (Sutherland & Russell 2005, Suffredini & Chanock 2006).

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Activation of the coagulation and inflammation cascade is associated with excessive production of thrombin and its inadequate removal by the fibrinolytic system produces alterations in microvascular circulation leading to multiple organ dysfunction syndrome and death (Schlichting & McCollam 2007). These cascades can be stimulated by an invasion of microorganisms or injury (Holmes et al. 2003). During injury, the cell produces cytokines, both pro-inflammatory [for example tumor necrosis factor-α (TNF), tumor necrosis factor-β TNFβ (lymphotoxin α - LTA), interleukin-1 (IL1) and IL6] and anti-inflammatory (such as IL10 and IL1RN) (Holmes et al. 2003).

In the present study, nine polymorphisms of selected pro and anti-inflammatory genes were investigated for their potential associations with either favorable clinical evolution or mortality in sepsis. The polymorphisms were selected based on a literature review that considered their potential effect on immune response and inflammation and included TNF-308, IL6-174, IL6-1753, IL6-2954, IL10-1082, IL10-819, Toll-like receptor-4 (TLR-4)-896, TLR-2-16933 and LTA.

The results showed differences in allele frequencies between patients and controls for two of the SNPs, suggesting their potential association with sepsis.

PATIENTS, MATERIALS AND METHODS

Casuistic - This study was approved by the Ethical Committee in Human Research (protocol 3795/2005) and the procedures were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 1983. The biological samples were obtained after informed consent forms were signed. The samples were processed anonymously.

The control samples, obtained from the Blood Donors Centre of the Hospital de Base at São José of Rio

Preto, São Paulo, Brazil, were collected between 2005-2006. The sepsis samples were obtained from 97 patients admitted between 2005-2007 to the ICU of the same hospital. The study was carried out in a university hospital where a majority of the patients and healthy donors were from the middle and lower middle class. The patients were classified as having sepsis, severe sepsis or septic shock according to the American College of Chest Physicians/Society of Critical Care Medicine Consensus criteria (Levy et al. 2003). The classification was made within the first 24 h after the patient's admission to the ICU. An Acute Physiology and Chronic Health Disease Classification System (APACHE) II score was assigned (Knaus et al. 1985) to patients with severe sepsis and a high risk of death (typically APACHE II ≥ 25 or multiple organ failure) and to those with severe sepsis and a low risk of death (e. g.: APACHE II < 20 or one organ failure). To calculate the score, data were collected over the first 24 h of ICU stay and the highest values were assigned to patients with abnormal vital signs. Patients with autoimmune diseases, diabetes, hepatitis and positive human immunodeficiency virus status were excluded.

Infectious agents - The infectious agents were identified by routine hospital testing. Two methods were used to identify the infectious agents: the manual method, in which samples were sown in plates containing EPMILE and Muller Hinton agar for growth, isolation and identification (Trabulsi & Alterthum 2008) and the automated method, in which agent identification and antibiotic sensitivity testing were carried out in a MicroScan® (Siemens) device.

Genomic DNA extraction - Genomic DNA was extracted from 2.5 mL of peripheral blood according to the protocol previously described (Miller et al. 1988). The purified DNA was stored at -20°C.

Polymorphism analysis - The polymorphisms were determined by three methodological strategies: real-time PCR using the TaqMan system (Applied Biosystems, Foster City, CA, USA) for the following polymorphisms: TNF-308, IL6-174, IL6-1753, IL6-2954, IL10-1082, IL10-819, TLR-4-896 and TLR-2-16933. Polymorphisms were analysed using the sequence detection software (SDS) v1.3 (Applied Biosystems) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for the LTA (Majetschak et al. 1999) gene polymorphism. Following sample amplification, the SDS software plots the results of the allelic discrimination run on a scatter plot of allele X vs. allele Y and the results of the amplification allow for viewing in both real-time and post-run. The real-time PCR reactions were performed in a total volume of 10 µL as follows: 60°C for 1 min, 95°C for 1 min, 40 cycles of 92°C for 1 min and 60°C for 1 min.

For RFLP analysis on the LTA polymorphism, the PCR reaction was standardized with Platinum Taq DNA polymerase (Invitrogen) and run under the following conditions: 96°C for 1 min, 35 cycles (94°C for 1 min, 54.2°C for 1 min, 72°C for 1 min) and 72°C for 10 min. The LTA amplicons were digested with the restriction enzyme *NcoI* for 16 h at 37°C. The LTA products were then analyzed by agarose gel (2%) electrophoresis. Information concerning the polymorphisms such as methodology, access number of the assay in GenBank (Applied Biosystems) and the primers used are listed in Table I.

