

Interleukin-8 but Not Interleukin-6 Variant May Affect Susceptibility to Brucellosis

Sadaf Asaei¹, Manoochehr Rasouli^{1*}, Ali Moravej²

¹Department of Immunology, Professor Alborzi Clinical Microbiology Research Center, Shiraz University of Medical Sciences, Shiraz, ²Department of Immunology, Fasa University of Medical Sciences, Fasa, Iran

ABSTRACT

Background: Increased levels of interleukin-8 (IL-8) and interleukin-6 (IL-6) in acute human brucellosis have been reported. Previous studies have shown that the production and level of IL-6 and IL-8 cytokines are associated with the polymorphism of the encoding genes. **Objective:** To investigate the probable association between IL-6 (-174 C/G) and IL-8 (-251 A/T) gene polymorphisms and susceptibility/resistance to brucellosis. **Methods:** The patient group included 196 patients suffering from *Brucella* infection and the control group consisted of 82 healthy animal husbandmen from the same geographical area. IL-8 (-251 A/C) and IL-6 (-174 C/G) gene polymorphisms were analyzed by PCR-RFLP and Allele Specific PCR (AS-PCR) respectively. **Results:** The frequency of -251 IL-8 AA genotype was significantly lower in the controls compared with that of the patients ($p=0.0051$), while the frequencies of other genotypes (AT and TT) and alleles (A and T) were not significantly different among the participants. No association was found between IL-6 (-174 C/G) polymorphism and brucellosis. **Conclusion:** This study indicates that the IL-8 -251 AA genotype may be considered as a genetic susceptibility factor for brucellosis.

Asaei S, et al. *Iran J Immunol*. 2013; 10(3):158-66

Keywords: Brucellosis, Interleukin-6, Interleukin-8, Polymorphism

*Corresponding author: Dr. Manoochehr Rasouli, Department of Immunology, Prof. Alborzi Clinical Microbiology Research Center, Namazi Hospital, Shiraz University of Medical Sciences, Shiraz, Iran, Tel: (+) 98 711-6474304, Fax: (+) 98 711-6474303, e-mail: rasoulim@sums.ac.ir

INTRODUCTION

Brucella, the causative agent of brucellosis, is an obligatory intracellular pathogen that can stay alive and replicate within phagocytic cells such as monocytes and macrophages (1). Brucellosis is a zoonotic disease that could be transmitted to humans through consumption of contaminated milk and dairy products, contact with products of conception, or sometimes through meat ingestion from the infected animals. Other portals of entry are conjunctivae, respiratory tract, and abraded skin, which are the more prevalent routes of infection especially in animal husbandmen. The disease is a worldwide distributed and its most important endemic regions are the Mediterranean countries, the Middle East, India and the Southern America (1). The mechanism of host resistance to *Brucella* spp. is incompletely understood, but similar to other intracellular bacteria; cell-mediated immunity seems to play a major role in immune response against *Brucella* infection (2). Some studies have shown that the cytokines produced by Th-1 cells are key mediators both for the protection against this infection and for the increase in the level of interferon- γ (IFN- γ) in acute human brucellosis (3). IFN- γ is a physiologic activator of macrophages to produce proinflammatory cytokines including interleukin-1 (IL-1), tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6) and interleukin-8 (IL-8) (4,5).

IL-6 is a multifunctional cytokine playing a major role in the inflammatory responses. This cytokine is produced by several cell types such as T lymphocytes, macrophages, fibroblasts and endothelial cells (6). Previous studies have shown that IFN- γ , IL-6 and TNF- α serum levels are increased in the patients with *Brucella* infection (7,8). It seems that serum IL-6 level depends on the nature of the bacterium, not simply on the bacterial number (6). Since LPS is a stimulator for IL-6 production (9) and *Brucella* spp., being Gram negative bacteria, are rich in LPS, IL-6 serum level in patients with brucellosis may be affected (6). It seems that IL-6 production by both splenic and peritoneal cells affect the number of activated macrophages in *Brucella* infection (6).

