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ORIGINAL ARTICLE

Relationship between γ-interferon gene polymorphisms and susceptibility to brucellosis infection

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ABSTRACT

Interferon-gamma (IFN-γ) is a pro-inflammatory cytokine that plays a pivotal role in the defense mechanism against Brucella infection. It was hypothesized that the IFN-γ in (+874 A/T in intron 1) TT and +5644 T/A, TT genotypes, which are reportedly associated with high IFN production, are associated with susceptibility to brucellosis in Iranian subjects. Genotyping of these IFN-γ variants by an allele-specific polymerase chain reaction method was performed in 281 subjects, comprising 153 patients with active brucellosis and 128 healthy controls. It was found that the +874 minor allele (A) and homozygote genotype (AA) were significantly more frequently present in brucellosis patients than in controls (OR = 2.588; 95% CI, 1.313–5.104; $P = 0.006$ for the AA genotype; OR = 1.575; 95% CI, 1.124–2.216; $P = 0.010$ for the A allele). However, the allelic and genotypic distribution of the IFN-γ polymorphism at position UTR5644 A>T did not differ significantly between patients and controls ($P > 0.05$). The distribution of haplotypes in this study suggests that the T/A haplotype (+874/UTR5644), which was present more frequently in controls than in patients, may protect subjects against Brucella infection. It is suggested that IFN-γ +874 AA genotype and A allele are risk factors for developing brucellosis infection in Iranian subjects.

Key words Brucellosis, gene polymorphism, interferon-gamma.

Brucella spp., short, nonmotile, nonsporulating, nonencapsulated, and gram-negative aerobic rods are important facultative intracellular pathogens of humans and livestock (1). Brucellosis is the most common bacterial zoonotic disease worldwide, over half a million people being infected annually (2). *Brucella* spp. are important intracellular human and animal pathogens associated with fascinating mechanisms of immune modulation. Despite control of this organism in many countries, it remains endemic in the Mediterranean and Middle Eastern regions including Iran, Turkey and the Arabian Peninsula (3–4). *B. melitensis* and *B. abortus* are the most frequent causes of human brucellosis in these geographical areas (5–7). The *Brucella* spp. cause brucellosis, the pathophysiological manifestations being arthritis, endocarditis and meningitis in humans, and spontaneous abortion in cattle (8). *Brucella* organisms invade cells of the reticuloendothelial system and can be sequestered in

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List of Abbreviations: *B.*, *Brucella*; CI, confidence interval; IFN, interferon; IL, interleukin; LD, linkage disequilibrium; NF-kB, nuclear factor-kB; OLP, oral lichen planus; OR, odds ratio; SNP, single nucleotide polymorphism; Th, T-helper; UTR, untranslated region.

macrophages at specific locations within the body, such as the spleen, brain, joints, heart, liver and bone marrow (9).

Both cell-mediated immunity and humoral responses are responsible for the clearance of *Brucella* infection (10, 11). Host protection against *Brucella* spp. primarily depends on cell-mediated immunity, involving mainly activated antigen-presenting cells (macrophages, dendritic cells) and CD4þ+ and CD8þ+ 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. (6). Brucella antigens induce production of Th1 cytokines in humans and the Th1 immune response has been proven to be essential for the clearance of Brucella infection (8). IFN-γ is a critical cytokine for host control of Brucella infection and a key mediator in conferring protection against Brucella infection both *in vivo* and *in vitro* (12). *In vitro* studies have shown that activation of macrophages with IFN-γ results in fewer intracellular *B. abortus* organisms; *in vivo* studies have also supported a role for IFN-γ in control of this infection (6). Patients with acute brucellosis display a Th1 type response with cell proliferation and production of IFN-γ, IL-2 and IL-12 (13), crucial lymphokines in the regulation and generation of the immune response (14).

There is a single-nucleotide polymorphism +874 (A/T) located at the 5′-end of a CA repeat at the first intron of human IFN-γ. The +874 T allele is linked to the 12 CA repeats, whereas the A allele is linked to the non-12 CA repeats. The specific sequence of the T allele provides a binding site for the transcription factor NF-kB. Because NF-kB induces IFN-γ expression, this T allele has been shown to correlate with strong IFN-γ expression, whereas the A allele correlates with weak expression (6).

Because it affects the ability of the 3′ UTR to control gene expression for processes such as nuclear export, polyadenylation, translation efficiency and mRNA degradation, the importance of SNPs in this region has been highlighted. It has been suggested that an A to T polymorphism at position 5644 in the 3′ UTR of the γ-IFN gene affects gene expression and production of IFN-γ by governing mRNA stability, localizing mRNA and regulating translation efficiency. Individuals homozygous for polymorphic allele T at site UTR5644 of IFN-γ gene reportedly produce greater amounts of IFN-γ than do those with the TA or AA genotypes (15).

