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# Interleukin-12 and Tumor Necrosis Factor-B Gene Polymorphisms as Genetic Susceptibility Factors for Brucellosis in Iranian Patients

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## Abstract

**Background:** Some reports suggest that the cytokine gene polymorphisms can contribute to the resistance or susceptibility to brucellosis. The aim of this study was to find out any probable association of genetic polymorphisms of Interleukin-12 (+1188 A/C) and TNF-β (+252 A/G) with susceptibility to the disease.

**Methods:** One hundred and ninety-six patients with brucellosis and 81 healthy farmers (controls) who owned infected animals and consumed their contaminated dairy products were included in this study. IL-12 (+1188 A/C) and TNF-β (+252 A/G) genotyping were carried out for all of the subjects.

**Results:** The results showed that the frequencies of IL-12 AA genotype and A allele were higher in controls than in patients while TNF-β AA genotype and A allele were significantly higher in patients compared to the controls.

**Conclusion:** These findings suggest that the inheritance of the above-mentioned genotypes and alleles can be considered as genetic factors conferring resistance or susceptibility to brucellosis.

**Keywords:** *Brucella*; Genetic polymorphisms; Interleukin-12; Tumor necrosis factor-β; Iran

## Introduction

Bacteria of the genus *Brucella* are gram-negative facultative intracellular pathogens that cause a severe infectious disease, brucellosis, in many animals and human as well.[1] The highest incidence of this zoonotic disease is in the developing countries and among people who have close contact with livestocks.[2] The organism enters macrophage-monocyte lineage cells, surviving and multiplying within them.[1] Consequently, acquired cell-mediated immunity (CMI), characterized by the activation of T-helper1 (Th1) lymphocytes and subsequent activation of macrophages, plays a crucial role in the protection against this infectious disease.[3] The major role of Th1 cells in *Brucella* immunity is the secretion of interferon-gamma (IFN-γ) to activate the bactericidal function of macrophages and cytolytic activity of cytotoxic T lymphocytes (CTLs) to eradicate the parasite.[3]

In this regard, cytokines such as IL-12 and TNF-α, which are secreted in the initial response and affect IFN-γ secretion or have synergetic effects with IFN-γ, can leave a strong impact on the outcome of the infection.[4-8] IL-12 is secreted by antigen presenting cells and plays a key role in cell-mediated immunity through regulating differentiation of naive T lymphocytes into Th1 cells and subsequently IFN-γ production by these cells.[9-13] However, it has been clarified that the early production of IL-12 is a T cell-independent function. The experiments with TNF receptor knockout mice indicate that TNF plays an important role in IL-12 early production.[4,5] TNF-α is also required for the maximal killing of *Brucella* by macrophage in the absence of IFN- γ activation.[14]

Physicians have long been aware of the markedly different immune responses of seemingly similar in-

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dividuals to the same inflammatory or infectious agents. The role of individual genetic differences as an explanation for these observations has been the subject of much speculation.[15-17] Several studies have identified some polymorphisms in cytokine gene regulatory regions that correlate with inter-individual variations of cytokine production in the immune response against pathogens.[18]

In this respect, TNF-α (positions -308G/A) and TNF-β (position +252 A/G) are the polymorphic sites whose roles in the production level of TNF-α were well-recognized.[19-21] As gene polymorphism role of TNF-α (-308G/A, -238G/A) in susceptibility to brucellosis had previously been studied,[22] one of the aims of the present study was to investigate the polymorphism role of TNF-β (+252 A/G) in susceptibility to this infection. It has been shown that the substitution of Guanine (G) or Adenine (A) at position +252 of TNF-β (LT-α) gene designates TNFB*1 (5.5 kb) and TNFB*2 (10.5 kb) alleles, respectively.[23] TNFB*1(G) allele is strongly associated with increased TNF-α production by peripheral blood mononuclear cells (PBMCs) in response to phytohemagglutinin (PHA), related to the increased gene transcription.[21,24] Gene polymorphisms in this locus are associated with the susceptibility to several autoimmune, inflammatory and infectious diseases.[25,26]