IL-8 is a chemokine inducing the migration of leukocytes to the site of infection. Main producers of IL-8 are monocytes and endothelial cells (4). Monocytes can produce more IL-8 in response to the IFN- γ produced by T-cells (4). It is shown that IL-8 serum level increases significantly in the patients with brucellosis in comparison with the normal individuals. These could indicate the functional role of IL-8 in the process and pathogenesis of brucellosis (10).

It is clarified that in infectious diseases, the rate of cytokine production and the effect of cytokine on immune cells are influenced by various factors such as the nature of the bacterium, the number of bacteria and the host genetic background (11). In this regard, several studies have revealed the important roles of gene polymorphisms on the susceptibility and/or resistance of different individuals to the same infectious diseases (12-16). Previous studies have shown that the production of IL-6 and IL-8 cytokines is affected by their gene polymorphisms (17-19). Taking into account the presence of these two cytokines in brucellosis and the role of different variants of the two genes in cytokine production, the present study attempts to study the association between IL-6 (-174 C/G) and IL-8 (-251 A/T) gene variants and susceptibility and/or resistance to brucellosis.

MATERIALS AND METHODS

Patients. The patient group included 196 (84 males and 112 females, age range 7-80 and Mean \pm SD = 30.5 \pm 1.2 years) persons suffering from *Brucella* infection who were registered in the health center of Fars province, Southern Iran, during a one year period in 2007. All of the patients were either farmers keeping animals infected with *Brucella* or had a history of consumption of contaminated raw milk and unpasteurized dairy products. Diagnosis of brucellosis was based on clinical signs and symptoms such as fever, night sweating, weakness, anorexia, weight loss, malaise, splenomegaly and lymphadenopathy in addition to positive serological tests (antibody titer higher than 1/160 for standard agglutination test and 2-mercaptoethanol test) at the time of infection. The control group consisted of 82 (38 males and 44 females, age range 5-74 and Mean \pm SD = 27.3 \pm 1.7 years) healthy animal husbandmen from the same geographical area as the patients. All of them had close contact with animals infected with *Brucella* and consumed their contaminated milk and dairy products, but they did not show any clinical manifestations after a six-month follow-up. Blood samples were taken from all patients and controls after obtaining informed written consents. This study was approved by the Research Ethics Committee of Shiraz University of Medical Sciences.

DNA Extraction and Genotyping. DNA was extracted from blood samples using the standard salting out method. IL-8 (position -251 A/C) gene polymorphism was analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). For each specimen, a PCR reaction was set up using specific pair of primers. Each PCR reaction contained 1X PCR buffer, 200 μ M dNTPs mix, 0.5 units *Taq* DNA polymerase (all from CinnaGen, Iran), 0.5 μ M specific primer set (Primm, Italy), 250 ng genomic DNA and 2.5 mM MgCl₂. PCR was performed under the following thermal conditions: an initial denaturation step for 2 min at 95°C followed by 35 cycles of a denaturation step for 1 min at 95°C, annealing for 1 min at 55°C, extension for 1 min at 72°C and a final extension for 10 min at 72°C. The 816 bp amplified products were digested with 3 units of *MfeI* (Fermentas, Lithuania) overnight at 37°C. T allele remained undigested (816 bp), whereas C allele yielded 2 bands of 296 bp and 520 bp. Finally, the digested products were run on a 2.5% agarose NA gel (GE Healthcare, USA) and studied on UV transilluminator after staining with ethidium bromide. Sequences of the primers are presented in Table 1.

IL-6 (position -174 C/G) gene polymorphism was analyzed using allele specific polymerase chain reaction. Human growth hormone gene was used as an internal control. PCR reactions were set up using two specific sets of primers amplifying a 109 bp sequence of IL-6 gene and a 220 bp sequence of HGH gene. DNA was amplified in a 10 μ l reaction. Each PCR reaction contained 1X PCR buffer, 3 mM MgCl₂, 200 μ M dNTPs mix, 0.5 units *Taq* DNA polymerase (all from CinnaGen, Iran), 1 μ M specific primers of IL-6, 0.1 μ M specific primers of HGH (Primm, Italy) and 250 ng of genomic DNA. PCRs were performed under the following thermal conditions: an initial denaturation step for 5 min at 95°C followed by 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 60°C, extension for 45 sec at 72°C and a final extension at 72°C for 5 min.