In the present study, we aimed at genotyping the SNPs of the promoter regions of IFN-γ T>A SNP at positions +874 (rs2430561) and 3′ UTR5644 A>T in a group of Iranian patients with brucellosis to assess the role that this cytokine might play in immune responses against *B. abortus* infection.

MATERIALS AND METHODS

Study subjects

In this case-control retrospective study, 153 patients (102 men and 51 women) with active brucellosis (age range 6–76 years; mean ± SD = 31.24 ± 16.6) and 128 healthy individuals as a control group (93 men and 35 women; age range 19–64 years; mean ± SD = 34.04 ± 13.69) were recruited by the Central Clinical Laboratory of Shahid Sadoughi University of Medical Sciences, Yazd, Iran. Blood samples were taken from all participants and stored in EDTA-containing tubes for DNA extraction. All patients were either milk farmers (including some with diagnosed infected animals) or had a history of consuming raw milk and unpasteurized dairy products.

The control group was composed of healthy blood donors with no history of brucellosis or genetic disorders. They were matched for age, sex and geographic area, had the same backgrounds as the cases and were at the same risk of exposure for brucellosis. Almost all patients had overt serious clinical disease and brucellosis was confirmed by clinical serology tests in all of them. Ethical approval for recruitment was obtained from the local Ethics Committee of Shahid Sadoughi University of Medical Sciences and informed consent was obtained from all participants.

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% CO_2 for 48 hr. Typical and well-isolated Brucella-like colonies are small, transparent, raised, convex, and have complete edges and smooth and glistening surfaces along the streak lines on macroscopic examination after Gram staining. In addition, oxidase, catalase, urease, and other biochemical reactions were performed to identify *Brucella* species (16). For patients whose blood cultures were negative, PCR assay was performed to detect the 16s rRNA gene (EMBL accession no. X13695) of the *Brucella* species. The 16S rRNA gene was amplified from reference and clinical isolates of Brucella by using the PCR protocol of Mukherjee *et al*. (17). The PCR employed 0.5 mM each of a forward and a reverse primer (18) as presented in Table 1.

Brucella melitensis was differentiated from *B. abortus* by several techniques including growth on variable levels of CO_2 and PCR of 16s rRNA. *B. abortus* needs CO_2 for growth, whereas *B. melitensis* does not. In addition, growth of *B. abortus* is inhibited by thionine dye, whereas *B. melitensis* is resistant to this dye.

Polymerase chain reaction using primers targeting highly conserved regions such as BCSP31 or 16S-rRNA is an established and powerful technique for identification

Table 1. Primer sequences and fragment size for IFN-γ polymorphisms

Location	Amplicon size (bp)	Primer sequence (5′- to -3′)
IFN-γ, +874		
+874-CP		TCAACAAAGCTGATACTCCA
+874-A	262	TTCTTACAACACAAAATCAAATCA
+874-T	262	TTCTTACAACACAAAATCAAATCT
Internal control-F	426	GCCTTCCCAACCATTCCCTTA
Internal control-R		TCACGGATTTCTGTTGTGTTTC
IFN-gamma, UTR5644		
UTR5644-CP		GTCTACAACAGCACCAGGC
UTR5644-F1	298	CCT TCCTATTTCCTCCTTCG
UTR5644-F2	298	ACCTTCCTATTTCCTCCTTCA
Internal control-F	796	TGCCAAGTGGAGCACCCAA
Internal control-R		GCATCTTGCTCTGTGCAGAT
Brucella 16s rRNA primers		
F4		TCGAGCGCCCGCAAGGG
R2		AACCATAGTGTCTCCACTAA

CP, consensus primer; F, forward primer; F1, sequence specific forward 1 primers; F2, sequence specific forward 2 primers; F4, sequence specific forward 4 primers; R, reverse primer; R2, sequence specific reverse 2 primers as per Romero *et al*. (18).

of genus *Brucella*. In our study, *B. abortus* were distinguished from *B. melitensis* by species-specific PCR in which fragments of BCSP31 and omp2b/2a were amplified by *B. abortus*-specific primers as described by Imaoka *et al*. (19). *B. melitensis* was detected by amplification of fragments of BCSP31, omp2b/2a and omp31 using the pairs of primers B4/B5, JRF/JPR-ab and omp31. A list of primers used for detection of *Brucella* species is shown in Table 2. The cycling conditions used were according to the protocol described by Imaoka *et al*. (19). It was found that 114 *Brucella* spp. were *B. melitensis* and 38 B. *abortus*.

