Original Research Paper

Study the Impact of T-helper 1 Cytokine (TNF-α) Polymorphisms on Susceptibility/Resistance to Brucellosis in Makkah Region

[1,2]Alaa B. Ismael, [1,3]Adil Mergani, [4,5]Salama A-H Mostafa and [4]Abdelmoneim M. Salim

[1]Department of Medical Biotechnology, Faculty of Applied Medical Sciences, Taif University, Turrabah, 21995, KSA
[2]Department of Animal Medicine, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44519, Egypt
[3] Department of Human Molecular Genetics, National Cancer Institute (NCI), Gezira University, Sudan
[4]Department of Medical Microbiology, Faculty of Applied Medical Sciences, Taif University, Turabah, 21995, KSA
[5]Immunobiology and Immunopharmacology Unit, Animal Reproduction Research Institute (ARRI), Giza, Egypt

Abstract: Polymorphisms in the regulatory regions of cytokine genes may not only increase susceptibility to some infectious diseases but also affect the course and prognosis of the disease. TNF-α is considered an important Th1 cytokines that plays critical roles in control of Brucella infection and in macrophage activation. In this study, we are going to analyze the relationship of two polymorphisms in TNF-α and the inherited susceptibility/resistance to brucellosis in population of Makkah region. A cases-control association study was conducted in 69 individuals with human Brucellosis and 112 healthy individuals. Genotyping of TNF- 308G>A and -857C>T polymorphism in both patients and healthy controls was done by PCR-RFLP method and were assessed for potential associations with susceptibility for human brucellosis and their mode of penetrance. The findings indicate an increased risk of TNF-α-308 A allele for human brucellosis reliable with the recessive genetic model of penetrance (Odd Ratio: 3.222, 95% CI: 1.008-5.702, P = 0.018). There is no association between susceptibility of human brucellosis and TNF-α-857 C/T polymorphism was observed. The protective role of TNF-α-857 C/T polymorphism against human brucellosis in this study population could not be excluded.

Keywords: Brucellosis, Tumor Necrosis Factor-Alpha, Polymorphism

Introduction

Brucella species are Gram negative, facultative intracellular bacteria that cause abortion and temporary infertility in domestic animals (cattle, goats, sheep and camel) and undulant fever, arthritis, osteomyelitis and endocarditis in humans (Benjamin and Annobil, 1992; Corbel, 1997). Brucellosis is an endemic disease, mainly in Middle East, Africa, Asia and Central and South America and continues to execute significant health problems and important economic losses (Pappas, 2007). In Saudi Arabia, although human cases of brucellosis were reported sporadically between 1956 and 1982, brucellosis has emerged as a major public health problem in early 1980s with increasing rates of annual admissions for brucellosis (Kambal *et al*., 1983; Madkour *et al*., 1985; Arrighi, 1986). This increasing in incidence rates of brucellosis has been strongly linked to the uncontrolled importation of potentially infected animals with brucellosis, the prevailing habit of ingesting raw milk or its products among the population with a nomadic background and widespread cattle breeding (Hafez, 1986).

There are four Brucella species that cause disease in humans; *B. melitensis*, *B. suis*, *B. abortus* and *B. canis*, in descending order of pathogenicity. Transmission of the disease to humans occurs through direct contact with infected animal parts, the consumption of infected, unpasteurized milk products and inhalation of infected aerosolized particles. Brucella invade the reticuloendothelial cells and can be sequestered in macrophages at specific locations within the body, such as spleen, brain, joints, heart, liver and bone marrow (Ficht, 2003). Innate immunity recognition receptors can detect components so-called Pathogen-Associated Molecular Patterns (PAMPs) to provoke a host

protective response (Janeway Jr and Medzhitov, 2002). Human protective immune response against Brucella infection depends on cell-mediated immunity. This involves mainly activated antigen-presenting cells (dendritic cells, macrophages) and CD4+, CD8+ T lymphocytes. This is mediated by a Th1 immune response and is considered to be critical for the efficiency of the protective anti-Brucella immune response (Zhan *et al.*, 1993; Rodriguez-Zapata *et al.*, 2010). The activated host antigen-presenting cells release interleukin-12 (IL-12), which causes the differentiation of Th0 cells into Th1 cells that secrete gamma interferon (IFN-γ) and up-regulates macrophage killing mechanisms. This action is realized by induction of hydrogen peroxide and superoxide anions (Huang *et al.*, 2005; Murphy *et al.*, 2001). In addition, CD8+ cytotoxic T cells that secrete IFN-γ are able to lysis *B. abortus* infected macrophages. This T-cell subpopulation is critical for protection against Brucella infection (Oliveira and Splitter, 1995). Besides environmental and pathogen factors, it is generally accepted that host genetic factors are major determinants of susceptibility to or outcome of infectious diseases in humans. Target gene studies have implicated several immunogenetic polymorphisms in human infectious diseases, cytokine genes and HLA being the most relevant ones (Adrian and Hill, 1998). Polymorphisms in the regulatory regions of cytokine genes may not only increase susceptibility to some infectious diseases but also affect the course and prognosis of the disease (Bidwell *et al.*, 1999; Bidwell *et al.*, 2001). Several studies reported an association between cytokine genes polymorphism and development of brucellosis. Two of the most important Th1 cytokines are TNF-α and IFN-γ, both play critical roles in control of Brucella infection and in macrophage activation (Jiang, 1993; Baldwin and Parent, 2002; Dornand *et al.*, 2002).