Table 1. PCR primers and conditions for IL-6 and IL-8 gene amplification.

Locus	Method	PCR Primers	Annealing Temp.(°C)	MgCl ₂ (mM)	Restriction Enzymes	Fragment Sizes (bp)
IL-8 (-251A/T)	RFLP	Forward: 5'-GATTCTGCTCTTATGCCTCCA-3'	55°C	2.5 mM	mfeI	AA:296 and 520
		Reverse: 5'-CCCAAGCTTGTGTGCTCTGCTGTC-3'				GG:816 AG: 296 , 520 and 816
IL-6 (-174 G/C)	ASO-PCR	Il-6 Common: 5'-TTTGTTGGAGGGTGAGGGTGG-3'	60°C	3mM	-----	109
		Il-6 G allele: 5'-CCTCAGAGACATCTCCAGTCg-3'				220
		Il-6 C allele: 5'-CCTCAGAGACATCTCCAGTCc-3'				
		HGH Forward: 5'-GCCTTCCCAACCATTCCTTA -3'				
		HGH Reverse: 5'-TCACGGATTTCTGTTGTGTTTC -3'				

Statistical Analysis. Allele and genotype frequencies were calculated by direct gene counting. Statistical analysis of the differences between the two groups was performed by χ^2 test using EPI Info 2000 and SPSS software version 16. Odds ratio and 95% confidence interval were calculated by EPI info 2000. Arlequin software package version 3.1 was used to determine Hardy-Weinberg equilibrium (HWE). In cases in which the frequency of genotypes did not meet HWE, Armitage's trend test was calculated (<http://ihg.gsf.de/cgi-bin/hw/hwal.pl>). p value less than 0.05 was considered significant.

RESULTS

The single nucleotide polymorphisms (SNPs) of IL-8 (-251 A/T) and IL-6 (-174 C/G) genes were analyzed in the controls and patients with brucellosis. Genotypes of IL-8 in the control group met Hardy-Weinberg equilibrium while the others did not. The frequency of AA genotype at position -251 of IL-8 was significantly lower in the controls compared with that of the patients ($p=0.0051$, OR=6.34, 95% CI=1.41-39.66, study power=96%). Although the frequencies of AT and TT genotypes at this position were higher in the patients compared with controls, p values did not show any significant differences between the two study groups ($p=0.204$ and $p=0.644$, respectively). Regarding alleles at position -251 of IL-8, there was no significant difference between the controls and patients ($p=0.118$, study power=36%).

The frequencies of IL-6 (-174) CG and GG genotypes were lower in the controls, compared with that of the patients ($p=0.697$, study power=54%; $p=0.894$, study power=3, respectively), while the frequency of CC was higher in the controls than that of the patients ($p=0.287$, study power=16%). Moreover, allele G at position -174 was more frequent in the patients while allele C was more frequent in the controls ($p=0.590$, study power=8%). However, statistical analysis showed that these differences are not significant.

DISCUSSION

Cell mediated immunity (CMI) and its related cytokines play crucial roles in protective immunity against intracellular organisms such as *Brucella* spp. (20). In *Brucella* infected individuals, IFN- γ modulates the activation of macrophages; increases their class II MHC molecules expression and enhances their antigen presentation ability, and promotes lymphocyte differentiation (21). IL-6 promotes Th2 differentiation and simultaneously inhibits Th1 polarization. IL-6 activates the transcription mediated by the nuclear factor of activated T cells (NFAT) leading to an increase in the production of IL-4 by naive CD4⁺ T cells and their differentiation into effector Th2 cells. It is shown that IL-6 has an anti-inflammatory activity (22) and its level increases in sera of patients with brucellosis (8). Besides, some studies have shown that the rates of cytokine production are related to SNPs in cytokine genes (11). In view of the aforementioned studies, one of the aims of this study was to determine the potential associations between IL-6 (position -174) gene polymorphism and susceptibility to brucellosis in Iranian population.

