

## Association of tumor necrosis factor- $\alpha$ -308 (G $\rightarrow$ A) polymorphism and susceptibility to brucellosis

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**Background and purpose:** A critical response against intracellular organisms such as *Brucella* spp. is production of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), which enhances the initial response of infected macrophages. Polymorphism in the TNF- $\alpha$  gene promoter has an effect on the level of TNF- $\alpha$  production. Therefore, this study investigated the possible association of G-308A polymorphism of the TNF- $\alpha$  gene and susceptibility to brucellosis.

**Methods:** Genotyping was performed on DNA extracted from the peripheral leukocytes of 260 patients with brucellosis and 217 healthy control participants using the sequence-specific primer polymerase chain reaction method.

**Results:** The TNF- $\alpha$ -308(A/A) homozygote was significantly higher in patients than in controls (14.2% vs 5.5%;  $p = 0.001$ ). Logistic regression analysis showed a significant association between the TNF- $\alpha$ -308 (A/A) genotype and brucellosis (odds ratio, 2.4; 95% confidence interval, 1.2-4.8;  $p = 0.01$ ).

**Conclusion:** The results of this study suggest that TNF- $\alpha$  (G-308A) polymorphism might be involved in susceptibility to brucellosis.

**Key words:** Brucellosis; Polymorphism, genetic; Tumor necrosis factor-alpha

### Introduction

Brucellosis is a zoonotic disease with a worldwide distribution, caused by intracellular bacteria from *Brucella* spp. The disease is endemic in many Middle East countries, including Iran, in which brucellosis is primarily due to *Brucella melitensis*. Due to the high clinical morbidity and considerable loss of productivity in animal husbandry, brucellosis is a serious public health problem and an important cause of economic loss in many developing countries [1-3].

One of the most important factors for susceptibility, severity, and clinical outcome of an infection is host

genetic factors. Human gene polymorphism has been shown to play a role in immune responses. Among the immune-related genes, cytokines are important. Some polymorphisms in cytokines and cytokine receptor genes may have functional significance by both directly and indirectly altering the level of gene expression and/or its function [4,5].

Some cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-12 (IL-12), and interferon- $\gamma$  (IFN- $\gamma$ ), can regulate the immune system and play key roles in brucellosis [3,6,7]. TNF- $\alpha$  production is an essential element to enhance the initial response of infected macrophages against intracellular pathogens [7]. Control of brucellosis in a susceptible mouse model relied on TNF- $\alpha$  as well as CD8+ T cells until IFN- $\gamma$  production resumed and clearance began [8]. TNF- $\alpha$  might also be important in resistance to *Brucella* spp.,

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depending on the timing, level, and persistence of its production in response to infection [6,9]. However, some studies indicated that the increasing serum TNF- $\alpha$  was not consistent and had questionable susceptibility to brucellosis [10-12].

There are several biallelic single nucleotide polymorphisms in the human TNF- $\alpha$  gene promoter. It is possible that these polymorphisms have an effect on the level of TNF- $\alpha$  production, which in turn might have an impact on inflammatory responses [13,14]. A single base change from guanine to adenine at position -308 in the promoter region of the gene has been described. Several studies have shown that the mutant allele is a functional variant as the A-308 allele increases the transcriptional activation of the gene [15,16]. The TNF- $\alpha$  A allele has been found to be a stronger transcriptional activator than the G allele, resulting in higher TNF- $\alpha$  levels, and has been associated with a variety of autoimmune disorders [13]. This study was performed to examine the contribution of TNF- $\alpha$ -308 polymorphism in susceptibility to *Brucella* infection.

## Methods

### Study population

260 patients with acute brucellosis and 217 healthy participants matched for age, sex, and geographical area were enrolled. The patients' mean ( $\pm$  standard deviation) age was  $38.8 \pm 20.2$  years. There were 142 men (54.6%) and 118 women (45.4%).

Whole blood samples of 5 to 10 mL were taken from the patients and controls. Brucellosis was diagnosed on the basis of clinical, bacteriological, and/or serological findings. For negative blood or bone marrow cultures, diagnosis was based on the clinical signs, together with demonstration of specific antibodies at significant titers or a 4-fold or greater increase in the initial titers in 2 paired samples taken 2 to 4 weeks apart. Significant titers were considered to be Wright's seroagglutination titer of  $\geq 1/160$  or Coombs anti-*Brucella* test titer of  $\geq 1/320$ . All participants gave informed consent and the study protocol was approved by the Ethics Review Committee of Hamadan University of Medical Sciences, Hamadan, Iran.

