Polymorphisms in vitamin D receptor genes and its relation with susceptibility to brucellosis: A case-control study

Hassan Mahmoudi (Hassanmahmoudi24@gmail.com)
Hamadan University of Medical Sciences Medical School
Massoud Saidijam
Hamadan University of Medical Sciences Medical School
Younes Mohammadi
Hamadan University of Medical Sciences School of Public Health
Mohammad Yousef Alikhani
Hamadan University of Medical Sciences Medical School

Research Article

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Abstract

Objective: One of the systemic infections is Brucellosis which is caused by facultative intracellular bacteria of the genus *Brucell*a. Vitamin D is a fat-soluble prohormone, that metabolizes enzymes and its intracellular receptor creates the active hormone and also mediate in responses of immune system.

Methods: Current research consists of 102 patients with brucellosis who were selected based on culture, PCR results serology, and clinical symptoms. The control group composed of 102 healthy people. The polymorphism of genes (*Bsm I, Fok I, Taq I, Apa I*) encoding VDR were assessed by the PCR-RELP method.

Results: The results showed that ff, tt, aa, and bb genotypes in *Fok I, ApaI, TaqI, and BsmI* were significant in case/control groups (*P-value* ≤ 0.0001). The genotype frequency AA in the control group is higher than that of the study group, while genotype frequency aa in the study group is more than the control. The odds ratio for brucellosis in individuals with ff genotype is 37 times higher than that of Ff genotype. Also, the odds ratio of brucellosis in individuals with genotype tt, aa, and bb was 12, 53, and 6 times higher than those of the Aa, Bb, and Tt genotypes.

Conclusion: The genotypes aa and ff in the positions of the *ApaI* and *FokI* are of higher importance. The brucellosis risk in individuals accompanied aa genotype at *Apa I* is 53 times higher than that of the genotype AA, in other words, AA and BB, TT and FF genotypes are protective against the disease.

Introduction

One of the most common zoonotic diseases with bacterial globally is brucellosis that annually infect near 500,000 people around the globe. In spite of all efforts made to manage the spread, Brucellosis is till date is a major health issue in different regions worldwide western Asia, America & Mediterranean regions. In many parts of the Middle East including Iran, the Saudi Arabia and turkey it remains an endemic situation [1]. Brucella genus shall result in development of brucellosis, and it shall be transmitted by the taking the contaminated daity products such as contaminated milk, respiratory tract, animal's infected meat and vaginal secretions [2]. It has been demonstrated that effectual immune response against Brucella or any other intracellular pathogens that needs stimulation of cellular immunity [3].

Brucella spp. is a microorganism with intracellular characteristic, so destructive role of brucellosis is due to cellular immunity [4]. Approximately more than 1 billion individual globally have severe to moderate vitamin D deficiency [4]. Vitamin D is a vigorous immunonutrient which via calcitriol-its core metabolite-regulate the macrophages immunomodulations, B and T lymphocytes and dendritic cells, which show the VDR (vitamin D receptor), their respond to calcitriol and production. 25-OH- VitD3 (Twenty-five-hydroxyvitamin D3) stimulates osteogenic activity and regulates calcium metabolism, and also involves in host defense against microbial or inflammatory invasions [4]. The vitamin D and VDR importance on the immune system regulation was supported in few researches. The 25-OH-VitD3 hormonal forms equalized anti-microbial peptides and elevate bacterial clearance in cells of immune system [5–7].

Immune cells have 25-OH-VitD3 (25-hydroxyvitamin D3) receptors, and patients with deficiency of vitamin D, have disruptions in specifically in macrophages and also immune system, [8–10].

Studies on genetic association demonstrated that near 65 percent of serum vitamin D shall be described due to genetic background. The 90 percent of genetic variability formed in the sense of single nucleotide polymorphisms (SNPs), SNPs in genes associated to vitamin D metabolism have been connected to influence the calcitriol serum levels, including 25-hydroxylase, vitamin D receptors (VDRs) and vitamin D binding protein [11]. Common VDR's SNPs are Taq I (rs731236), FokI (rs2228570), Apa I (rs7975232) and Bsm I (rs1544410). Amongst them, Taq I, Bsm I and Apa I polymorphisms are located in VDR gene's 3'end that leads to silent mutation linked with elevated VDR mRNA stability. In opposite, location of Fok I SNP is at the start codon that makes a shorter in size that will be significantly for long term activity [12]. Therefore, the goal of current research is to study the association between vitamin D receptor (VDR) genes polymorphism in brucellosis patients referred to Sina Hospital, Hamadan, Iran compared to the normal controls.