TNF-α is a pro-inflammatory cytokine that is activated during the acute phase immune response to extracellular pathogens. TNF-α production appears to be necessary for full expression for macrophage anti-brucella activity (Jiang *et al.*, 1993). Studies in susceptible mice model established that effective control of brucellosis is relies on TNF-α as well as CD8+ T cells until IFN-γ production resumed and clearance initiated (Baldwin and Parent, 2002). In addition to, being important in resistance to brucella, TNF-α may be associated with immunopathology of the disease, depending on the timing (Caballero *et al.*, 2000a). Genetic polymorphisms that affect production levels of certain cytokines may determine the severity, risk or protection in some infectious diseases like brucellosis. In this study, we are going to analyze the relation of the well characterized TNF-α polymorphisms and the association with the alteration in cytokine expression

level, immune responses and predisposition/resistance to human brucellosis among population of Makkah region.

Materials and Methods

Study Design and Selection of Patients and their Controls

A case-control prospective study was carried out to investigate the impact of Th-1 cytokine, TNF-α, polymorphisms on resistance/susceptibility to human brucellosis disease among Saudi population at Makkah region. All patients who have recruited to general hospitals during the period of study subject selection and are presenting with clinical signs and symptoms (e.g., fever, malaise, night sweating, weakness, weight loss, myalgia, splenomegaly, lymphadenophathy and arthralgia), suggestive of active brucellosis. They had been examined by specialist and confirmed by a positive serological test in a single high titers (≥ 1/160) of Standard Agglutination Test (SAT) and/or positive blood cultures. They were selected, after their acceptance to participate in the study. A group of controls composed of healthy individuals with no history of brucellosis or genetic disorders. They are matching for geographic area, age and sex and have the same backgrounds as the cases. They are at the same risk of exposure for brucellosis and have SAT ≤ 1/80. They were selected for the study after accepting to participate in the study.

Screening of TNF-α Promoter Polymorphisms by PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

Genomic DNA was extracted from peripheral blood leukocytes using standard methods as described by the manufacture (Micromix660 DNA extraction kit, Talent, Italy). Two well characterized Single Nucleotide Polymorphisms (SNPs) in the TNF-α promoter region were screened in this study. One of them is transition replacement of adenine for guanine at positions -308 and the second polymorphism is transition replacement of thymine for cytosine at position -857. All PCR reactions were run under conditions previously described (Fargion *et al.*, 2001; Soga *et al.*, 2003). Primer sequences for the polymorphism at position -857(rs1799724) are sense 5′-AAG TCG AGT ATG GGG ACC CCC CGT TAA-3′ and anti-sense 5′-CCC CAG TGT GTG GCC ATA TCT TCT T-3′, those for the gene polymorphism at position -308 (rs361525) are forward 5′-GGG ACA CAC AAG CAT CAA GG-3′ and reverse 5′-GGG ACA CAC AAG CAT CAA GG-3′.

DNA samples were PCR amplified in 25-μL total volume containing 200 μmol/l deoxynucleoside triphosphate, 10 μmol/l each primer, 1.5-mmol/l magnesium chloride, a 2-μL DNA sample and 2 U of Taq polymerase. Then, in a total volume of 15 μL, two

μL PCR products were digested overnight at 37°C with Hinc II to detect the SNP at position -857 and at 37°C with NcoI to detect the SNP in the -308 gene allele. The Digested DNA was loaded on a 8% Non-denaturaning polyacrylamide gel (SeaKemÒ GTGÒ agarose, FMC BioProducts) and electrophoresed at 140 V for one hour. Then the gel will be stained in 1 μg mL^{-1} ethidium bromide solution for 10-15 min and visualized with UV light under Gel Documentation System (GDS) and the genotypes of each study subject for each polymorphism were determined accordingly (Fig. 1 and 2).

