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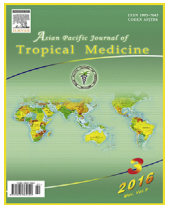

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Relationship between *CD14*-159C/T gene polymorphism and acute brucellosis risk

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ABSTRACT

Objective: To investigate the association between the cluster of differentiation 14 (*CD14*)-159C/T (rs2569190) gene polymorphism and susceptibility to acute brucellosis in an Iranian population.

Methods: The study included 153 Iranian patients with active brucellosis and 128 healthy individuals as the control group. Genotyping of the *CD14* variant was performed using an amplification refractory mutation system-polymerase chain reaction method.

Results: The prevalence of *CD14*-159 TT and CT genotypes were associated with increased risk of brucellosis [odds ratio (*OR*) = 1.993, 95% confidence interval (95% *CI*) = 1.07–3.71, *P* = 0.03 for CT; *OR* = 3.869, 95% *CI* = 1.91–7.84, *P* = 0.01 for TT genotype. Additionally, the minor allele (T) was significantly more frequently present in brucellosis patients than in controls (61% *vs.* 45%, respectively), and was a risk factor for brucellosis (*OR* = 3.058, 95% *CI* = 1.507–6.315, *P* = 0.01).

Conclusions: The findings provided suggestive evidence of association of the *CD14*-159C/T gene polymorphism with susceptibility to acute brucellosis in the Iranian population.

1. Introduction

Brucellosis (or Malta fever, Gibraltar fever, Cyprus fever, Danube fever, undulant fever, Mediterranean fever and Bang's disease) is one of the most frequent zoonotic infections worldwide, transmitted to humans by eating contaminated, unpasteurized dairy products, direct contact with infected animal, and through the inhalation of infected aerosolized particles [1–4]. The majority of cases arise in the Mediterranean countries of Europe and Africa, Mexico, and Central and South America, India, Central Asia and Middle East including Iran [4].

Brucella spp. (pleomorphic, gram-negative, non-spore-forming coccobacillus bacteria) causes severe debilitating and disabling disease that is accompanied with manifestations such as fever, weakness, sweating, weight loss, headache, persisted joint pain, neurological complications, endocarditis and testicular or bone abscess formation [1,2,4,5]. *Brucella* spp. are facultative intracellular bacteria that have the ability to evade from killing mechanism of immune system and multiply within in organs with frequent macrophages such as liver, spleen, lung, bone marrow and synovium (reticuloendothelial system) [4,6]. Among all species of *Brucella* genus, three species including *Brucella melitensis*, *Brucella abortus*, and *Brucella suis* are the most commonly associated with human disease [7].

Cluster of differentiation 14 (CD14) is a glycosyl-phosphatidylinositol-anchored molecule that is mainly expressed on cell membrane surfaces of monocyte/macrophage lineage [8,9], and acts as a pattern recognition receptor in innate immunity for detection of variety of ligands such as bacterial products, microbial cell wall components and even whole bacteria. Particularly, CD14 is the specific receptor for the lipopolysaccharide (LPS) of gram-negative bacteria [10,11].

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LPS is the major component of the outer membrane of gram-negative bacteria and is called 'bacterial endotoxin' [12]. LPS is the main virulence factor of *Brucella* and has a role in cell entry and immune evasion of the infected cell [6]. LPS also has an ability to induce the production of interleukin (IL)-12 resulting in promotion of T helper (Th) 1 responses and inhibition of Th-2 responses in the host [13]. CD14 as a co-receptor for the toll-like receptor (TLR) and together with TLR4 forms a complex that activates the innate immune system and starts a variety of effector functions including secretion of pro-inflammatory cytokines such as IL-1, IL-6, tumor necrosis factor-α, nitric oxide, oxygen radicals and complement components as well as cell proliferation, subsequent binding to LPS [8,13–15]. Nevertheless, high production of these molecules may cause intense harmful consequences including severe inflammatory responses, septic shock and death [15]. Moreover, a soluble type of CD14 (sCD14) is produced by monocytes, hepatocytes and endothelial cells and is present in the circulation and other body fluids. sCD14 is derived both from secretion of CD14 and from enzymatically cleaved glycosyl-phosphatidylinositol-anchored membrane type of CD14 [10,16]. Since sCD14 plasma levels increases during inflammation and infection, it is believed that sCD14 is an acute-phase protein, which is increased in several inflammatory processes [8,15,17].

The human *CD14* gene is located on chromosome 5q31.1 [14]. Several single nucleotide polymorphisms (SNPs), including -2451G/C (rs2569192), -1855A/G (rs3138076), -1619A/G (rs2915863) and -159C/T (rs2569190), have been identified in promoter region of the *CD14* gene [18,19]. One of the most extensively studied SNPs, the -159C/T (rs2569190), includes a substitution of C → T resulting in elevated transcriptional activity and expression levels of *CD14* [14,20].

