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Levels of Interleukin-(IL)-12p40 are Markedly Increased in Brucellosis Among Patients with Specific *IL-12B* Genotypes

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Abstract

Brucellosis remains a major zoonosis worldwide. Brucella antigens induce the production of T-helper 1 (Th1) cytokines such as interleukin-12 (IL-12) in humans. We aimed to investigate the association of two single nucleotide polymorphisms (SNPs) in the gene encoding the IL-12p40 cytokine (IL-12B) with brucellosis and to examine the functionality of these SNPs through measuring serum levels of IL-12p40. We genotyped *IL-12B* gene rs3212227, A>C; rs6887695 G>C polymorphisms in a case-control study on a total of 281 subjects including 153 patients with active brucellosis and 128 healthy controls, using RFLP and serum IL-12p40 levels, were assessed by ELISA. The rs3212227 minor allele (C) and homozygote genotype (CC) were more frequent in controls compared with patients with brucellosis ($P = 0.006$, OR = 0.608, 95%CI = 0.429–0.861 for the C allele; $P = 0.024$, OR = 0.443, 95% CI: 0.218–0.900 for the CC genotype). Comparison of *IL-12B* genotypes and serum levels of the IL-12p40 revealed that rs3212227 AA genotype, with higher frequency in patients than in controls, was associated with increased levels of the cytokine ($P = 0.0001$). Furthermore, the distribution of haplotype and genotype combinations in our study suggested that rs3212227C/rs6887695C haplotype or CC/GC or CC/CC genotype combinations may protect controls against Brucella infection by contributing to a functional downregulation of the serum IL-12p40 production *in vivo*, as shown by ELISA ($P < 0.05$). Overall, our study demonstrated that rs3212227 A variant was associated with higher levels of serum IL-12p40 and could possibly contribute to an inherited predisposition to brucellosis.

Introduction

Brucellosis is the most common bacterial zoonotic disease worldwide, with over half a million infected people annually [1]. Despite its control in many countries, it remains endemic in the Mediterranean and Middle Eastern regions including Iran, Turkey and the Arabian Peninsula [2, 3]. Brucella melitensis and Brucella abortus are the most frequent cause of human brucellosis in these geographical areas [4–6]. The Brucella spp. results in brucellosis with pathophysiological manifestations of arthritis, endocarditis and meningitis in humans and spontaneous abortion in cattle [7]. Brucella organisms invade cells of the reticuloendothelial system and can be sequestered in macrophages at specific locations within the body, such as spleen, brain, joints, heart, liver and bone marrow [8].

Both cell-mediated immunity and humoral responses are responsible for the clearance of Brucella infection. Host protection against Brucella spp. depends on cell-mediated immunity, involving mainly activated antigen-presenting cells (macrophages, dendritic cells) and CD4+, CD8+T-lymphocytes [5, 7]. The pattern of T-lymphocyte cytokine secretion is considered to be critical for the effectiveness of the protective anti-Brucella immune response. [5]. Brucella antigens induce the production of T-helper 1 (Th1) cytokines in humans; thus, Th1 immune response is essential for the clearance of Brucella infection [8]. Studies conducted to date have revealed that Brucella abortus can induce the release of proinflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-12 and TNF-α in a variety of cell types [9]. One of the most important Th1 cytokines is IL-12, which plays a pivotal role in macrophage activation and in control of Brucella infection both *in vitro* and *in vivo* [10].

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IL-12 is a regulatory cytokine that connects the innate and adaptive host response to Brucella spp. and exerts its effects mainly through induction of interferon gamma (IFN-γ) [11]. It is a potent inducer of IFN-γ production by both resting and activated T cells and natural killer (NK) cells; moreover, it directs the generation of Th1 responses. This cytokine also enhances the cytolytic activity of a number of effector cells, including T cells, NK cells and macrophages, and stimulates the proliferation of activated T and NK cells [10, 12].