Automated DNA sequencing - The sequencing reaction was carried out according to a protocol described previously (Sanger et al. 1992) using an automated DNA sequencer (Applied Biosystems Inc, Model 3100). Genotypes were confirmed by this method.

Statistical analysis - Statistical analyses were performed with Minitab®15.1.30.0 (Minitab Inc) for Windows. The genotypes were classified in contingency tables with death (survivors vs. non-survivors), the severity of the clinical condition (sepsis vs. severe sepsis

TABLE I

Information on polymorphisms studied: GenBank access number of single nucleotide polymorphisms (SNP), identification (ID) of assays (Applied Biosystems), primers and methods

Polymorphisms	Region	SNP ID (NCBI)	Assay ID	Primers	Methods
TNF	-308	rs1800629	C_7514879_10		Taqman SNP genotyping assay
LTA	+250	Noncoding *Nco*I NC_000006.11	*	sense: CCGTGCTTCGTGCTTTGGACTA antisense: AGAGGGGTGGATGCTTGGGTTC	PCR, NcoI digestion/DNA sequencing
IL6	-174	rs1800795	*		
	-1753	rs2069840	C_15804104_10		Taqman SNP genotyping assay
	-2954	rs1548216	C_7449206_10		
IL10	-1082	rs1800896	*		Taqman SNP genotyping assay
	-819	rs1800871	*		
TLR-2	-16933	rs4696480	C_27994607_10		Taqman SNP genotyping assay
TLR-4	-896	rs4986790	C_11722238_20		Taqman SNP genotyping assay

IL: interleukin; LTA: tumor necrosis factor-β; NCBI: National Center for Biotechnology Information; TLR: toll-like receptor; TNF: tumor necrosis factor-α. Asterisks mean validated assays by Applied Biosystems.

vs. septic shock), illness (patients vs. controls) and bacteria detected in blood culture (bacteria vs. other infectious agents). Group comparisons were performed using a likelihood ratio test for independent samples. Associations in patient groups were analyzed by Pearson's Chi-square or Fisher's exact test when recommended. The adopted significance level was 0.05.

A multiple binary logistic regression model for disease prediction was applied to the dataset; data were adjusted for all polymorphisms (except TNF and LTA because of their low allelic frequencies) and for gender, age and ethnicity (Caucasians vs. non-Caucasians). For comparison, variable selection was made by likelihood ratio forward and backward stepwise methods. Significance levels of variables entering in the model and being eliminated from it were 0.05 and 0.10, respectively.

Hardy-Weinberg equilibrium - The Hardy-Weinberg equilibrium was analyzed for each SNP using the Genepop program, version 3.4. A p value of 0.05 was indicative of disequilibrium (Norton & Neel 1965, Crow 1999, Morton et al. 2001).

RESULTS

The demographic data of the 97 septic patients and the 207 healthy individuals (control group) are presented in Table II. Of the 97 patients, 56.7% did not survive and 43.3% survived. In both groups, there was a high prevalence of males and Caucasians. The mean age was 57 [standard deviation (SD) = 18] years in the patient group and 26 in the control group (SD = 12).

Among the patients, the main sites of infection were lungs (41.23%) or abdomen (29.9%) and 12.4% were diagnosed with sepsis, 65% with severe sepsis and 22.6% with septic shock. The surviving patients differed from the non-survivors mainly with regard to the severity of the disease (Table II). The microorganisms identified in most of the blood cultures were Gram-negative bacteria (29.1%) (Table II).

The amplification curves and allelic discrimination plots from real-time PCR analyses showing the genotypic groups for IL10-819, TLR-2 and IL6-1753 are displayed in Figs 1-3, respectively. The TaqMan genotyping system and RFLP alleles were confirmed and validated by automated DNA sequencing (data not shown).

Table III shows all of the p values and statistical analyses. A near-significant p value was observed for IL6-1753 (p = 0.07) and TLR-2 (p = 0.083) when patient death was considered, which may suggest a trend towards an association of these polymorphisms with mortality in sepsis. The p value was probably not significant due to the small sample size (n = 97). The frequencies of each polymorphism from 304 individuals (97 patients and 207 controls) are presented in Table III. All polymorphisms studied are in agreement with Hardy-Weinberg equilibrium (Leal 2005, Cox & Kraft 2006, Trikalinos et al. 2006).