Table 2. Frequencies of IL-6 and IL-8 genotypes and alleles in patients with brucellosis and controls.

Genotypes and alleles	Patient Group n(%)	Control Group (%)	X ²	P Value*	OR (95%CI)	Study Power (%)
IL-6(-174)						
Genotypes						
CC	8 (4.20)	6 (7.30)	1.13	0.287	0.56 (0.17-1.88)	16
CG	53 (37.90)	21 (25.60)	0.15	0.697	1.012 (0.60-2.11)	54
GG	129 (67.90)	55 (67.10)	0.02	0.894	1.04 (0.58-1.87)	3
Alleles						
C	69 (18.16)	33 (20.12)	0.29	0.590	0.88 (0.54-1.44)	8
G	311 (81.84)	131 (78.88)				
IL-8(-251)						
Genotypes						
TT	64 (33.70)	30 (36.60)	0.21	0.644	0.88 (0.50-1.57)	7
AT	100 (52.60)	50 (61.00)	1.61	0.204	0.71 (0.41-1.24)	25
AA	26 (13.70)	2 (2.40)	6.67 ^s	0.0051	6.34 (1.41-39.66)	96
Alleles						
T	228 (60.00)	110 (67.07)	2.44	0.118	0.74 (0.49-1.10)	36
A	152 (40.00)	54 (32.93)				

*Each P value is the result of comparing corresponding row with the sum of other related rows.

The results of this study revealed that there was no significant difference in IL-6 gene polymorphism between the patients with brucellosis and the healthy controls. Similarly, the study by Karaoglan *et al.* indicated that there was no significant difference between the frequencies of IL-6 alleles and genotypes among Turkish patients infected with *Brucella* and healthy ones (17). Contrary to our study, Budak *et al.* found high producer -174 IL-6 variants (GG and CG) were more common in brucellosis patients in comparison to that of the healthy controls (18). They recommended that IL-6 gene polymorphisms may affect susceptibility to brucellosis and can be considered as a genetic risk factor for developing the fulminant disease (18). The lack of similarity between our results and Budak's might be due to the number of the participants enrolled in both studies. Budak genotyped only 40 patients and 49 controls while the number of our patients and controls were 196 and 82, respectively; and Karaoglan studied IL-6 polymorphism on 85 patients and 85 controls. In addition to the number of the participants, genetic varieties among these different ethnic groups may be another reason of these differences. Budak collected blood samples from Bursa (the Northwest of Turkey), which is populated by different ethnic groups from Bulgaria, Greece and North East of Black Sea. Then, this admixture could be another reason for lack of similarity between Budak's study on the one side and Karaoglan's study and ours on the other side.

Cytokines and chemokines produced by various cells play major roles in the immunity to infectious diseases. IL-8 is a member of the CXC chemokines inducing chemotaxis of neutrophils. This cytokine is produced by a variety of cell types in response to other pro-inflammatory cytokines such as TNF- α (18), and bacterial or viral products in the areas of inflammation (23). One of the aims of the present study was to determine the effect of various genotypes of IL-8 gene promoter polymorphism on the susceptibility/resistance to brucellosis. We found that -251 IL-8 AA genotype is more frequent in the patients than in the controls. A previous study has shown that IL-8 A allele (-251) is associated with the increase in IL-8 production after stimulation with lipopolysaccharide or cytokines such as IL-1 β and TNF- α (24). Refik and colleague showed that the patients with brucellosis had significantly elevated serum levels of IL-6 and IL-8, compared with those of the healthy controls (10). They also clarified that the serum level of IL-8 is higher in the acute brucellosis than in the subacute brucellosis. This research group concluded that the extent of IL-8 elevation depends on the severity and clinical patterns of the disease (10). According to the above-mentioned findings, we suggest that individuals inheriting AA genotype have 6.34 times more chance of developing brucellosis (OR=6.34) when they are exposed to the bacteria. Due to the lack of study in this field, it was not possible to compare our results with others. Then, further analysis of IL-8 gene may help identify more functional polymorphisms associated with altered IL-8 production and susceptibility to *Brucella*. However, several studies have shown the association between IL-8 -251 A/T polymorphism and different infectious and non-infectious diseases (13,22,25-27). Farshad *et al.* tried to find the potential association between the prevalence of -251 IL-8 gene variants and *Helicobacter pylori* infected and non-infected patients who suffered from gastritis and/or ulcerative gastritis (13). Okada *et al.* hypothesized that IL-8 polymorphisms might be associated with serum levels of anti-p53 antibody (25). They demonstrated that -251 IL-8 AA genotype was associated with higher anti-p53 antibodies than those of the reference range (25). Kamali-Sarvestani and colleagues carried out a case-control study on the breast cancer patients with invasive ductal carcinoma and healthy women