IL-12, a heterodimer composed of IL-12p35 and IL-12p40 subunits, was shown to play an important role in protective immune response against Brucella infection. In its bioactive form, IL-12 with a molecular mass of 70 kDa (IL-l2p70) is secreted mainly by monocytes and macrophages, but also by several other cell types including dendritic cells, B cells and polymorphonuclear leucocytes. Furthermore, in comparison with the cytokine (IL-l2) p70 bioactive form, the 40-kDa subunit is secreted at higher levels and is shown to contribute to the intracellular defence against bacterial infections like Brucella infection and in favouring Th1 polarization [5, 11].

IL-12 p35 and p40 subunits are encoded by *IL-12A* and *IL-12B* genes located on chromosomes 3p12–q13.2 and 5q31–33, respectively. The +1188A→C substitution (rs3212227) in the 3′UTR of *IL-12B* has been reported to influence gene expression and is associated with several Th1-mediated diseases [11, 13, 14]. A further common variant (rs6887695) located 60 kilobases (kb) upstream of the *IL-12B* coding region has been associated with several pathological conditions including multiple sclerosis [15], inflammatory bowel disease (IBD) [16], Graves' disease [17], psoriasis [18]. Thus, these SNPs of IL-12B are candidates as some of the genetic factors in Brucellosis. In this study, we examined for the first time the association of two polymorphisms in the *IL-12p40* gene, rs3212227 A/C and rs6887695 G/C and the cytokine serum levels with susceptibility to brucellosis in an Iranian population.

Materials and methods

Patients. In the current case-control retrospective study, 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, were participated. After obtaining written informed consent, a blood sample was taken from all participants and the sera were separated and stored at 70 °C until the ELISA analysis, and the remaining WBC pellet were collected in EDTA-containing tubes for DNA extraction. All the patients were from Yazd province, Iran, and were either milk farmers (including diagnosed infected animals) or had a history of consuming raw milk and unpasteurized dairy products. Table 1 demonstrates demographic characteristics of patients and their clinical complications. Brucellosis was diagnosed according to the clinical manifestations including fever, night sweating, weakness, malaise, weight loss, splenomegaly, lymphadenopathy, myalgia and arthralgia, positive blood cultures and serological tests, defined as Wright titre $\geq$ 1/160 plus mercaptoethanol test (2ME) $\geq$ 1/80 or Coomb's wright $\geq$ 1/320. Almost all patients had overt serious clinical disease, and their disease was confirmed after clinical serology tests. The control group composed of healthy blood donors with no history of brucellosis and genetic disorders and matched for age, sex and geographical area. The controls come from the same background population as cases and are at the same risk of exposure to brucellosis.

Genotyping of two variants, rs3212227 and rs6887695. Genomic DNA was extracted from the whole blood by a 'salting-out' method as described previously [19]. Two selected SNPs in the *IL-12B* gene were genotyped by a restriction fragment length polymorphism (RFLP) method as described previously [17] with minor modifications (The optimum annealing temperature for the rs6887695 genotyping was changed from 63 °C to 61 °C). The rs3212227 is an A/C SNP in the 3′UTR, and rs6887695 is a G/C SNP located 60 kb upstream from the

Table 1 Demographic and clinical characteristics of patients.

	Number (%) Total = 153
Age	31.24 ± 16.60
Sex	
Men	102 (66.66)
Women	51 (33.33)
Fever	99 (64.70)
Myalgia	38 (24.83)
Anorexia	85 (55.55)
Headache	58 (37.90)
Malaise	70 (45.75)
Low back pain	35 (22.87)
Fatigue	65 (42.48)
Sweating	93 (60.78)
Weight loss	53 (34.64)
Arthralgia	84 (54.90)
Paresthesia	29 (18.95)
Palpitations	26 (16.99)
Nausea	23 (15.03)
Rash	18 (11.76)
Dysuria	17 (11.11)
Blood culture (positive)	105 (68.62)
Brucella species	
Brucella melitensis	114 (74.17)
Brucella abortus	38 (25.83)
Clinical complications	
Arthritis	22 (14.37)
Endocarditis	2 (1.30)
Spondylitis	4 (2.61)
Neurobrucellosis	7 (4.57)
Meningitis	1 (0.65)
Mortality	6 (3.92)