Both forward and backward stepwise methods showed the same results. The adjustment was performed using complete data from 294 individuals with age selected for as a risk factor [with odds ratio (OR) 1.11 and 95% confidence interval 1.08-1.13]. The adjusted mul-

TABLE II

Patient and control demographic, clinical data and list of microorganisms identified in blood cultures from patients

	Patients	Controls
Total of individuals (n)	97	207
Ethnic group (%)		
Caucasian	77.3	63.2
Intermediate/Hispanic	15.5	31.6
African	4.1	3.3
Asian	3.1	1.9
Gender (%)		
Female	34	35
Male	66	65
Age (mean; SD)	18-90 (57.3; 18.5)	18-77 (26.5; 12.2)
Classification of patients (%)		
Sepsis	12.4	-
Severe sepsis	65	-
Septic shock	22.6	-
Infection focus (%)		
Abdominal	30	-
Urinary tract	5.1	-
Central nervous system	4.1	-
Respiratory tract	41.2	-
Others	19.6	-
Non-survivors		
Total of individuals (n)	56	-
Gender (%)		
Female	34	-
Male	66	-
Age (mean; SD)	18-90 (59.9; 18.3)	
Classification of patients (%)		
Sepsis	14.3	-
Severe sepsis	51.8	-
Septic shock	33.9	-
APACHE score (mean)	6-35 (19.4)	-
Survivors		
Total of individuals (n)	41	-
Gender (%)		
Female	34.1	-
Male	65.9	-
Age (mean; SD)	18-90 (53.88; 18.5)	
Classification of patients (%)		
Sepsis	12.2	-
Severe sepsis	80.5	-
Septic shock	7.3	-
APACHE score (mean)	5-34 (15)	-
Microorganisms (%)		
Acinetobacter (multiresistant)	5.3	-
Burkholderia cepacia	1	-
Enterococcus sp.	1	-
Staphylococcus sp.	6.3	-
Candida albicans	4.2	-
Candida krusei	1	-
Candida tropicalis	2	-

↳

	Patients	Controls
Escherichia coli	2	-
Gram negative	9.5	-
Gram positive	4.3	-
Gram positive and negative	9.4	-
Klebsiella sp.	2	-
Providencia sp.	2	-
Pseudomonas sp.	6.3	-
Stenotrophomonas sp.	1	-
Not identified[a]	42.7	-
APACHE score (mean) (all patients)	5-35 (17.6)	-

a: absence of identification in the patient handbook; APACHE: Acute Physiology and Chronic Health Disease Classification System SD: standard deviation. The agents were not recovered or microorganisms were not identified in those patients.

tiple binary logistic regression model had a specificity of 91% and sensitivity of 76%. The other variables showed no significance because of low allelic frequency.

Using the logistical regression method adjusted for sex and age, two genes showed significant results: IL10-819 [CC] (p = 0.028; OR = 2.33) and TLR-2 (p = 0.022; OR = 2.84) with deleterious characteristics for genotype AT, using the genotype CT as the reference group. In the same analysis using the CC genotype as the reference, the CT genotype displayed significant results (p = 0.028; OR = 0.43) indicating the presence of a protective factor for sepsis.

DISCUSSION

The sample size of patient groups in the current study may be considered small for the analyses of polymorphisms. One factor that limited this study was the difficulty in obtaining clinical samples. RFLP was an efficient detection method which allowed for the interpretation and analysis of results. All alleles were DNA sequenced and the genotypes were confirmed to avoid errors in allele identification. Genotyping with real-time PCR using the TaqMan system was a fast and efficient method. However, the interpretation of allelic discrimination plots must be done carefully and it is essential to use gold standard alleles and known genotypes identified by automated DNA sequencing.

The study was conducted using healthy volunteers as the control group because patients hospitalized in the ICU may have caused confusion in the diagnosis. The high incidence of inflammation and shock seen in patients, their weakened immune systems and many others factors can influence their general status making it difficult to foresee whether they will develop sepsis. Despite adjustments using a multiple binary logistic regression model for prediction that considered all polymorphisms within the complete dataset and patient age, results were non-significant. Although the groups (survivors, non-survivors, controls and total patients) contained more males than females, the statistical analysis showed no significant results in terms of patient gender.