Another cytokine involved in the immunity against *Brucella*, with its production affected by gene polymorphism, is IL-12. Interlukin-12 p70 is composed of two disulfide-linked subunits; p35 and p40 which are encoded by the IL-12A and IL-12B genes, respectively.[27] Several promoter, intron and 3' untranslated region (UTR) polymorphisms were identified at IL-12B gene.[28,29] A *TaqI* (A/C) single nucleotide polymorphism (SNP) in the 3' UTR of the IL-12B gene at position 1188 was recently found to be functional,[28,30] and associated with many diseases.[30-34]

Considering the important roles of these two cytokines in immunity against *Brucella*, this study aims at investigating the relationship between IL-12 and TNF-β gene polymorphisms and susceptibility to brucellosis.

## Materials and Methods

One hundred and ninety six patients (96 males and 100 females, between 7-80 years of age) suffering from acute brucellosis were selected using a systematic random sampling method among 600 cases registered in the Health Center of Fars Province, southern Iran, an endemic area for brucellosis. Having gone to their places of residence and obtained their informed written consents approved by the Ethics Committee of Shiraz University of Medical Sciences, the researchers collected blood samples from the patients. Brucellosis was diagnosed based on clinical signs and symptoms (e.g. fever, night sweating, weakness, malaise, weight loss, splenomegaly, lymphadenophaty, myalgia and arthralgia), serological tests, and/or positive blood cultures. The diagnostic criteria for a positive serological test were single high titers (≥1/160) of standard agglutination test (SAT), confirmed by a high titer (≥1/160) of 2-mercaptoethanol test (2ME) at the time of infection.

The control group consisted of 81 randomly-selected healthy shepherd (40 males and 41 females, between 5-74 years of age) who had close contact with animals infected with *Brucella* and consumed their milk and dairy products. They were from the same area as the patients and all had SAT≤1/80 and 2ME≤1/20. They did not exhibit any clinical manifestations after a six-month follow up. Brucellosis in their animals was confirmed by the serological tests carried out in the laboratory of Fars Province Veterinary Administration, Shiraz, Iran.

Genomic DNA was extracted from EDTA anticoagulated venous blood using salting out method.[35] SNPs at IL-12 (+1188) and TNF-β (+252) were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) as described previously[36] with some modification. Briefly, PCR reaction for IL-12 was performed in 10 μL volume using 1 μL of 10X PCR buffer (Cinnagen-Iran), 250 ng genomic DNA, 0.5 pM of each primer (forward; 5'-TTTGGAGGAAAAGTGGAAGA-3', reverse; 5'-AACATTCCATACATCCTGGC-3'), 2.5 mM MgCl2, 200 μM of each dNTP (Cinnagen-Iran) and 1 unit of *Taq* DNA polymerase (Cinnagen-Iran) under the following conditions: a denaturation step for 5 min. at 95°C followed by 35 cycles of a denaturation for 30 sec. at 95°C, annealing for 30 sec. at 60°C, extension for 1 min at 72°C and a final extension for 5 min at 72°C in a thermocycler (5530 Mastercylcler, Eppendorf, Germany). The 300 bp amplified products were digested with 2 units of *TaqI* endonuclease (Fermentas) overnight at 65°C. The A allele remained undigested, whereas C allele yielded 2 bands of 166 and 134 bp. PCR digested products were separated on 1.5% agarose gel and identified by ethidium bromide staining.

Also, the TNF-β (+252 A/G) polymorphism was detected using PCR-RFLP method. The 782 bp PCR

product was generated in 10 µL cocktail containing 1 µL of 10X PCR buffer, 250 ng DNA template, 0.5 pM of each primer[16] (forward; 5'-CCGTGCTTCGTGGTTTGGACT-3' and reverse; 5'-AGAGGGGTGGATGCTTGGGTT-3'), 1 mM MgCl2 and 1 unit of *Taq* DNA polymerase, and 200 µM of each dNTP. The amplification protocol was carried out under the conditions as follows: a denaturation step for 5 min at 95°C, followed by 35 cycles of a denaturation at 95°C for 30 sec, annealing at 66°C for 30 sec, extension at 72°C for 1 min. A final extension was performed for 5 min at 72°C. The PCR products were digested with 2.5 units of *NcoI* endonuclease overnight at 37°C. Digested PCR products were analyzed using electrophoresis on 1.5% agarose LE gel and staining by ethidium bromide. The digestion revealed the fragments of 586 and 196 bp for the G allele and 782 bp for the A allele.