IL-12B coding region. The forward and reverse primers used for each polymorphism were as follows (5′–3′): for rs3212227 (F: CACAACGGAATAGACCCAAAA, R: GG CAACTTGAGAG-CTGGAAA) and rs6887695 (F: GCTT CAGGCTTCACCAGTCT, R: GAAGCAACACCCCCTA GGTC). Thermocycling was carried out using the following conditions: 95 °C for 2 min, 35 cycles of 95 °C for 20 s, 60 °C for 20 s, 72 °C for 30 s, followed by a 5-min extension at 72 °C. For the rs6887695 polymorphism, annealing temperature was 63 °C. The rs3122227 and rs6887695 PCR products (15 μl) were further digested with restriction enzymes TaqI and HphI, respectively, and then visualizing on a 3% agarose gel.

Cytokine assay. Serum IL-12p40 levels were measured by an ELISA kit (IBL, Hamburg, Germany) with a lower limit of detection of 10 pg/ml. The analyses were performed in duplicates and according to the manufacturer's procedure. Also for defining of the serum levels of this cytokine, standard samples with known concentration of cytokine expressed as pg/ml were utilized.

Statistical analysis. All statistical analyses were performed using the SPSS software for Windows, version 15.0 (SPSS Inc, Chicago IL, USA). Data were expressed as mean ± SD. Comparisons between groups were analysed by Student's t-test, ANOVA and χ^2 test when appropriate for quantitative variables. The association between genotypes and brucellosis was assessed by computing the odds ratio (OR) and 95% confidence intervals (95% CI) from logistic regression analyses. For genetic comparisons, differences in allele, genotype and haplotype frequencies were evaluated using the χ^2 test. The Hardy–Weinberg equilibrium was tested with the χ^2 test for any of the SNPs under consideration. Linkage disequilibrium and frequencies of haplotypes in the controls and patients were estimated using HAPSTAT version 3.0 software (Tammy Bailey, Danyu Lin and the University of North Carolina, Raleigh, NC, USA) [20]. A two-tailed P-value < 0.05 was considered to be statistically significant.

Results

Frequency of rs3212227A/C and rs6887695G/C IL-12B polymorphisms

The allele and genotype frequencies of *IL-12p40* gene rs3212227 (+1188A/C) and rs6887695G/C positions in patients with active brucellosis and controls are listed in Table 1. It has been established that the OR lower than 1 along with a $P < 0.05$ is associated with lower risk of disease (protective factor), whereas the OR above 1 along with a $P < 0.05$ is associated with higher risk of disease (risk factor). A significant difference was found between two groups regarding allelic and genotyping distribution of the rs3212227A/C variant ($P = 0.030$). The frequency of CC genotypes in the control group was higher than in the cases showing a statistically significant difference (OR = 0.443, 95% CI: 0.218–0.900, $P = 0.024$). Furthermore, frequency of C allele at rs3212227 position in patients was reduced once compared with the control group, demonstrating the protective role of C variant against brucellosis (OR = 0.608, 95% CI: 0.429–0.861, $P = 0.006$). On the other hand, the allelic and genotypic distribution of the *IL-12B* polymorphism at position rs6887695G/C was not significantly different between patients and controls (OR = 0.740, 95% CI: 0.398–1.376, $P = 0.342$ for the CC genotype; OR = 0.788, 95% CI: 0.564–1.100, $P = 0.173$ for the C allele). Furthermore, in the current study, the *IL-12B* gene polymorphisms were analysed according to the patient's clinical complications and disease severity at the very first time of patients' referral for specialist treatment; however, no significant association between these features and IL-12p40 genotypes was found (the data were not shown).