Genotyping of gamma-interferon variants +874 T>A and UTR5644 A>T

Genomic DNA was extracted from whole blood by a "salting-out" method as described previously (20). The quality of the isolated DNA was checked by electrophoresis on 1% agarose gel, quantitated spectrophotometrically and stored at −20°C till further use. IFN-γ +874 and UTR5644 SNPs were genotyped by allele-specific PCR as described previously (21, 22). All analyses were performed blindly with respect to the patients' characteristics, and two separate reactions were carried out on each sample.

All primer sequences and fragment sizes are listed in Table 1. PCR was performed using commercially available PCR premix (AccuPower PCR PreMix; Bioneer, Daejeon, Korea) according to the manufacturer's instructions. In brief, 1 μL template DNA (∼100 ng/mL), 1 μL of each primer (10 pmol/mL) and 16 μL DNase-free water were added to AccuPower PCR PreMix. For the IFN-γ +874 SNP, T- or A-allele-specific forward primers were used in combination with a single reverse primer in separate PCR reactions. Amplification was performed with an initial denaturation step at 95°C for 1 min; 10 cycles of 95°C for 15 s, 60°C for 50 s, and 72°C for 40 s; 95°C for 20 s, 56°C for 50 s, and 72°C for 50 s for 25 cycles, with a final extension of 5 min at 72°C in the last cycle. Each reaction was verified on 2% agarose gel. Products size was 262 bp for either of the T or A alleles. Internal control primers, with which a 426 bp human growth hormone sequence was amplified, were used to check for successful PCR amplification.

Genotyping of IFN-γ UTR5644 SNP was performed using two sequence-specific forward primers and one consensus reverse primer with an expected product size of 298 bp. Control primers were used to amplify a 796 bp fragment of the DRB gene as an internal positive amplification control. Thermocycling was carried out using the following conditions: an initial denaturation at 94°C for 2 min, followed by 10 cycles of amplification at 96°C for 20 s and annealing at 64°C for 50 s, with extension for 40 s at 72°C, followed by 20 cycles of denaturation at 96°C for 20 s, annealing at 61°C for 50 s, with extension for 40 s at 72°C.

Statistical analysis

All statistical analyses were performed using SPSS software for Windows, version 18.0 (SPSS, Chicago, IL, USA). Associations between genotypes and brucellosis were assessed by computing the OR and 95% CI from logistic

Table 2. Primers used for detection of *B. abortus* and *B. melitensis*

Target gene	Primer ame	Sequence (5′- to -3′)	Target length (bp)	GenBank accession no.	Location
BCSP31	B4 (S)	TGG CTC GGT TGC CAA TAT CAA	244	M20404	789–809
	B5 (AS)	CGC GCT TGC CTT TCA GGT CTG		M20404	1012–992
omp2	JPF (S)	GCG CTC AGG CTG CCG ACG CAA	186	U26438	2110–2130
	JPR-ab (AS)	CAT TGC GGT CGG TAC CGG		U26438	2295–2276
	JPR-ca (AS)	CCT TTA CGA TCC GAG CCG GTA	187	U26439	2296–2276
Omp31	1S (S)	GTT CGC TCG ACG TAA CAG CTG	249	AF366073	2296–2276
	1AS (AS)	GAC CGC CGG TAC CAT AAA CCA		AF366073	446–466

regression analyses. P values below 0.05 were considered statistically significant. The Hardy–Weinberg equilibrium was tested with the χ^2 test for any of the SNPs under consideration. LD and frequencies of haplotypes in the controls and patients were estimated using SNPStats software (23).

RESULTS

Study subjects

Table 3 shows relevant patient characteristics and their clinical complications. Brucellosis was diagnosed based on clinical manifestations (including fever, night sweats, weakness, malaise, weight loss, splenomegaly, lymphadenophathy, myalgia and arthralgia) and positive blood cultures. In patients whose blood cultures were negative, PCR assay of Brucella 16s rRNA gene, clinical manifestations, serological tests (Wright titer $\geq$ 1/160 plus mercaptoethanol test $\geq$ 1/80 or Coombs Wright $\geq$ 1/320) were used to confirm brucellosis infection. Of the 153 brucellosis patients, 105 had positive blood cultures and the remaining subjects tested positive for brucellosis by PCR assay. All 48 clinical strains were identified as belonging to the genus Brucella by PCR assays of 16s rRNA gene.