Material & Methods

Study Subjects

In this case control project, 102 individuals with brucellosis, and 102 healthy persons who referred to Sina hospital or Imam Khomeini clinic in Hamadan University of Medical Sciences are registered, and all groups were sorted based on habitat, age and gender. All participants signed an informed consent before entering into the research. The protocol of current study was compatible with the Declaration of Helsinki and approved by Research Ethics Committee of Hamadan University of Medical Sciences (Ethical committee ID: IR.UMSHA.REC.1396.266).

The study was done from April 2020 to May 2021. brucellosis Patients by an infectious disease physician based on clinical symptoms (arthralgia, splenomegaly, fever sweating, hepatomegaly, malaise, focal complication), were included in the study, which was confirmed by positive serology tests (Wright ≥ 1/80 and 2ME ≥ 1/40) and positive blood by culture or PCR methods for Brucella spp [4, 10].

The control group, was randomly selected, did not show any clinical symptoms and confirmed by negative serological test for Brucella. The control and patient groups were from the same geographical areas.

PCR-RFLP

PCR was performed at a final volume as much as a mixture of 25 µl consisting 12.5 µl of mastermix (Fermentas Co), 2 µl total DNA extracted, 1 µl of each specific primers and distilled water; and the mixture was processed in a thermocycler (Eppendorf Co) [13]. PCR products were digested by Apa I, Fok I, Taq I, and Bsm I, and restriction enzymes (Nedy Fan Co, Iran). For PCR-RFLP, 15 µl reactions were prepared. Restriction enzymes were used according to manufacturer's instruction. Briefly, 5 µl of PCR product, 1 µl

of enzyme buffer, 0.5 µl of restriction enzyme, and sterile distilled water up to 15 µl were used. The micro-tubes were incubated for four hours at 37°C. The digested products were isolated on 1.5% agarose and visualized under UV light. The primer sequences used are listed in Table 1.

Table 1
The PCR primers for amplify the vitamin D receptor single nucleotide polymorphisms

Vitamin D receptor SNPs	Primer Sequences (5'- 3')	Size (bp)	Reference
Apa I (rs7975232)	F- CTGCCGTTGAGTGTCTGTGT	242	(15)
	R- TCGGCTAGCTTCTGGATCAT		
Bsm I (rs1544410)	F- CCTCACTGCCCTTAGCTCTG	209	(15)
	R- CCATCTCTCAGGCTCCAAAG		
Taq I(rs731236)	F- CTGCCGTTGAGTGTCTGTGT	242	(15)
	R- TCGGCTAGCTTCTGGATCAT		
Fok I (rs10735810)	F- GGCCTGCTTGCTGTTCTTAC	174	(15)
	R- TCACCTGAAGAAGCCTTTGC		
SNPs: Single nucleotide polymorphisms;			

Dna Extraction

Blood samples genomic DNA extracted by the salting out method [14]. In short, 0.5 ml of blood samples with 1% Triton X-100, 1 ml of erythrocyte lyses solution (320 mM saccharose, 5 mM MgCl2, 10 mM Tris HCl [pH = 7.5]) were mixed and centrifuged at 15000×g for 2 min. This step was repeated 3x. 400 microliters of lyses buffer (10 mM Sodium Acetate [pH = 8], 10 mM Tris-Hcl, 10mM EDTA, 1% SDS) and proteinase K (10 mg/ml) were added to the pellet and mixed. After incubation at 55°C for 20 min, 100 µL of ammonium acetate (7.5 M) was added and centrifuged for 10 min. Finally, 200 µL of absolute ethanol was added to the supernatant and after centrifuging, the pellets were kept in 50 µL of TE buffer and stored at -20°C for PCR [15].

Statistical Analysis

The analysis of the data performing by SPSS version 20 and statistical tests, such as adjusted logistic regression models at 95% confidence interval (CI) and odds ratio and independent Pearson's chi-square (P – value < 0.05).

Results

Aim of current project was to study the genes polymorphism encoding VDR in patient's diagnosed brucellosis & normal individuals over a period of 2019–2020. Total number of studied persons was 102 brucellosis patients, and 102 of control group. The mean of age in brucellosis group was 43.91 ± 16.46 years and in control group, the mean of age was 36.12 ± 8.72 years. All groups were matched on the basis of age and there was significant statistical difference among the groups ($P < 0.001$).

Patients with brucellosis, and control group were matched on the basis of gender distribution ($P < 0.001$), and habitat too ($P < 0.001$). In brucellosis group, 77 (75.49%) male and 25 (24.50 0%) female and in the control group 79 (77.45%) male and 23 (22.54%) female was studied. The Demographic participant's characteristics are showed in Table 2.