Linkage disequilibrium and haplotype analysis of IL-12B polymorphisms

None of the SNPs had genotype frequencies that deviated significantly from Hardy–Weinberg equilibrium in the studied control groups ($P > 0.05$). Linkage disequilibrium was tested by calculating Lewontin's Delta′ coefficient and the correlation coefficient r2 [21], and it was found that the *IL-12B* rs3212227A/C was in low linkage disequilibrium with rs6887695G/C (D′ = 0.177, r2 = 0.021). Four haplotypes of the *IL-12B* gene comprised two alleles of each polymorphism site (Table 2). The C/C haplotype found to be protective against brucellosis with higher frequency in controls than in patients, and the difference between two groups was statistically significant (OR = 0.488, 95% CI = 0.319–0.746, $P = 0.0008$).

Table 3 demonstrates the frequency of nine genotype combinations in patients and controls. The frequency of patients carrying genotype combinations rs3212227CC/ rs6887695GC or CC/CC was significantly reduced once compared with healthy controls, suggesting that carriage of these genotype combinations may protect controls from brucellosis (OR = 0.172, 95% CI: 0.042–0.711, $P = 0.015$ for the CC/GC; OR = 0.342, 95% CI: 0.118–0.992, $P = 0.048$ for the CC/CC). However, no significant difference in the frequency of subjects carrying other genotype combinations was observed between patients and controls ($P > 0.05$). Furthermore, as demonstrated in the Table 3, maximum and minimum levels of serum IL-12p40 in patients with brucellosis are observed in AA/GG and CC/CC carriers, respectively.

Serum Levels of IL-12p40 according to genotypes at rs3212227 and rs6887695 positions

The analyses of genotypes and their corresponding serum levels of IL-12p40 protein from the patient and control

Table 2 Comparison of *interleukin-12B* gene polymorphisms between the patients and the controls.

Polymorphism	Patients n (%)	Control n (%)	[a]Odds Ratio (95% CI)	[a]*P*-value
Position rs3212227 Genotypes				
AA	78 (51.0)	47 (36.7)	Ref.	–
AC	57 (37.3)	55 (43.0)	0.630 (0.374–1.060)	0.082
CC	18 (11.8)	26 (20.3)	0.443 (0.218–0.900)	0.024
Alleles				
A	213 (69.6)	149 (58.2)	Ref.	–
C	93 (31.4)	107 (41.8)	0.608 (0.429–0.861)	0.006
Position rs6887695 Genotypes				
GG	61 (39.9)	40 (31.3)	Ref.	–
GC	55 (35.9)	53 (41.4)	0.708 (0.406–1.234)	0.223
CC	37 (24.2)	35 (27.3)	0.740 (0.398–1.376)	0.342
Alleles				
G	177 (57.8)	133 (51.9)	Ref.	–
C	129 (42.2)	123 (48.1)	0.788 (0.564–1.100)	0.173

[a]Adjusted for Age and Sex.

Table 3 Distribution of haplotypes of *interleukin-12B* gene polymorphisms in patients with brucellosis and control groups.

Haplotypes	Patients	Controls	[a]$\chi 2$	[a]*P*-value	[a]Odds Ratio	[a]95% CI
A/C	0.28	0.22	2.12	0.17	1.42	0.88–1.87
A/G	0.42	0.35	0.09	0.12	1.33	0.96–1.73
C/C	0.14	0.26	10.86	0.01	0.38	0.29–0.75
C/G	0.16	0.16	0.01	0.84	0.95	0.58–1.52

[a]Adjusted for Age and Sex.

groups are summarized in Table 4. The genotype rs3212227 AA was present in 87 of 153 patients with brucellosis, and its mean IL-12p40 level was 170.1 ± 42.6 pg/ml, which was much higher than that of AC or CC genotypes ($P = 0.0001$). In respect to rs6887695, although GG carriers showed a relatively increased IL-12p40 serum level (160.5 ± 44.4 pg/ml) in comparison with GC or CC carriers, this tendency was not statistically significant ($P = 0.094$). However, no significant difference regarding serum IL-12p40 levels among genotypes of the two loci, rs3212227 and rs6887695, was found in controls ($P = 0.580$ and $P = 787$).