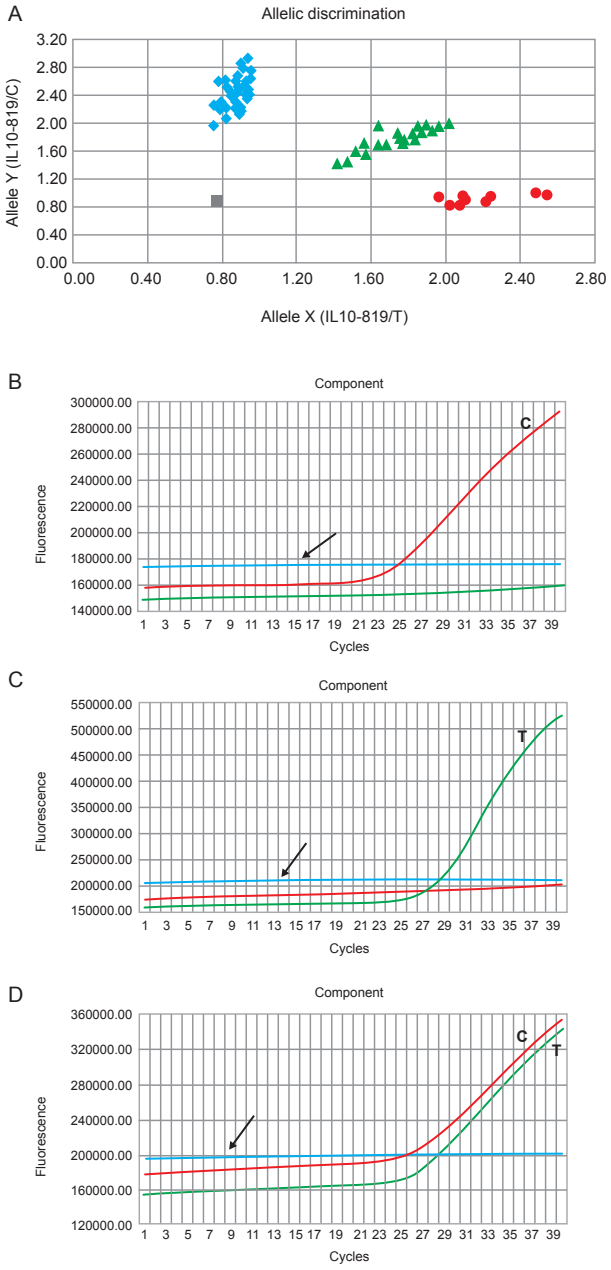

Fig. 1: allelic discrimination plots and amplification curves of the IL10-819 [C/T] single nucleotide polymorphisms-alleles by real-time polymerase chain reaction. A: allelic discrimination plots (C/C: ♦; T/T: ●; C/T: ▲; ■: negative control); B-D: amplification curves for the three genotypes: the line ROX is shown by arrows, the red line represents the allele C and the green one allele T.

The TLR-4 gene polymorphism showed no association with mortality or susceptibility to sepsis, a result that corroborates the data published previously (Child et al. 2003, Jessen et al. 2007). On the other hand, Shalhub et al. (2009) found that variation within the TLR-4 gene is associated with severity of post-traumatic sepsis, but this divergence could be explained by the difference in patient number (n = 598). In the present study, the Brazilian population studied (n = 294) showed an absence of