(26). They showed that -251 IL-8 T/A genotype is associated with the development of the invasive ductal carcinoma type of the breast cancer (26). Frade *et al.* investigated the probable role of -251 IL-8 genotypes on the susceptibility to visceral leishmaniasis (VL), but they did not find any relationship between IL-8 gene polymorphisms and susceptibility to VL (27).

In conclusion, this study indicated that -251 IL-8 AA genotype may influence increased susceptibility to brucellosis in a sample of Iranian population and may be considered as a genetic factor for sensitivity to this infectious disease.

ACKNOWLEDGEMENTS

Our special thanks are due to Miss. Esmat Kazemi for her linguistic copy editing. We also thank Health Center of Fars Province for their help with finding the patients. This study was financially supported by Grant No. 84-10 awarded by Clinical Microbiology Research Center, Shiraz University of Medical Sciences, Shiraz, Fars, Iran.

REFERENCES

- 1 Corbel MJ. Brucellosis: an overview. *Emerg Infect Dis.* 1997; 3:213-21.
- 2 Serre A, Bascoul S, Vendrell JP, Cannat A. Human immune response to *Brucella* infection. *Ann Inst Pasteur Microbiol.* 1987; 138:113-7.
- 3 Golding B, Scott DE, Scharf O, Huang LY, Zaitseva M, Lapham C, et al. Immunity and protection against *Brucella abortus*. *Microbes Infect.* 2001; 3:43-8.
- 4 Bosco MC, Gusella GL, Espinoza-Delgado I, Longo DL, Varesio L. Interferon-gamma upregulates interleukin-8 gene expression in human monocytic cells by a posttranscriptional mechanism. *Blood.* 1994; 83:537-42.
- 5 Akira S, Taga T, Kishimoto T. *Interleukin-6 in biology and medicine.* *Adv Immunol.* 1993; 54:1-78.
- 6 Saunders BM, Liu Z, Zhan Y, Cheers C. Interleukin-6 production during chronic experimental infection. *Immunol Cell Biol.* 1993; 71:275-80.
- 7 Rodríguez-Zapata M, Matías MJ, Prieto A, Jonde MA, Monserrat J, Sánchez L, et al. Human Brucellosis Is Characterized by an Intense Th1 Profile Associated with a Defective Monocyte Function. *Infect Immun.* 2010; 78: 3272-9.
- 8 Akbulut H, Celik I, Akbulut A. Cytokine levels in patients with brucellosis and their relations with the treatment. *Indian J Med Microbiol.* 2007; 25:387-90.
- 9 Van Snick J. Interleukin-6: an overview. *Annu Rev Immunol.* 1990; 8:253-78.
- 10 Refik M, Mehmet N, Durmaz R, Ersoy Y. Cytokine profile and nitric oxide levels in sera from patients with brucellosis. *Braz J Med Biol Res.* 2004; 37:1659-63.
- 11 Koen Vandebroek. Cytokine gene polymorphism in multifactorial conditions. Taylor & Francis Group; 2006. p. 83-93.
- 12 Moravej A, Rasouli M, Kalani M, Asaei S, Kiany S, Najafipour S, et al. IL-1 β (-511T/C) gene polymorphism not IL-1 β (+3953T/C) and LT- α (+252A/G) gene variants confers susceptibility to visceral leishmaniasis. *Mol Biol Rep.* 2012; 39:6907-14.
- 13 Farshad S, Rasouli M, Jamshidzadeh A, Hosseinkhani A, Japoni A, Alborzi A, et al. IL-1 β (+3953 C/T) and IL-8 (-251 A/T) gene polymorphisms in *H. pylori* mediated gastric disorders. *Iran J Immunol.* 2010; 7:96-108.
- 14 Kalani M, Rasouli M, Moravej A, Kiany S, Rahimi HR. Association of interleukin-15 single nucleotide polymorphisms with resistance to brucellosis among Iranian patients. *Tissue Antigens.* 2011; 78:352-8.
- 15 Rasouli M, Kalani M, Moravej A, Kiany S. Interleukin-18 single nucleotide polymorphisms contribute to the susceptibility to brucellosis in Iranian patients. *Cytokine.* 2011; 54:272-6.