Thus far, the *CD14*-159C/T gene polymorphism has been examined in several inflammatory and infectious diseases including tuberculosis [8,9,21], brucellosis [22], *Chlamydia pneumoniae* infection [23,24], *Helicobacter pylori* infection [25], hepatitis B and C [26,27] and severe acute respiratory syndrome [28], but the results have been inconsistent and conflicting. Therefore, the current study aimed to evaluate the possible association between the *CD14*-159C/T (rs2569190) gene polymorphism and susceptibility to acute brucellosis in an Iranian population.

Table 1
Clinical characteristics of brucellosis patients.

	Number	Percent (%)
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 spp.:		
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

2. Materials and methods

2.1. Study population

The current case-control retrospective study included 153 patients (102 men and 51 women) suffering from active brucellosis, age range 6–76 years old 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 sample from each participant was collected in EDTA-containing tubes for DNA isolation after taking an informed consent from all patients and healthy individuals. All patients were either milk farmers (including diagnosed infected animals) or had a history of consuming raw milk and unpasteurized dairy products. Demographic characteristics of patients and their clinical complications are shown in 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 [29–32]. The control group included healthy blood donors with no evidence 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 spp. were cultivated on 5% sheep blood-agar plates and incubated at 37 °C in the contact to 5%–10% of CO_2 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 [33]. Serological tests, defined as wright titre ≥1/160 plus mercaptoethanol test ≥1/80 or coomb's wright ≥1/320 certified the brucellosis infection. The complete details of the organism identification methods have been described previously in papers by this research team [29–32].

2.3. Genotyping of CD14-159C/T (rs2569190) SNP

Genomic DNA was extracted from the peripheral blood leukocytes by the 'salting-out' method as explained previously [34]. The quality of the isolated DNA was checked by electrophoresis on 1% agarose gel, quantitated spectrophotometrically and stored at −20 °C still further use.

The *CD14*-159C/T SNP, rs2569190, was genotyped using an amplification refractory mutation system-polymerase chain reaction method as described previously by Karhukorpi *et al* [25,35]. This allele-specific, single-tube polymerase chain reaction

method uses four primers including two allele specific primers (cfors for the C allele and trevs for the T allele) and two outer primers (cdp1 and cdp2) for the control band. An additional mismatch was inserted at the penultimate 3′ nucleotide (underlined) of the allele-specific primers to increase the specificity of the PCR reaction. The primer sequences were as follows: cfors: 5′-CTC CAG AAT CCT TCC TGT TAC GAC-3′, trevs: 5′-TGT AGG ATG TTT CAG GGA GGG GTA-3′, cdp1: 5′-TTG GTG CCA ACA GAT GAG GTT CAC-3′, cdp2: 5′-TTC TTT CCT ACA CAG CGG CAC CC-3′. The PCR conditions for the *CD14*-159C/T (rs2569190) polymorphism were as followed: an initial denaturation at 95 °C for 5 min; 30 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min; the final extension step proceeded at 72 °C for 5 min [25,35]. The PCR products were separated by standard electrophoresis on 2% agarose gel containing ethidium bromide. The PCR products included a 381 bp band for the T allele, a 227 bp band for the C allele and a 561 bp band for the control.

2.4. Statistical analysis

All statistical analyses were performed applying the 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 interval (95% *CI*) from logistic regression analyses. *P* values below 0.05 were considered statistically significant. The Hardy–Weinberg equilibrium was tested with the χ^2 test for the SNP under consideration.

3. Results

The *CD14* polymorphism, -159C/T, was successfully genotyped in 153 brucellosis patients and 128 control subjects. No deviation from Hardy–Weinberg equilibrium was observed in the studied case and control groups ($P > 0.05$). The genotype and allele frequency of the SNP in the studied groups were shown in Table 2.

The results showed that the *CD14* polymorphism, -159C/T, was associated with the risk of brucellosis in our population. At genotypic level, the *CD14* CT, and TT genotypes were more frequent in the patients group compared to cases, and were associated with increased risk of brucellosis in the codominant model (*OR* = 1.993, 95% *CI* = 1.07–3.71, *P* = 0.03 for CT; *OR* = 3.869, 95% *CI* = 1.91–7.84, *P* = 0.01 for TT genotype). Additionally, the CT + TT *vs.* CC genotype in the dominant model was a risk factor for brucellosis (*OR* = 2.514, 95% *CI* = 1.34–4.73, *P* = 0.02). Likewise, in the recessive model, the TT genotype *vs.* CC + CT was associated with the risk of brucellosis (*OR* = 2.379, 95% *CI* = 1.33–4.27, *P* = 0.02). At allelic level, the minor allele (T) was significantly more frequently present in brucellosis patients than in controls (61% *vs.* 45%, respectively), and was a risk factor for brucellosis (*OR* = 3.058, 95% *CI* = 1.507–6.315, *P* = 0.01).