### Tumor necrosis factor- $\alpha$ genotyping

Genotyping was performed by the sequence-specific primer polymerase chain reaction (SSP-PCR) method. For each polymorphic site, 1 PCR was carried out on

a DNA template with a pair of specific primers, the additional control primers, and PCR reaction. The internal control primers were used for all reactions to amplify a 428-bp segment of the human growth hormone gene to check for successful PCR amplification.

Amplification was carried out using the PCR Techne Flexigene apparatus (Roche Applied Science, Mannheim, Germany) in a total volume of 50  $\mu$ L containing 0.2 ng genomic DNA, 10 pmol allele-specific primers (TNF- $\alpha$ -308 common 5'-TCTCGGTTTCT TCTCCATCG-3', TNF- $\alpha$ -308G 5'-ATAGGTTTT GAGGGGCATGG-3' and TNF- $\alpha$ -308A 5'-AATAG GTTTTGAGGGGCATGA-3') and 10 pmol internal control primers (HGH1 F: 5'-GCCTTCCCAAC CATTCCCTTA-3', HGH2 R: 5'-TCACGGATTCT GTTGTGTTTC-3'), 200  $\mu$ M deoxyribonucleotide triphosphate, 10 mM trisaminomethane hydrochloride, pH 8.3, 50 mM potassium chloride, 1.5 mM magnesium chloride, and 0.5 U Taq DNA polymerase. PCR performed without DNA templates represented the negative controls.

The reaction was carried out as follows: initial denaturation was at 96°C for 2 min, followed by 10 cycles of amplification at 96°C for 20 sec, annealing at 64°C for 50 sec, followed by 20 cycles of denaturation at 96°C for 20 sec, annealing at 61°C for 50 sec, extension at 72°C for 40 sec, and a final soak at 10°C. The PCR reactions were performed in triplicate for each participant.

The genotyping was performed blinded to the clinical diagnosis; the agarose gel results were reported by an investigator who was unaware of the origin of the samples. Independent quality control analysis was performed by a laboratory technician. For the G-308A variant, this consisted of PCR and genotyping of a random selection of 20% patient and control samples to test for any differences with initial genotype data. Repetition of random samples genotyping revealed no differences with the initial data in all tested samples.

### Agarose gel electrophoreses and image scanning

The PCR products, together with approximately 4  $\mu$ L 100bp DNA ladder as a molecular weight marker (MBI, San Francisco, CA, USA), were electrophoresed on 2% agarose gel with ethidium bromide, at 120 V for 45 min. After electrophoresis, the agarose gel was scanned and imaged by the Alphaimager TM 2200 instrument (Alpha Innotech Corporation, San Leandro, CA, USA) and each sample was genotyped.

### Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) for Windows (Version 11.0; SPSS, Chicago, IL, USA). The consistency of genotype frequencies with Hardy-Weinberg equilibrium was checked using chi-squared test. The results of the gene polymorphism studies were analyzed by comparison of allele frequencies (ratio of test allele to total alleles). Frequencies of allele and genotype distribution were analyzed using chi-squared test. Odds ratios (OR) were calculated for disease susceptibility in carriers of specific alleles. The 95% confidence intervals (CI) for the OR were also calculated.  $p$  Values of  $<0.05$  (2-tailed) were considered significant.

### Results

All participants were screened for -308G/A locus variability. In the control group, distribution of TNF- $\alpha$  alleles and genotypes did not show a significant deviation from Hardy-Weinberg equilibrium ( $p = 0.07$ ). The distribution of allelic and genotypic frequencies of TNF- $\alpha$ -308 polymorphism in the patients and controls are shown in Table 1. A significant difference in A/A genotype frequency was observed between healthy and affected participants (14.2% vs 5.5%;  $p = 0.001$ ). However, the frequency of the mutant -308A allele was not different for patients compared with healthy controls (30.6% vs 28.3%;  $p = 0.6$ ). To reveal the effect of -308A/A genotype on disease susceptibility, the study population was regrouped based on the presence of a mutant genotype of TNF- $\alpha$  (e.g., TNF- $\alpha$ -A/A308 vs combined TNF- $\alpha$ -G/A308 and TNF- $\alpha$ -G/G308). As expected, logistic regression analysis illustrated a significant relationship of -308A/A genotype carriers in the patients with brucellosis (OR, 2.4; 95%CI, 1.2-4.8;  $p = 0.01$ ).