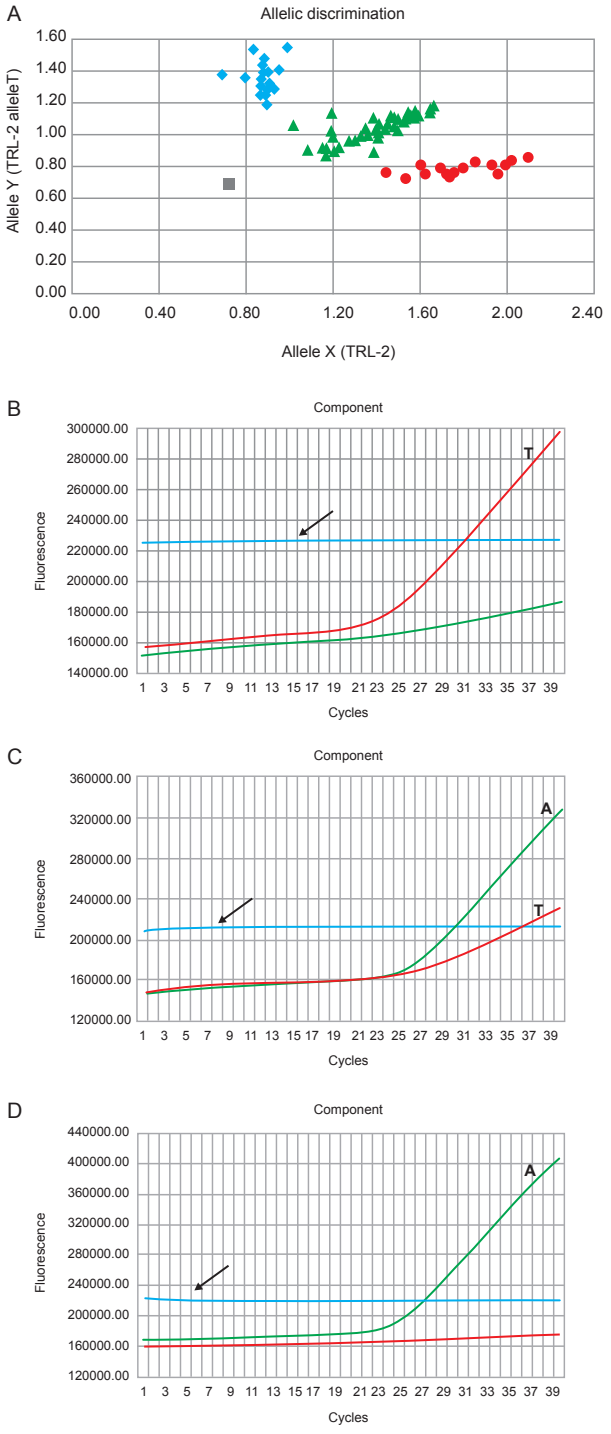

Fig. 2: allelic discrimination plots and amplification curves of the TLR-2-16933 [A/T] single nucleotide polymorphism-alleles by real-time polymerase chain reaction. A: allelic discrimination plots (T/T: ♦; A/A: ●; A/T: ▲; ■: negative control; B-D: amplification curves for the three genotypes: the line ROX is shown by arrows, the red line represents the allele T and the green one allele A.