4. Discussion

Our study provided data on the association between one important SNP in the 5′ flanking region of the *CD14* gene and acute brucellosis using genotype analysis. The results showed that the prevalence of −159 homozygote TT genotype as well as T allele were much higher in brucellosis patients than in controls (36.6% *vs.* 19.5% and 61% *vs.* 44%, respectively). The carriers of the TT genotype and T allele were at 3.8-fold or 3-fold higher risk of brucellosis, respectively, than those with the CC genotype or C allele.

Several studies have been conducted to show the impact of the *CD14*-159C/T SNP on the risk of infectious diseases [9,18,21–23]. To the best of our knowledge, there is only one study assessing the association of *CD14*, -159C/T polymorphism and brucellosis [22]. Haidari *et al* [22], in their study on brucellosis in a Western Iranian population have shown that the frequency of the TT genotype was higher in the patients than in controls (34.5% *vs.* 15.5%). They suggested that the homozygousity for T allele of promoter of *CD14* gene may confer an increased risk of brucellosis, which supports our findings. Moreover, our results regarding *CD14*-159C/T polymorphism are in agreement with several infectious studies on pulmonary tuberculosis [9,18,21] and *C. pneumoniae* infection [23]. In contrast, other studies reported no association between *CD14*-159C/T SNP and *Chlamydia trachomatis* infection [36], *Campylobacter jejuni* infection [37] and severe acute respiratory syndrome [28].

Most *Brucella* species are highly virulent causing an acute infection in their hosts. The LPS of this bacterium is one of the essential components for the first steps in the establishment of an intracellular replication niche, in which *Brucella* survives and multiplies [7]. LPS is a potent stimulator of monocytes and macrophages [25]. Activation of macrophages is one of the early events in the innate immunity to intracellular pathogens. CD14 is a high-affinity receptor for bacterial LPS and is expressed mainly by cells of monocyte/macrophage lineage. The binding of LPS to CD14 results in cellular activation and initiates a range of effector functions including

Table 2
The genotype and allele frequencies of *CD14*-159C/T SNP between brucellosis patients and controls.

SNPs	Allele/genotype	Brucellosis patients		Controls		*OR* (95% *CI*)	*P* value
		Number	Percent (%)	Number	Percent (%)		
CD14-159C/T Models							
Codominant	CC	22	14.4	38	29.7	Ref.	–
	CT	75	49.0	65	50.8	1.993 (1.07–3.71)	0.03
	TT	56	36.6	25	19.5	3.869 (1.91–7.84)	0.01
Dominant	CC	22	14.4	38	29.7	Ref.	–
	CT + TT	131	85.6	90	70.3	2.514 (1.34–4.73)	0.02
Recessive	CC + CT	97	63.4	103	29.7	Ref.	–
	TT	56	36.6	25	19.5	2.379 (1.33–4.27)	0.02
Alleles	C	119	38.8	143	55.4	Ref.	–
	T	187	61.2	115	44.6	3.058 (1.507–6.315)	0.01

cytokine secretion and cell proliferation [8,19]. CD14 as a co-receptor for the TLR is transferring LPS to the TLR4/MD-2 signaling complex. Engagement of this complex results in the activation of innate immunity mechanisms such as the release of proinflammatory cytokines and subsequently severe inflammatory responses as well as in worse circumstances of septic shock and death [15,19]. Some studies have been performed to explain the impact of the *CD14*-159C/T SNP on CD14 protein expression [8,38]. The *CD14*-159C/T polymorphism is located at a GC box close to the transcription start site, which serves as a binding site for Sp1-Sp2/Sp3 transcription factors that are involved in the regulation of *CD14* gene transcription [15]. The T variant of this SNP is associated with high expression of *CD14* on the surface of monocytes and macrophages, which further increases the inflammatory response [39,40]. Thus, this SNP could be a genetic factor for inter-individual differences in susceptibility to infectious diseases like brucellosis. Our results highlight the role of CD14 in the inflammatory response and the effect of its promoter polymorphism on the host immune defense.

In conclusion, our study provided evidence that the *CD14*-159, CT and TT genotypes may serve as risk factors for brucellosis infection in our population. Further studies on larger populations with different ethnicities are required to validate our findings. More inclusive understanding of the basic mechanisms underlying infections pathogenesis may lead to the development and application of efficient products for the treatment of brucellosis.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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