Discussion

It has been shown that IL-12 plays an important role in protective immune response against Brucella infection. IL-12 is a Th1-related cytokine that plays an important role in both the innate and the adaptive immune system [22]. It has been postulated that Th1 cytokines confer resistance, while Th2 cytokines facilitate the development of brucellosis [23]. Early production of IL-12 within the 1st h of infection has been reported, particularly in the case of intracellular bacteria and parasites. In murine models, endogenously produced IL-12 has been observed to contribute to the control of the host response against infections with intracellular organisms such as Chlamydia pneumoniae [24], Mycobacterium tuberculosis [24], Leishmania major [25], Legionella pneumophila [26], B. abortus [27]. In our study, it was found that IL-12p40 levels are elevated in patients with active brucellosis compared with healthy subjects. In accordance with the results, there are several reports suggesting increased IL-12p40 levels in some infectious diseases including severe malaria [28, 29], tuberculosis (TB) [30, 31] and brucellosis [5, 32]. In Brucellosis, increased IL-12p40 levels and normal levels of Th2-related cytokines, in untreated patients with brucellosis patients and remarkable reduction in the serum IFN-γ and IL-12p40 levels after effective treatment of the patients, in the absence of significant variations in the Th2 cytokines, indicated that Brucella infective microorganisms are the critical inducers of the Th1 response in human [5, 33].

In the current study, we found that the rs3212227 CC genotype was more frequently observed in the control group than in the patients (20.3% versus 11.8%). However, the rs3212227 AA genotype was more prevalent in the patients than in the healthy subjects (51.0% versus 36.7%), and the maximum serum levels of the IL-12p40 (170.1 ± 42.6) were observed in patients with the AA genotype in comparison with the cytokine levels in the remaining genotypes (Table 4). From the data, it can be inferred that the rs3212227 C allele, associated with lower IL-12 production, confers protection against brucellosis infection.

To the best of our knowledge, no study has been conducted on *IL-12B* gene polymorphisms and brucellosis; therefore, we were not able to compare our results with other investigations on brucellosis. But, there are several researches in regard to *IL-12B* gene polymorphisms and infectious and non-infectious diseases among various populations. Our results regarding the protective role of rs3212227 C allele parallel reports on Type 2 diabetic [34] and Breast Cancer[35]. In opposition to our results, the C allele was associated with higher risk of a wide range of diseases of immune dysregulation, including psoriasis[18, 36], ankylosing spondylitis [37], nasopharyngeal [38], cervical [39, 40] and oesophageal cancers [41]. However, no association was found between this variant and Chronic obstructive pulmonary disease (COPD) [42], pulmonary tuberculosis [14], Type I Diabetes[43], Acute coronary syndrome (ACS), pre-eclampsia [44], psoriasis [45] and IBD [16].

Table 4 The corresponding serum levels of interleukin-12p40 protein (pg/ml) among different genotypes at rs3212227 and rs6887695 positions from the patient group and the control group.

	Patients			Control		
	Frequency	Serum level (pg/ml)	P	Frequency	Serum level (pg/ml)	P
rs3212227						
AA	78	170.1 ± 42.6	0.0001	47	22.03 ± 7.29	0.580
AC	57	139.3 ± 20.7		55	21.69 ± 8.47	
CC	18	120.3 ± 35.3		26	21.04 ± 8.10	
rs6887695						
GG	61	160.5 ± 44.4	0.094	40	20.67 ± 8.81	0.787
GC	55	154.9 ± 35.6		53	20.63 ± 8.26	
CC	37	142.2 ± 40.2		35	21.75 ± 8.81	
Total	153	154.1 ± 40.8		128	20.95 ± 7.95	