Table 3. Relevant clinical and other characteristics of brucellosis patients

	Number (%) Total = 153
Age	**31.24 ± 16.60 years**
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**	
B. melitensis	**114 (74.17)**
B. 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)**

Frequency of gamma-interferon +874 T>A and UTR5644 A>T genetic polymorphisms

The allele and genotype frequencies of two IFN-γ variants, +874 T>A and UTR5644 A>T, in patients with active brucellosis and controls are listed in Table 4. It has been established that OR < 1 along with $P < 0.05$ is associated with lower risk of disease (protective factor), whereas OR > 1 along with $P < 0.05$ is associated with higher risk of disease (risk factor) (24). A significant difference was found between the two groups regarding allelic and genotyping distribution of the position at +874 T>A. The frequency of AA genotype was significantly greater in patients than in controls (OR = 2.58; 95% CI, 1.31–5.10; $P = 0.006$). Furthermore, the frequency of A allele at +874 T>A position was associated with an increased risk of brucellosis, the frequency being 47.4% and 36.6% in patients and controls, respectively (OR = 1.57; 95% CI, 1.12–2.22; $P = 0.01$). On the other hand, the allelic and genotypic distribution of IFN-γ polymorphism at position UTR5644 A>T did not differ significantly between patients and controls (OR = 0.88; 95% CI, 0.52–1.49; $P = 0.64$ for the TT genotype; OR = 0.95; 95% CI, 0.68–1.34; $P = 0.79$ for the T allele). Additionally, IFN-γ gene polymorphisms were analyzed according to the patients' clinical complications and disease severity: no significant association between these features and IFN-γ genotypes was found (data not shown).

Linkage disequilibrium and haplotype analysis of γ-interferon polymorphisms

None of the SNPs had genotype frequencies that deviated significantly from Hardy–Weinberg equilibrium in the studied control groups ($P = 0.97$ and 0.07 for the variants at positions +874 T>A and UTR5644 A>T, respectively). LD was tested by calculating Lewontin's Delta′ coefficient and the correlation coefficient r^2 (25) and it was found that the IFN-γ +874 T>A was in a low degree of LD ($D' = 0.52$; $r = 0.12$) with UTR5644 A>T variant. Four haplotypes of IFN-γ involved two-alleles of each polymorphism site (Table 5). With an increased frequency in controls than in patients, the T/A haplotype (for the +874/UTR5644 positions, respectively) was found to be protective against brucellosis (OR = 0.71; 95% CI = 1.02–2.87; $P = 0.042$).

Table 4. Comparison of IFN-γ gene polymorphisms in brucellosis patients and controls

IFN-γ polymorphism	Patients $n = 153$ (%)	Controls $n = 128$ (%)	Odds ratio (95% CI)	*P*-value	Odds ratio (95% CI)†	*P*-value†
Position +874 genotypes						
TT	52 (34.0)	52 (40.6)	Ref.	–	Ref.	–
AT	57 (37.3)	59 (46.1)	0.966 (0.569–1.640)	0.898	0.953 (0.560–1.623)	0.860
AA	44 (28.8)	17 (13.3)	2.588 (1.313–5.104)	0.006	2.541 (1.284–5.029)	0.007
Alleles						
T	161 (52.6)	163 (63.7)	Ref.	–		
A	145 (47.4)	93 (36.3)	1.578 (1.124–2.216)	0.010		
Position UTR5644 genotypes						
AA	67 (43.8)	53 (41.4)	Ref.		Ref.	
AT	57 (37.3)	51 (39.8)	0.956 (0.499–1.831)	0.892	0.871 (0.515–1.474)	0.608
TT	29 (19.0)	24 (18.8)	0.884 (0.525–1.490)	0.644	0.912 (0.473–1.758)	0.782
Alleles						
A	191 (62.4)	157 (61.3)	Ref.	–		
T	115 (37.6)	99 (38.7)	0.954 (0.678–1.344)	0.794		

†Adjusted for age and sex.

DISCUSSION

Brucella is a facultative intracellular pathogen that has the capacity to survive and multiply within phagocyte host cells (26). Most cases of brucellosis are caused by two distinct serotypes *B. abortus* and *B. melitensis*. Clinical manifestations and complications of the disease are so diverse that the disease is sometimes called the great imitator, like tuberculosis. As with any other infectious disease, apart from virulence and route of transmission of microorganism, host immunity is an important determinant of disease progression. Eventual elimination of the microorganism depends on activation of macrophages with development of Th1 type cell-mediated immunity (27). The most striking defense against intracellular Brucella is mediated through the cytokine IFN-γ, which is relevant for generating macrophages. Furthermore, cytokines such as IL-2, IL-10 and IL-12 influence acquired cellular resistance and specifically contribute to the control of Brucella multiplication. These cytokines seem to act via an IFN-γ-dependent pathway (6, 15).