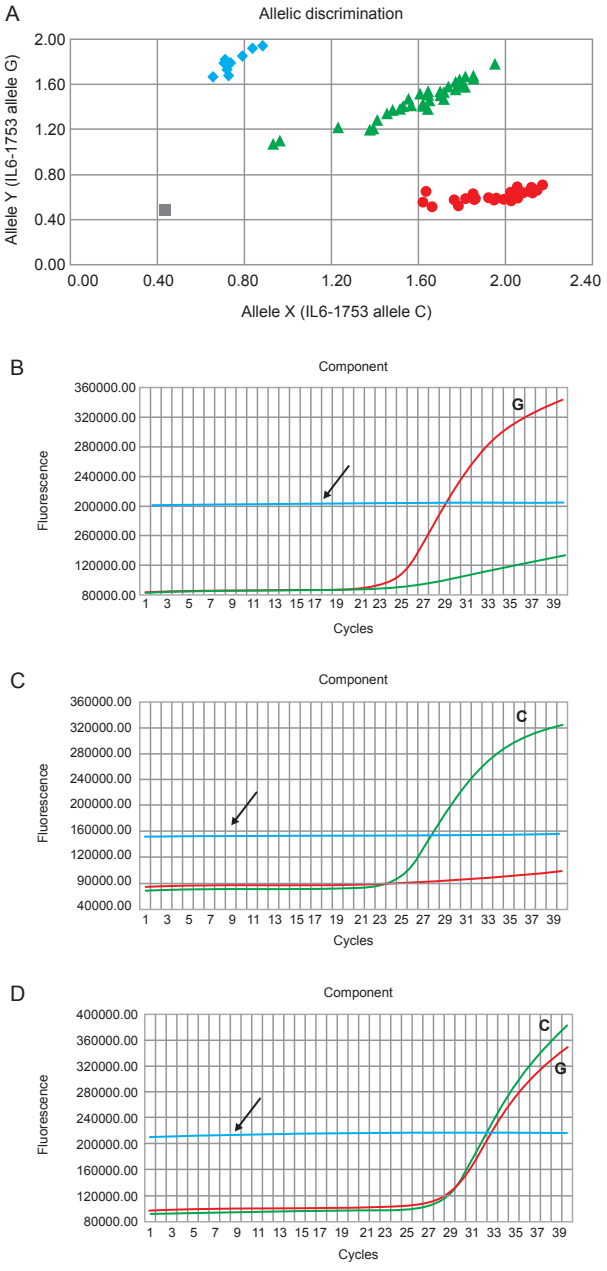

Fig. 3: allelic discrimination plots and amplification curves of the IL6-1753 [C/G] single nucleotide polymorphism-alleles by real-time polymerase chain reaction. A: allelic discrimination plots (G/G: ♦; C/C: ●; C/G: ▲; ■: negative control; B-D: amplification curves for the three genotypes: the line ROX is shown by arrows, the red line represents the allele G and the green one allele C.

the TLR-4 genotype GG, similar to genotypic distribution observed by other researchers (Arbour et al. 2000, Michel et al. 2003, Rylander & Michel 2005).

IL10 is a regulatory cytokine that has anti-inflammatory properties to counterbalance pro-inflammatory mediators. Over-expression of IL10 was proposed to induce immunosuppression in bacterial sepsis (Schaaf et al. 2003). These data corroborate with the result attained for the IL10-819 [CC] polymorphism, showing a significant increase in sepsis severity ($p = 0.028$; OR = 2.33) in comparison to the reference genotype CT by the logistical regression method. In the same analysis, when the reference genotype was CC, the genotype CT ($p = 0.028$; OR = 0.43) displayed significant results indicating the presence of a protective factor for sepsis. These data

TABLE III

Frequency and genotype distribution observed for the nine polymorphisms in Brazilian patients and control groups. Chi-square p values and results of other statistical analyses calculated for the association between polymorphisms and death, severity, illness and bacteremia

Gene region	Patients n (%)		Controls n (%)		Death[a]	Severity[b]	Illness[c]	Bacteria Infection[d]
IL10-1082	n = 97		n = 207					
	A/A	44 (45.4)	A/A	82 (39.6)	0.799	0.832	1.0[e] 0.284[f]	0.89
	A/G	44 (45.4)	A/G	103 (49.8)				
	G/G	9 (9.2)	G/G	22 (10.6)				
IL10-819	n = 97		n = 207					
	C/C	47 (48.4)	C/C	82 (39.6)	0.147	0.194	0.84[e] 0.028[f]	0.77
	C/T	38 (39.2)	C/T	102 (49.3)				
	T/T	12 (12.4)	T/T	23 (11.1)				
TNF	n = 97		n = 206					
	A/A	0 (0)	A/A	3 (1)	0.345	0.989	0.89[e] 0.999[f]	0.66
	A/G	23 (23.7)	A/G	57 (27)				
	G/G	74 (76.3)	G/G	146 (72)				
TLR-4 -896	n = 97		n = 205					
	A/A	88 (90.7)	A/A	178 (86)	0.942	0.556	1.0[e] 0.397[f]	0.60
	A/G	9 (9.3)	A/G	26 (12.6)				
	G/G	0 (0)	G/G	1 (0.4)				
TLR-2-16933	n = 97		n = 205					
	A/A	21 (21.6)	A/A	49 (23.8)	0.083	0.858	1.0[e] 0.022[f]	0.21
	A/T	56 (57.8)	A/T	108 (53)				
	T/T	20 (20.6)	T/T	48 (23.2)				
IL6-174	n = 97		n = 207					
	G/G	49 (50.5)	G/G	94 (45.4)	0.111	0.274	1.0[e] 0.447[f]	0.52
	G/C	39 (40.2)	G/C	96 (46.4)				
	C/C	9 (9.3)	C/C	17 (8.2)				
IL6-2954	n = 97		n = 205					
	G/G	1 (1)	G/G	2 (1)	-	-	1.0[e] 0.318[f]	0.44
	G/C	16 (16.5)	G/C	29 (14)				
	C/C	80 (82.5)	C/C	174 (85)				
IL6-1753	n = 97		n = 205					
	G/G	9 (9.3)	G/G	16 (7.7)	0.070[g]	0.441	1.0[e] 0.972[f]	0.67
	G/C	53 (54.7)	G/C	103 (49.8)				
	C/C	35 (36)	C/C	86 (41.5)				
LTA (controls n = 165)	n = 97		n = 165					
	G/G	6 (6.6)	G/G	20 (12)	0.15	0.38	0.86[e] 0.473[f]	0.82
	A/G	39 (40.2)	A/G	60 (36)				
	A/A	52 (53.6)	A/A	85 (52)				

