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TNF-α -238, -308, -863 polymorphisms, and brucellosis infection

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Background: *Brucella abortus* is an intracellular bacterium that affects humans and domestic animals. Tumor necrosis factor-alpha (TNF-α) has been shown as a key player in the induction of cell-mediated resistance against Brucella infection. We aimed to evaluate the possible influence of the TNF-α promoter polymorphisms (-308 G/A, -238 G/A, and -863 C/A) on the susceptibility of human brucellosis.
Methodology: A total of 153 patients with active brucellosis and 128 healthy individuals were recruited. All subjects were genotyped for the polymorphisms in the TNF-α gene by Allele-Specific polymerase chain reaction analysis.
Results: Our results showed that the TNF-α -308 GG genotype was significantly more frequently present in controls than in brucellosis patients (91% vs. 75%), thus was a protective factor against developing brucellosis (OR = 0.313, $p = 0.001$). In contrast, the -308 GA genotype (OR = 3.026, $p = 0.002$) and minor allele (A) (OR = 3.058, $p = 0.001$) as well as AAG haplotype (OR = 4.014, $p = 0.001$) conferred an increased risk of brucellosis. However, the -238 G/A and -863 C/A polymorphisms were not associated with the risk of brucellosis at both allelic and genotypic levels ($p > 0.05$).
Conclusion: Our study revealed that the TNF-α -308 A allele or GA heterozygosity or AAG haplotype were associated with an increased risk of brucellosis in our population.
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1. Introduction

A gram-negative, facultative and intracellular bacteria, *Brucella* spp. can result in chronic zoonotic infections in humans (Eze, Yuan et al. 2000) [1]. The *Brucella* spp. causes brucellosis with pathophysiological manifestations of arthritis, endocarditis, and meningitis in humans, and spontaneous abortion in cattle [2]. Human brucellosis is an extremely weakening disease, which influences over half a million infected people annually [3]. The disease exists worldwide, but is especially common in the Mediterranean and Middle Eastern regions including Iran, Turkey and the Arabian Peninsula [4–8].

The T helper (Th)1/Th2 balance has been known to be involved in the susceptibility or resistance to the brucellosis infection. Brucella infections result in Th1 cellular immune response, the development of which is under the control of major cytokines like tumor necrosis factor-alpha (TNF-α), gamma interferon (IFN-γ) and IL-12, produced at the onset of the infection [3]. These cytokines play a key role in the regulation of the immune response against brucellosis infection, and mediate production of many pro- and anti-inflammatory signals [9] TNF-α is a potent pleiotropic proinflammatory and immunoregulatory cytokine, which contribute to the initiation, up-regulation, and perpetuation of the inflammatory response. TNF-α is produced by many cell types including neutrophils, fibroblasts, NK cells, T and B cells, and macrophages infiltrating tissue as the part of host defense against brucellosis infection (1, 11-12, 16). Increased levels of serum TNF-α has been observed in several infectious diseases including brucellosis [7,10], advanced tuberculosis (TB) [11], acute-phase Mediterranean spotted fever [12] and malaria.

The TNF-α gene lies in the class III region of the major histocompatibility complex (MHC) and is located on the human

Abbreviations: TNF-α, tumor necrosis factor-alpha; Th, The T helper; IFN-γ, gamma interferon; TB, tuberculosis; MHC, major histocompatibility complex; SNPs, single nucleotide polymorphisms; G, guanine; A, adenine; HBV, hepatitis B virus; AS-PCR, Allele-Specific polymerase chain reaction; HWE, Hardy–Weinberg equilibrium; LD, Linkage disequilibrium.

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chromosome 6p21.3 [10]. Variation between individual TNF-α concentrations has been attributed to single nucleotide polymorphisms (SNPs) in the TNF promoter region [13–15]. Several SNPs in the TNF-α promoter region have been identified, which are thought to affect TNF-α production. The best documented of the TNF-α promoter SNPs are -238G/A (rs361525), -308G/A (rs1800629), and -863C/A (rs1800630). The SNPs at positions -308 and -238 of the TNF-α gene promoter involve the substitution of guanine (G) for adenine (A) and the formation of two alleles (-308G/-238G and -308A/-238A) and three genotypes GG, GA, and AA [13,14]. Multiple in vivo and in vitro studies have shown that the allele A of TNF-α -308 and -238 polymorphisms was associated with increased expression of the TNF-α gene [16,17]. However, the C-863A (rs1800630) polymorphism which involves a C to A exchange at position -863 of the TNF-α gene promoter was correlated with lower TNF-α levels [18].

Thus far, the TNF-α polymorphisms have been examined in several infectious diseases including TB [17,19], hepatitis B virus (HBV) and HCV [20–22], *Helicobacter pylori* [23] and brucellosis infections [24], but the results have been inconsistent and contradictory. Therefore, the current study was aimed to examine the possible association of three genetic polymorphisms in TNF-α (-308 G/A, -238 G/A and -863 C/A) gene and the risk of brucellosis in an Iranian population.