Because it induces monocyte/macrophage activation and reduces bacterial multiplication inside infected autologous macrophages, IFN-γ is essential for an efficient response to Brucella infection (6). IFN-γ, a representative Th1-type cytokine, enhances macrophage activation and induces cellular immunity; thus it plays a pivotal role in defense against viruses and intracellular pathogens and in induction of immune-mediated inflammatory responses (28). Cytokines that affect the functionality of IFN-γ render the host susceptible to severe disseminated disease (29). Thus, persons with a genotype associated with a high or intermediate IFN-γ producer status would be expected to show innate resistance to brucellosis. Thus, it is logical that this genotype would be significantly less common in patients than in matched controls (30).

The IFN-γ functional SNP at position +874 (T/A) is located at the 5′-end of a CA repeat at the first intron of human IFN-γ gene. It has been demonstrated that the transcriptional factor NF-kB binds preferentially to DNA containing the γ-IFN +874 T allele and reinforces expression of the IFN-γ gene (29). Thus, +874 (T/A) A/A correlates with low production, T/A with intermediate production and T/T with high production. In the current study, we found that the IFN-γ +874 AA genotype and A allele act as risk factors for developing brucellosis infection in Iranian subjects. We observed the +874 AA genotype more frequently in brucellosis patients than in controls (28.8% vs. 13.3%); this was also true for the IFN-γ +874 A

Table 5. Frequency of haplotypes of IFN-γ gene polymorphisms in patients with brucellosis and control group

Haplotype	Patients	Controls	*P*-value	OR	95% CI
+874/UTR5644					
A/A	0.3815	0.3048	–	1	–
T/T	0.2835	0.3282	0.099	1.40	0.94–2.08
T/A	0.2427	0.3085	0.042	0.71	1.02–2.87
A/T	0.0924	0.0585	0.99	1.00	0.43–2.33

allele (47.4% vs. 36.3%). Although we did not investigate whether patients who are homozygous for the +874A allele are low producers of IFN-γ, lower production of IFN-γ might underlie their susceptibility to brucellosis (30).

Our data regarding IFN-γ polymorphism at the +874 position parallel several studies on brucellosis (30, 31), pulmonary tuberculosis (32–37), HIV-1/AIDS infection (38), retinochoroiditis toxoplasmosis (39), intrauterine HBV infection (40), American tegumentary leishmaniasis (41), chronic hepatitis C (42) and chronic hepatitis B and C virus infection (43). Rasouli and Kiany (31) and Bravo *et al.* (30) showed that the frequency of the +874A allele is higher in brucellosis patients than in controls (34% vs. 19%), suggesting that individuals who are homozygous for this allele may be more susceptible to the infection (30). In opposition to our results and those of several other studies, Karaoglan *et al*. reported that the + 874 TT variant, which is associated with IFN-γ high producer genotype, was significantly more frequent in patients than in controls, whereas the +874 AA, the IFN-γ low producer genotype was more common in controls (44).

With respect to another variant, IFN-γ UTR5644 A>T, our study failed to show any significant differences in allelic and genotypic distribution of this polymorphism across the groups. In accordance with our results, others have reported no association between the IFN-γ UTR5644 A>T variant and brucellosis infection (15, 45). Davoudi *et al*. found no significant correlation between brucellosis and IFN-γ UTR5644 A>T genotypes in a relatively small groups of Iranian subjects (42 brucellosis cases and 162 controls); however, they observed that the AT genotype is slightly more common in patients (15). Similarly, Hedayatizadeh-Omran *et al*. showed that allelic and genotype frequencies of G5644A polymorphism of IFN-gamma gene did not differ significantly between brucellosis patients and controls; however, they reported higher prevalence of the 5644A allele in patients with focal brucellosis (45).

In our study, the distribution of haplotypes suggests that the T/A haplotype (for the +874/UTR5644 positions, respectively), which occurs more frequently in controls than in patients, may protect healthy subjects from Brucella infection, possibly through contributing to a functional downregulation of serum IFN-gamma production *in vivo*, as reported previously (15, 44).

In summary, our findings suggest that there is an association between the IFN-γ +874 A/A genotype and A allele and brucellosis in the studied population. Because attaining a better understanding of brucellosis immunology is a priority for the development of new therapeutic and vaccination strategies, these results need to be confirmed in larger patient cohorts and subjects from different ethnic groups need to be studied.

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DISCLOSURE

The authors declare that they have no conflicts of interest to disclose.

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