a: survivor x non-survivor; *b*: sepsis x severe sepsis x septic shock; *c*: control x patient; *d*: bacterial infection x other type of agent; *e*: value of likelihood ratio test for independent samples; *f*: value of logistical regression method adjusting for sex and age; *g*: value of Fisher's exact test; IL: interleukin; LTA: tumor necrosis factor-β; TLR: toll-like receptor; TNF: tumor necrosis factor-α; -: no statistical analysis due to small sample size.

are very important because there is a lack of information on the involvement of IL10-819 in sepsis.

The absence of significant p values for the IL10-1082 polymorphism is not consistent with results previously reported by some authors that found an association of these polymorphisms with septic shock, infection or mortality, which was observed in a great number of patients with severe pancreatitis, Epstein-Barr virus infection or pneumonia (Helminen et al. 2001, Gallagher et al. 2003, Lowe et al. 2003, Wattanathum et al. 2005, Zhang et al. 2005). In the present study, the patients were not selected according to specific primary infection or agent, a fact that probably influenced the results.

In the LTA (+250 G/A) analysis no association was observed corroborating the findings of Rauchschwalbe et al. (2004) and Gong et al. (2005). Furthermore, Schueller et al. (2006) suggested the likelihood that this polymorphism does not serve as a prognostic marker for elevated sepsis risk in preterm infants. Some studies demonstrate that LTA [AA] individuals are high producers of TNF in vitro (Mølvig et al. 1990, Pociot et al. 1993). A previous study showed that individuals with the LTA [AA] polymorphism had a significantly reduced survival rate along with high TNF concentrations in comparison to heterozygous patients (Stuber et al. 1996). In agreement with the literature, our study indicates the importance of future studies with a greater quantity of methodologies and larger numbers of patients. The significantly negative result found for the polymorphism (-308) of the TNF gene promoter region in relation to sepsis severity is in agreement with Kovar et al. (2007) and it suggests that this polymorphism does not increase the risk of sepsis.

The polymorphism of gene TLR-2 [AT] showed a significant p value ($p = 0.022$; OR = 2.84) with deleterious characteristics. This may suggest an association of this polymorphism with mortality in sepsis. Recently, this polymorphism was associated with high prevalence of infections by Gram-positive bacteria in patients with sepsis (Sutherland et al. 2005b, Verstak et al. 2007).

There are few reports in the literature about an association of an IL6 polymorphism with sepsis, although it has been associated with other diseases such as cancer (Wilkening et al. 2006). In fact, Müller-Steinhardt et al. (2007) found that the allele IL6-174GG is associated with low levels of IL6 and the allele -174C is associated with increased IL6 secretion. These findings corroborate the results of Michalek et al. (2007), who demonstrated an association between a high IL6 serum level and mortality in septic patients. The IL6-1753 polymorphism showed a near-significant p value ($p = 0.07$), which may be important in the progression of sepsis and mortality, but this data must be confirmed in a larger number of patients. This observation supports the findings reported by Sutherland et al. (2005a).

The present study has different characteristics when compared to other studies on polymorphisms and sepsis: the nine polymorphisms were evaluated in the same population (patient and control group) and the patients were not selected according to clinical or pathological status or any other features. This characteristic of the sample set may have influenced the results, which are divergent from the literature, but supports the potential role of IL10 and TLR-2 in sepsis. To our knowledge, this is the first study of these nine genetic polymorphisms and genetic sepsis conducted in Brazil.

The study of polymorphisms in relation to outcome in sepsis patients is relatively recent and larger samples will have to be analyzed to improve our knowledge of genetic variants and their effects on the clinical course of this illness. However, the current results obtained show that polymorphisms play a major role in sepsis and will possibly be helpful in prognosis and in the targeting of studies on the physiopathology of sepsis.

The polymorphisms IL10-819 and TLR-2 are potentially associated with sepsis, demonstrating the importance of these genes in the development of or susceptibility to sepsis.

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