- 16 Kamali-Sarvestani E, Rasouli M, Mortazavi H, Gharesi-Fard B. Cytokine gene polymorphisms and susceptibility to cutaneous leishmaniasis in Iranian patients. *Cytokine*. 2006; 35:159-65.
- 17 Karaoglan I, Pehlivan S, Namiduru M, Pehlivan M, Kiliçarslan C, Balkan Y, et al. TNF- α , TGF- β , IL-10, IL-6 and IFN- γ gene polymorphisms as risk factors for brucellosis. *New Microbiol*. 2009; 32:173-8.
- 18 Budak F, Göral G, Heper Y, Yilmaz E, Aymak F, Baştürk B, et al. IL-10 and IL-6 gene polymorphisms as potential host susceptibility factors in Brucellosis. *Cytokine*. 2007; 38:32-6.
- 19 Köhidai L, Csaba G. Chemotaxis and chemotactic selection induced with cytokines (IL-8 and TNF- α) in the unicellular tetrahymena pyriformis. *Cytokine*. 1998; 10:481-6.
- 20 Skendros P, Pappas G, Boura P. Cell-mediated immunity in human brucellosis. *Microbes Infect*. 2011; 13:134-42.
- 21 Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon- γ : an overview of signals, mechanisms and functions. *J Leukoc Biol*. 2004; 75:163-89.
- 22 Diehl S, Rincón M. The two faces of IL-6 on Th1/Th2 differentiation. *Mol Immunol*. 2002; 39:531-6.
- 23 Cassatella MA. Neutrophil-derived proteins: selling cytokines by the pound. *Adv Immunol*. 1999; 73:369-509.
- 24 Ohyauchi M, Imatani A, Yonechi M, Asano N, Miura A, Lijima K, et al. The polymorphism interleukin 8-251 A/T influences the susceptibility of Helicobacter pylori related gastric diseases in the Japanese population. *Gut*. 2005; 54: 330-5.
- 25 Okada R, Rahimov B, Ahn KS, Abdiev S, Malikov Y, Bahramov S, et al. Interleukin-8 T-251A Polymorphisms was associated with positive anti-p53 antibodies in Uzbekistan population. *Nagoya J Med Sci*. 2009; 71:151-6.
- 26 Kamali-Sarvestani E, Aliparasti MR, Atefi S. Association of interleukin-8 (IL-8 or CXCL8) - 251T/A and CXCR2 +1208C/T gene polymorphisms with breast cancer. *Neoplasma*. 2007; 54:484-9.
- 27 Frade AF, Oliveira LC, Costa DL, Costa CH, Aquino D, Van Weyenbergh J, et al. TGF β 1 and IL8 gene polymorphisms and susceptibility to visceral leishmaniasis. *Infect Genet Evol*. 2011; 11:912-6.