Functional characterization of *IL-12B* rs3212227 A/C variants is unclear, and the reports are inconsistent and much debating. Our study revealed that the A variant of this polymorphism, which was more prevalent among patients with brucellosis, was associated with elevated serum levels of the IL-12p40, thus may contribute to an inherited susceptibility to brucellosis. Similarly, Morahan *et al.* [46] observed that the rs3212227AA genotype was associated with a significantly elevated expression of IL-12 in Epstein–Barr virus-transformed human cell lines. Additionally, the association between the rs3212227 A variant and increased levels of the cytokine was also reported [35, 43, 46–49]. However, the rs3212227 A variant was shown to be correlated with reduced levels of IL-12p40 in several other researches [37, 50]. This low IL-12B response in these individuals could be partly due to the influence of other genetic polymorphisms affecting the IL-12B gene expression or due to linkage disequilibrium of the 1188C allele with other common polymorphisms in IL12B that were recently found to be associated with brucellosis. In fact, the interaction between host and pathogen is so complex that natural control of the bacterial infection and its resulting disease can almost never be determined by one single gene, although expression of an allelic variant at one particular locus may significantly affect the pathogenesis of the disease in some persons.

With respect to another variant, rs6887695, our study failed to show any significant differences in allelic and genotypic distribution of this polymorphism across the groups. We also found no correlation between rs6887695 genotypes and IL-12p40 levels in either of two groups including patients with brucellosis and healthy subjects, although the GG carriers showed higher levels of the cytokine among patients with brucellosis. Although the exact reason for the lack of a functional association between rs6887695 genotypes and IL-12p40 levels reported here remains to be determined, it is possible that additional polymorphic variants, such as rs3212227, *IL-12B* [14], or variation in other genes that influence IL-12 production (e.g. IL-10 and IL-4) may be responsible for mediating IL-12 generation [51]. In accordance with our results, no association was found between rs6887695 variant in Graves' disease [52], psoriasis [45, 53] and Crohn's disease [54].

Furthermore, we tried to examine *IL-12B* rs3212227 A/C and rs6887695 G/C combination of genotypes (Table 3). Analysis of combination genotype clarified a higher frequency of CC/GC or CC/CC combinations in the controls than in the patients. The CC/GC and CC/CC combination carriers hold lower levels of IL-12p40 in comparison with other combination carriers in the patient group. Meanwhile, the distribution of haplotype in our study suggested that rs3212227C/rs6887695C haplotype may protect controls against Brucella infection possibly through contributing to a functional downregulation of the serum IL-12p40 production *in vivo*, as shown by ELISA ($P < 0.05$).

On the other hand, in this study, serum IL-12p40 level was not associated with *IL-12B* polymorphism, 3212227 A/C, in healthy controls. A plausible explanation is that the IL-12p40 expression is inducible, and its expression is up-regulated after stimulation and such stimulation in healthy donors should be missing [12]. We postulate that the increased production of IL-12p40 by activated inflammatory cells such as macrophages, monocytes, dendritic cells and neutrophils after stimulation with the bacteria may lead to more evident differences between genotypes in patients with brucellosis.

Furthermore, the data of our study showed that distribution of SNPs of IL-12B in patients with brucellosis was associated with disease susceptibility, rather than severity of brucellosis. Also, IL-12p40 level was at high and approximately equal levels in the patients' sera, with no differences resulting from severity of disease, whereas the cytokine level was affected by IL-12B genotypes. However, as our research was a retrospective study, we were not able to follow-up the outcome or severity of the disease after patients' first referral for treatment, and this is the

limitation of our study. In fact, the severity mentioned in the manuscript means the severity of the brucellosis infection at the time of patient's first visit for treatment, without knowing the course of disease after the first referral. Although, the majority of patients with brucellosis who participated in our study had severe and acute disease and were at relatively equal stage of disease.

Overall, On the basis of the results, it was shown that the frequencies of the rs3212227 C allele and CC genotype were significantly higher in controls than in the patients and both were associated with a reduced level of IL-12p40, so it can be suggested that the inheritance of the rs3212227 C variant may be one of the factors which can result in resistance to brucellosis among Iranian subjects. As reaching to a better understanding of brucellosis, immunology is a priority for the development of new therapeutic and vaccination strategies; thus, the results need to be confirmed in larger patient cohorts, as well as extending the work to subjects from different ethnic groups.

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Declaration of interest

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Declaration of conflicting interests

The Authors declare that there is no conflict of interest.

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