Genotypic and allelic frequencies were estimated by counting method. Associations were analyzed using Chi Square test with the level of significance set at <0.05 by EPI 2000 and SPSS software (version 13, Chicago, IL, USA). Odds ratio and 95% confidence interval were calculated. Analysis for deviations from Hardy-Weinberg equilibrium was performed based on the Pearson ($\chi 2$) test.

## Results

Genotyping of variants at position +252 in TNF-β and +1188 in IL-12B genes were performed on 196 Iranian patients with acute brucellosis and 81 healthy controls from the same region. The results of the assessment of IL-12B (+1188 A/C) and TNF-β (+252 A/G) genotypes and allele frequencies are demonstrated in Table 1 for the subjects in both groups. The distribution of each of genotypic variants met the conditions of Hardy-Weinberg equilibrium. For IL-12 gene polymorphism, the frequency of AA genotype showed a significant increase in the controls in comparison with the frequency observed in the patients (81.5% vs. 65.3%; $p$=0.007, OR=0.43, 95% CI=0.22-0.84). In addition, a significantly higher frequency of carriers of the variant +1188 A allele was observed in shepherds in comparison to that in the patients (88.9% vs. 79.6%; $p$=0.009, OR=0.49, 95% CI=0.27-0.87). Similarly, a significant difference was observed in the distribution of TNF-β (+252 A/G) genotypes between the two groups. The frequency of homozygosity of TNF-β +252 A allele was significantly higher in patients with acute brucellosis, compared to that in the controls (63.3% vs. 40.7%; $p$=0.0005, OR=2.51, 95% CI=1.43-4.41). In patients, there was a significant over-presentation of the TNF-β A allele, too (79.6% vs. 66%; $p$=0.0007, OR=2.0, 95% CI=1.31-3.07).

## Discussion

The outcome of an inflammatory response is dictated

**Table 1:** The frequencies of IL-12 and TNF-β genotypes and alleles in patients with brucellosis and controls

| | **Patient group No. (%)** | **Control group No. (%)** | ***P* value** |
|---|---|---|---|
| IL-12(+1188) Genotype (phenotype) | | | |
| AA (high IL-12) | 128 (65.3) | 66 (81.5) | 0.007 |
| AC (moderate IL-12) | 56 (28.6) | 12 (14.8) | 0.015 |
| CC (low IL-12) | 12 (6.1) | 3 (3.7) | 0.418 |
| Total | 196 | 81 | |
| Allele | | | |
| A (high IL-12) | 312 (79.6) | 144 (88.9) | 0.009 |
| C (low IL-12) | 80 (20.4) | 18 (11.1) | |
| TNF-β (+252) | | | |
| Genotype (phenotype) | | | |
| AA (low TNF-α) | 124 (63.3) | 33 (40.7) | 0.0005 |
| AG (moderate TNF-α) | 64 (32.7) | 41(50.6) | 0.005 |
| GG (high TNF-α) | 8 (4.0) | 7 (8.7) | 0.124 |
| Total | 196 | 81 | |
| Allele | | | |
| A (low TNF-α) | 312 (79.6) | 107 (66.0) | 0.0007 |
| G (high TNF-α) | 80 (20.4) | 55 (34.0) | |

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

by a variety of factors, including the pathogenecity, duration of the stimulus and balance between the pro-inflammatory and anti-inflammatory responses. It has been suggested that multiple host genetic factors are involved in the development of proper immune responses. The recent reports have shown that SNPs of some cytokines influence their expressions or functional levels.[37,38] Therefore, gene polymorphisms of cytokines and their receptors are attractive candidates as genetic factors in immune-mediated diseases and have been reported to be associated with susceptibility to some inflammatory and infectious diseases.[28-33] In a similar vein, in the present study, the influence of genetic variations in IL-12 and TNF-β genes on the susceptibility to brucellosis was investigated. To the best of our knowledge, this is the first report on the association of IL-12B and TNF-β gene polymorphisms with brucellosis.