Table 2
Demographic characteristics of brucellosis patients and control group

Variables	Cases	Controls	p-Value
Age mean ± SD (years)	16.46 ± 43.91	8.72 ± 36.12	< 0.001
Gender No (%)	77 (75.49)	79 (77.45)	< 0.001
Male	25 (24.50 0)	23 (22.54)	
Female			
Occupation No (%)	60 (58.82)	6(5.88)	< 0.001
Rancher and farmer	4 (3.92)	0(0.0)	
Butcher	26 (25.49)	19 (18.62)	
Housewife	12 (11.76)	77 (75.49)	
Other			
Place of residence No (%)	4(3.7)	72(70.58)	< 0.001
Urban	103 (96.3)	30(29.42)	
rural			
Level of Education No (%)	95(93.13)	8(7.84)	< 0.001
High school	6(5.88)	80(8.43)	
Diploma	1(0.98)	14 (13.72)	
Bachelor			
History of brucellosis No (%)	60 (58.82)	0(0.0)	< 0.001
Treatment history No (%)	60 (58.82)	0(0.0)	< 0.001
Consumption history of unpasteurized dairy products No (%)	99 (97.05)	0(0.0)	< 0.001
Livestock contact history No (%)	102 (100)	0(0.0)	< 0.001

The frequencies of vitamin D receptor gene polymorphisms compared between healthy controls and patients with brucellosis (Table 3).

Table 3
Comparison of frequencies of vitamin D receptor gene polymorphisms between patients with brucellosis and healthy controls

Polymorphism	Genotypes	brucellosis patients no.	Controls	P-value	Odds ratio (95% CI)
Apa I	aa (%)	49(48.0)	2(2)	P = 0.000	OR = 53.90 95% CI: 12.40-234.254
	Aa (%)	18(17.6)	23(22.5)	P = 0.147	OR = 6.630 95% CI: 0.826–3.59
	AA (%)	35(34.3)	77(75.5)		
Taq I	tt (%)	28(27.5)	3(2.9)	P = 0.000	OR = 12.20 95% CI: 3.517–42.35
	Tt (%)	22(21.6)	31(30.4)	P = 0.823	OR = 0.928 95% CI: 0.448–1.78
	TT (%)	52(51.0)	68(66.7)		
Bsm I	bb (%)	25(24.5)	5(4.9)	P = 0.000	OR = 6.630 95% CI: 2.358–18.64
	Bb (%)	31(30.4)	36(35.3)	P = 0.672	OR = 1.142 95% CI: 0.618–2.11
	BB (%)	46(45.1)	61(59.8)		
Fok I	ff (%)	47(46.1)	2(1)	P = 0.000	OR = 37.386 95% CI: 8.64–161.7
	Ff (%)	10(10.8)	30(29.4)	P = 0.18	OR = 0.583 95% CI:0.266–1.282
	FF(%)	44(43.1)	70 (34.3)		

Discussion

The goal of the current project was to look for links between polymorphisms in brucellosis and polymorphism vitamin D variants *ApaI, BsmI, TaqI and Fok I* located VDR genes in brucellosis patients referred to Sina Hospital, Hamadan, Iran. Vit D is an immune-modulator molecule that, by corresponding VDR, shall regulate responses of T cells cytokine [16].

In vitro studies performed to display that vit D suppress proliferation of T cells and leading to inhibition in production of IFN-c (interferon gamma, IL-17 and interleukin-2 (IL-2), IL-23, IL-12 and IL-6 [17–19]. Additionally, the 25(OH) 2D3 immunosuppressive activity is showed by a suppression in the differentiation, maturation, survival and activation of dendritic cells, leading to T-cells hypo-responsiveness [20]. Vitamin D receptor has found in microphages, monocytes, effector/memory T-cells,

dendritic [21]. This recommends that functional polymorphisms in the VDR gene shall regulate the immune system and susceptibility to brucellosis in each individual.

Results from this case-control study of patients with brucellosis suggest that polymorphism of receptor of vitamin D may be related to an elevated chance of brucellosis, particularly for ff genotype. Using a case–control research, a prominent relation between VDR gene & brucellosis was found, which to our knowledge has not been reported previously.

After adjustment for confounding factors such as age, sex individuals carrying the “ff” genotype had a 37-fold increased risk compared with those with “Ff” genotype for brucellosis. Also, the odds ratio of brucellosis in individuals with genotype tt, aa, and bb was 12, 53, and 6 times higher than those of the, Aa, Bb, and Tt genotypes. The results of this preliminary study show that frequency of VDR Fok1 polymorphism, genotype ff is %46.1 in brucellosis patients in our population. Many such studies and reviews have been previously published on related between polymorphism vitamin D receptor in metabolic disease and neck and oral and breast cancer but very few of them have focused on the effects of vitamin D polymorphism in causing infection disease in individuals. Other meta-analysis of study demonstrated that the polymorphism of Fok1 of the VDR gene is related to an elevated breast cancer risk (ff vs. Ff + FF, OR: 1.09, 95%CI: 1.02 to 1.16, p = 0.007) [22].