### Discussion

Protective immunity to intracellular bacteria such as *Brucella* spp. is mediated mainly by cellular immunity, characterized by the activation of T lymphocytes and macrophages to increase killing of these organisms. Macrophage-derived cytokines play an important role in the induction of immunity to these intracellular bacterial infections [6]. TNF- $\alpha$  has been demonstrated to be essential for resistance to such pathogens as *Listeria* spp. [17] and mycobacteria [18]. By TNF- $\alpha$  or IL-12 depletion in vivo, Zhan et al have shown that both cytokines are involved in resistance to *Brucella* infection [6]. The importance of TNF- $\alpha$  in brucellosis can be better understood by its inhibition in *Brucella* evasion from the immune system [19,20]. However, Zhan et al suggested that TNF- $\alpha$  and IL-12 act as a double-edged sword, contributing to both immunity and immunopathology, although their roles are quite different [6]. A delicate balance is needed to maximize their effect of enhancement of immunity and to minimize their effect of involvement in pathology. Such a balance for TNF- $\alpha$  has also been linked to leishmaniasis and tuberculosis. Although TNF- $\alpha$  is essential for the control of *Mycobacterium* and *Leishmania* infection [18,21], a role for TNF- $\alpha$  in the pathogenesis of the toxic syndrome, tissue necrosis, and cachexia accompanying tuberculosis and in the tissue destruction in mucocutaneous leishmaniasis is strongly suggested [18,22].

This study showed that, although allele frequency in the 2 groups wasn't statistically significant, the TNF- $\alpha$ -308(A/A) genotype contributes to susceptibility to brucellosis. This finding is in line with a growing body of evidence showing that TNF- $\alpha$ -308(A/A), directly or indirectly with other genes, is important

**Table 1.** Frequency distribution of allele and genotype of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )-308 polymorphism in patients with brucellosis and healthy control participants.<sup>a</sup>

TNF- $\alpha$ G3-08A polymorphism	Brucellosis No. (%)	Controls No. (%)	$p^b$
Allele			
-308G	361 (69.4)	311 (71.7)	0.6
-308A	159 (30.6)	123 (28.3)	
Genotype			
G/G	138 (53.1)	106 (48.8)	0.001
G/A	85 (32.7)	99 (45.6)	
A/A	37 (14.2)	12 (5.5)	

<sup>a</sup>Values are for patients or controls positive for each allele or genotype.

<sup>b</sup> $p$  Values were calculated by chi-squared test from  $3 \times 2$  and  $2 \times 2$  contingency tables for genotypes and alleles, respectively.

in infectious diseases. Previous studies have shown the association of TNF- $\alpha$  promoter polymorphism with various diseases in which TNF- $\alpha$  is known to be important and is present at high levels in serum, such as cerebral malaria [23], lepromatous leprosy [24], mucocutaneous leishmaniasis [25], and fatal meningococcal infection [26]. The fact that the -308A allele is associated with high TNF- $\alpha$  expression [15,16] and increased susceptibility to diverse illnesses such as those mentioned above, that are associated with high serum TNF- $\alpha$  levels, prompted the idea that this polymorphism predisposes to overproduction of TNF- $\alpha$ , which would favor the development of such diseases.

Such polymorphism has been studied in brucellosis by other groups [9]. However, Caballero et al did not find any relationship between the -308(A/A) genotype and brucellosis [9]. These researchers only showed the association of the -308(A/G) genotype and the -308A allele with brucellosis and concluded that the -308 G/A genotypes might be involved in susceptibility to brucellosis [9]. However, some researchers have shown a significant effect for the TNF- $\alpha$ -308(G/G) genotype and susceptibility to diseases [27], although others have found no significant association between TNF- $\alpha$  polymorphism and susceptibility to brucellosis [28]. The discordance in the results of these studies of the association of gene polymorphism and disease susceptibility may be attributed to differences in ethnicity, patient and/or control cohort selection or size, disease classification or status, or methods of statistical analysis [4]. Sample size is an important factor in genetic polymorphism studies. Therefore, the discrepancies that occurred between these studies might be due to a low study population. In Caballero et al's study, 59 patients with brucellosis were genotyped [9], Davoudi et al studied 47 patients [27], and Budak et al evaluated only 40 patients with brucellosis [28]. Discrepancies between studies have also been seen for TNF- $\alpha$  levels in the serum of patients with brucellosis. TNF- $\alpha$  has been shown to be significantly increased in the serum of patients with acute brucellosis [10], but the levels were undetectable in another study [11], or unchanged in patients with brucellosis compared with healthy controls [12].

As Zhan et al have suggested that the TNF- $\alpha$  balance is needed for control of infection and reducing the pathology of the infection [6], it is possible that homozygosity for the -308A allele may produce high levels of TNF- $\alpha$ , which has an immunopathologic

effect in brucellosis. This study suggests that polymorphism at position -308 in the promoter region of TNF- $\alpha$  may be involved in susceptibility to brucellosis. With regard to the different results in this area, further simultaneous study of other populations and other gene polymorphisms is required.

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