IL-12 plays its important role by the induction of type 1 immune response, the protective immunity against intracellular bacteria such as *Brucella*.[39,40] Then, it seems that the increase in the production of IL-12 in response to *Brucella* infection may cause resistance to the development of full-blown disease and can probably control the organism. As shown in Table 1, IL-12 A allele, which is associated with the high production of IL-12,[41] was significantly more frequent in the controls compared to that in patients. Furthermore, AA genotype was significantly more frequent in the controls compared to that in the patients. Based on the results, we speculate that the individuals who inherit the AA genotype may produce higher levels of IL-12 at crucial points of the infection process which can cause a more effective cell-mediated immunity against brucellosis. In contrast, individuals who inherit C allele homo- or heterozygously (CC and AC) produce lower levels of the cytokine (IL-12) that is not sufficient to prevent the bacterial invasion.

Initial studies evaluated many exogenous cytokines for activating phagocytes for anti-*Brucella* activities and IFN- γ was found significantly more effective at enhancing intracellular control than any others.[9] This cytokine requires the presence of endogenous TNF-α for maximal controlling. TNF-α is also required for maximal killing of *Brucella* by macrophage in the absence of IFN- γ activation.[16] During the first 24 hours after infection, phagocytosed *Brucella* is killed regardless of the presence of IFN-γ, but is heavily dependent upon TNF-α.[5] Zhan *et al*. showed that mice lacking the receptors for TNF (TNFR -/- mice) were severely deficient in IL-12 production following the infection with *B.abortus.20* Then, it can be deduced that TNF-α has an essential role for the production of IL-12. TNF-α may be acting by at least two pathways to promote the early production of IL-12. First, TNF-α in an autocrine fashion along with bacterial products promotes the secretion of IL-12 by the macrophages. Secondly, TNF synergizes with IL-12 to activate NK cells to produce IFN-γ which in turn upregulates IL-12 production by macrophages.[6-8] There are several genetic polymorphic sites which are known to be associated with the production level of TNF-α, including; TNF-α (-308G/A, -238G/A) and TNF-β (+252 A/G). In some previous studies, TNF-α polymorphisms at positions -308 and -238 were studied and an association with susceptibility to brucellosis was shown. In the present study, to our knowledge for the first time, attempts were made to investigate the relationship between TNF-β (+252 A/G) and susceptibility to brucellosis among the population living in southern Iran, an endemic area for brucellosis.

Investigation of TNF-β (+252 A/G) gene polymorphism in patients with brucellosis and healthy controls showed that the frequency of AA genotypes, which is associated with the lower production of TNF-α,[21] was significantly higher in the patients than in the controls. Also, the distribution of A allele, which is associated with the low production of TNF-α,[21] was higher in the patients. Based on the results and the above-mentioned roles of TNF-α in controlling the *Brucella* infection, it could be deduced that patients who inherit A allele as homozygous (AA) may produce lower levels of TNF-α which results in lack of proper immune response at the early stage of infection with *Brucella* and so the occurrence of a full-blown disease. But in individuals with AG and GG haplotypes (controls), due to more TNF-α production, the bacteria are controlled and the disease does not develop.

Association of IL-12 and TNF-β gene polymorphisms with several diseases among Iranian population has been studied. Movahedi et al. studied the relationship between IL-12 (+1188) gene polymorphism and susceptibility to asthma. They considered the presence of CA genotype ($p$=0.003) as genetic susceptibility factor for the development of asthma.[42] Kamali-Sarvestani and his colleagues conducted some studies to find any probable association between TNF-β gene polymorphism and susceptibility to cutaneous leishmaniasis[16] and recurrent pregnancy loss.[43] Their findings revealed no association between the polymorphism and the diseases.

In conclusion, based on the present findings, it could be suggested that IL-12 (+1188) AA genotype

and A allele are influential factors in the resistance against brucellosis. Also, TNF-β (+252) AA genotype and A allele could be considered as genetic factors for the susceptibility to the disease.

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**Conflict of interest:** None declared.

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