2. Materials and methods

2.1. Study population

In this case-control retrospective study, we used 153 patients (102 men and 51 women) suffering from active brucellosis, age range 6–76 years and mean ± SD = 31.24 ± 16.6 and 128 healthy individuals as the control group (93 men and 35 women), age range 19–64 years and mean ± SD = 34.04 ± 13.69. Blood samples from all participants were collected in EDTA-containing tubes for DNA isolation. All the patients were either milk farmers (including diagnosed infected animals) or subjects with a history of consuming raw milk and unpasteurized dairy products. Demographic characteristics of patients and their clinical complications have been shown in Supplementary Table 1. Brucellosis was diagnosed according to the clinical manifestations (including fever, night sweating, weakness, malaise, weight loss, splenomegaly, lymphadenophathy, myalgia and arthralgia) and positive blood cultures as described previously [1,25–27]. The control group involved healthy blood donors with no record of brucellosis and genetic disorders and matched for age, sex, and geographic area. The control subjects had the same background as patients and were at the same risk of exposure for brucellosis.

2.2. Culture and identification of organism

Brucella strains were grown on 5% sheep blood-agar plates and incubated at 37 °C in the presence of 5–10% of CO2 conditions for 48 h. A typical and well-isolated Brucella like colony is tiny, transparent, raised, and convex, with an entire edge and smooth and glistening surface along the streak lines by examining macroscopically by Gram's stain [28]. Serological tests, defined as wright titre ⩾1/160 plus mercaptoethanol test ⩾1/80 or coomb's wright ⩾1/320 were confirmative of the brucellosis infection. The inclusive details of organism identification methods have been described previously in papers by this research team [1,25–26].

2.3. Genotyping of TNF-α (-308 G/A, -238 G/A and -863 C/A) variants

DNA extraction from the peripheral blood leukocytes was performed by the 'salting-out' method as described previously [29]. The purity of the isolated DNA was checked by electrophoresis on 1% agarose gel, quantitated spectrophotometrically and stored at −20 °C till further use.

All TNF-α gene polymorphisms (-308 G/A, -238 G/A and -863 C/A) were genotyped using Allele-Specific polymerase chain reaction (AS-PCR) method as described previously [30–31]. The AS-PCR uses two sequence-specific forward primers (F1 and F2) and one common reverse primer (R) in separate PCR reactions. The cycling conditions for three TNF-α SNPs consisted of a 1-min denaturation step at 95 °C; 10 cycles of 15 s at 95 °C and 50 s at 65 °C and 40 s at 72 °C; and 20 cycles of 20 s at 95 °C, 50 s at 59 °C, and 30 s at 72 °C, followed by cooling to 4 °C 96 °C for 1 min, followed by five cycles of 96 °C for 25 s, 70 °C for 45 s, and 72 °C for 25 s; 21 cycles of 96 °C for 25 s, 65 °C for 50 s, 72 °C for 30 s; and four cycles of 96 °C for 30 s, 55 °C for 60 s, extension at 72 °C for 90 s and hold at 15 °C [31]. PCR products were separated by standard electrophoresis on 2.5% agarose gel containing ethidium bromide. All primer sequences and amplicons size were listed in Supplementary Table 2.

2.4. Statistical analysis

All statistical analyses were computed by SPSS software for Windows, version 18.0 (SPSS Inc, Chicago IL, USA). The association among genotypes and brucellosis was calculated by reckoning the odds ratio (OR) and 95% confidence intervals (95% CI) from logistic regression analyses. *P*-values below 0.05 were considered statistically significant. The Hardy–Weinberg equilibrium (HWE) was calculated with the χ^2 test. Linkage disequilibrium (LD) and frequencies of haplotypes in the controls and patients were analyzed using SNPStats software [32].

3. Results

3.1. Genotype frequencies of TNF-α polymorphisms

All SNPs were successfully genotyped in 153 brucellosis patients and 128 control subjects. No deviation from HWE was observed in the studied control groups ($p > 0.05$). The genotype and allele frequencies of the three SNPs in the studied groups are shown in Table 1.

Among three TNF-α SNPs (-308 G/A, -238 G/A and -863 C/A) the -308 G/A gene polymorphism was associated with predisposition to brucellosis infection. The TNF-α -308 homozygote genotype (GG) was significantly more frequently present in controls than in brucellosis patients (91% vs. 75%), thus was a protective factor against developing brucellosis (OR = 0.313, 95%CI = 0.146–0.659, $p = 0.001$). However, the TNF-α heterozygote genotype (GA) was more prevalent in patients compared with controls (24% vs. 9%) and was associated with increased risk of brucellosis (OR = 3.026, 95% CI = 1.499–6.108, $p = 0.002$). Likewise, the -308 G/A minor allele (A) was significantly more frequently present in brucellosis patients than in controls (13% vs. 5%), and was a risk factor for brucellosis (OR = 3.058, 95% CI = 1.507–6.315, $p = 0.001$).

On the other hand, the allele and genotype frequencies of the other two TNF-α SNPs (-238 G/A and -863 C/A) did not differ significantly between brucellosis patients and the controls, and were not associated with the risk of brucellosis ($p = 0.805$, $p = 0.524$ for minor alleles of 238 and -863, respectively).

3.2. LD and haplotype association analysis of TNF-α polymorphisms

LD was analyzed by calculating Lewontin's Delta′ coefficient and the correlation coefficient r^2 [33]. Pairwise LD between the SNPs TNF-α (-308 G/A, -238 G/A and -863 C/A) was calculated

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