The study results done produced Zeljic et al., showed that genetic heterozygous variant in the CYP24A1 gene (rs2296241) is linked with diminished risk of oral cancer. After adjustment by the potential confounders, their results demonstrated a 0.281-fold decline for the risk oral cancer amongst CYP24A1 heterozygote genotype compare to wild type [23].

Other projects have recommended that the potential character of vitamin D in the rheumatoid arthritis development, systemic lupus erythematosus, type 1 diabetes, Crohn’s disease, and multiple sclerosis [24–26]. Mostowska et al., showed that the VDR F/f and F/F genotypes frequency of Fok I was statistically different between patients with renal disease and patients without this symptom OR = 3.228 (1.534–6.792, p = 0.0014 and association of the VDR TaqI (rs731236), Apa I (rs7975232), Fok I (rs2228570) and Bsm I (rs1544410) polymorphisms with clinical symptoms of SLE [27].

Our study did demonstrate that the SNPs such as *TaqI, FokI, ApaI and BsmI* VDR are risk factors of brucellosis patients in the Hamadan population, but we discovered an involvement of the polymorphism of FokI in brucellosis patients. Additionally, to analyze the detailed role of these SNPs in brucellosis, this study must be replicated in other independent cohorts.

The Fok I polymorphisms of VDR have also been recognized as risk factors of some autoimmune diseases, including Behcet’s, RA, Addison’s diseases psoriasis and Graves [24].

Results study Alicia K. Wilbur et al., indicate that, the Fok-I F allele shields patients with Tuberculosis infection, while the Taq I t allele saves in case of active disease but not infection. To be exact, FF homozygotes are 17 times more likely to test positive for exposure to Tuberculosis, but no more likely to

have ever been diagnosed with active Tuberculosis. TT individuals are 42 times less likely to mount a delayed-type hypersensitivity response, and the T allele was significantly more likely to have been transmitted to offspring who have been diagnosed with active Tuberculosis [28].

Polyxeni Nicolaidou et al., show that the allelic frequencies and genotypic distribution differed between Hypocalcemic vitamin D-resistant rickets carriers and their respective controls regarding *Bsm* I and *Taq* I polymorphisms. The bb genotype and the T allele (presence of *Bsm* I and absence of *Taq* I polymorphisms) were less frequent in the Hypocalcemic vitamin D-resistant rickets carrier group than in the control group in a statistically significant manner ($p = 0.029$ and $p = 0.025$, respectively) [29].

Morrison et al. suggested that *Bsm* I and *Taq* I VDR polymorphisms define differential transcriptional VDR activity or mRNA stability in vitro. Since then, attempts have been made to associate these VDR polymorphisms with bone-related diseases and decreased calcium absorption. Particularly, postmenopausal women with the BB and tt genotypes have a significantly greater bone loss at the lumbar spine, decreased function of VDR and elevated 1, 25 (OH) 2D3 levels [30].

Tajik et al., demonstrated that comparison of genotype and allele frequencies for the above VDR gene polymorphic sites revealed no significant difference between patient and control groups.

The current research has focused only on four genes related vitamin D in Iranian population. The relation between the polymorphisms vitamin D pathway genes and brucellosis must be deeply studied; this project is the first study to analysis the relation between brucellosis susceptibility in the Iranian population and four single nucleotide polymorphism of metabolic pathway genes of vitamin D (*TaqI, ApaI, BsmI, FokI*) [31].

Current research had common limitations. First of all, the study did not exclude the potential influence of some confounding factors such as, the sample size might not be sufficient. So, repeated research with larger sample size in different ethnic groups should be considered to further explore and verify the role of metabolic pathways of vit D in brucellosis development.

Conclusions

This project provided the proof that the genotypes aa and ff in the positions of the *Fok I* and *Apa I* are of higher importance. The brucellosis risk in individuals posing aa genotype at *Apa I* is 53 times higher than that of the genotype AA, in other words, AA and BB, TT and FF genotypes are protective against the disease.

Declarations

Author Contributions: MYA and FK cooperated in the design of the study and selecting the patients. HM and YM contributed to sampling and data analysis. MYA and AK prepared the first draft. MYA and MS

contributed to preparing and revising the manuscript. MYA and HM contributed to revising the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Hamadan University of Medical Sciences, Hamadan, Iran (IR.UMSHA.REC.1396.266).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declared no conflict of interest.

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