Statistical Analysis

The Statistical Package for the Social Science (SPSS for Windows) was used for statistical analysis. *T-test* was used for comparing the distribution of a variable between cases and controls groups. Genotypes and alleles distributions among the patients and controls were determined. The magnitude of the polymorphisms association with predisposing to human brucellosis was assessed by logistic regression analysis using SNPS tats for its penetrance in different genetic models of inheritance; allelic, co-dominant, dominant, over dominant and recessive models. Odds ratios will be calculated with 95% confidence intervals using SNPS tats online software (http://bioinfo.iconcologia.net/snpstats/start.htm). $P < 0.05$ values were considered as significant.

Results

Sixty nine individuals with human brucellosis and 112 healthy individuals were selected for this cases-control association study. Forty-five (65.2%) of the patients were male and 24 (34.8%) were female. The distribution of patients according their age is showing in Fig. 1 with an age mean of 42±22.17 years. Fifty-two (75.4%) were Saudi Arabian and the rest of the patients were from other different nationalities which are Sudanese 7(10.1%), Egyptian 4(5.8%), Pakistanian 4(5.8%) and Indian 2(2.95%).

Genotype and Allele Distributions in Brucellosis Patients and Healthy Subjects

Genotyping of TNF-α-308G>A and -857C>T polymorphism in both patients and healthy controls was done by PCR-RFLP method as showing in Fig. 2 and 3 respectively. The assessment of genotypes and allele frequencies of TNF-α-308G>A and -857C>T polymorphisms are demonstrated for the subjects in both groups in Table 1 and Table 2 respectively. The distribution of each of genotypic variants met the conditions of Hardy-Weinberg equilibrium. For TNF-α-308 gene polymorphism, The allele frequencies in control group (n = 112) were 0.83 for TNF-α-308G (TNF1) and 0.17 for TNFα-308A (TNF2) and the allele

frequencies in 69 human brucellosis subjects were 0.83 for TNF-α-308G (TNF1) and 0.17 for TNFα-308A (TNF2). However, the distribution of TNFα-308 genotypes and alleles did not differ significantly between patients and controls (P = 0.116 and P = 0.996 respectively) (Table 1). While, for TNFα-857 gene polymorphism, The allele frequencies in control group (n = 112) were 0.91 for TNF-α-857C and 0.09 for TNF-α-857T and the allele frequencies in 69 human brucellosis subjects were 0.93 for TNFα-857C and 0.07 for TNF-α-857T with no significant differences in the genotypes and alleles frequencies between patients and controls (P = 0.0.640 and P = 0.482 respectively) (Table 2).

Assessment of Effects of TNFα -308 and -857 Gene Polymorphisms on Susceptibility to Human Brucellosis

The magnitude of the polymorphisms association with predisposing to human brucellosis in study population was assessed by logistic regression analysis using SNPS tats for its penetrance in different genetic models of inheritance; allelic, co-dominant, dominant, over dominant and recessive models (Table 1 and 2). Accordingly, the penetrance of TNF-α -308 G/A was more consistent with recessive genetic model rather than other genetic models (OR = 3.003, 95% CI 0.278-5.702, P = 0.018). This indicates that having TNF-α-308 A/A genotype is associated with high risk for human brucellosis under recessive genetic model in study population. While, for TNF-α-857 C>T gene polymorphism, as shown in Table 2, none of the tested genetic models was found to be consistent with its penetrance.

Results of statically test for deviation from Hardy-Weinberg equilibrium in control and infected cases of Brucella were presented in Table 3.
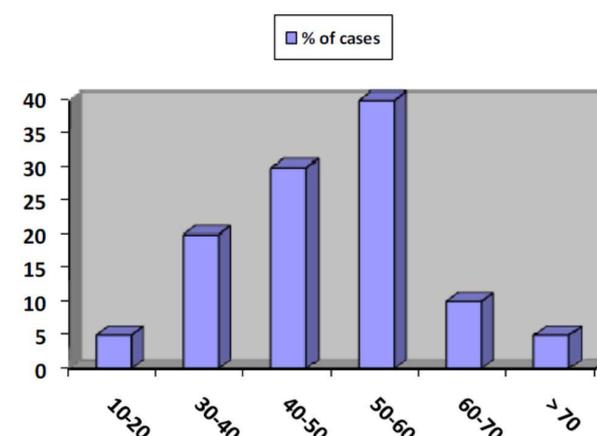

Fig. 1. Distribution of Brucella infected cases according to their age. The age mean is 42±22.17 years
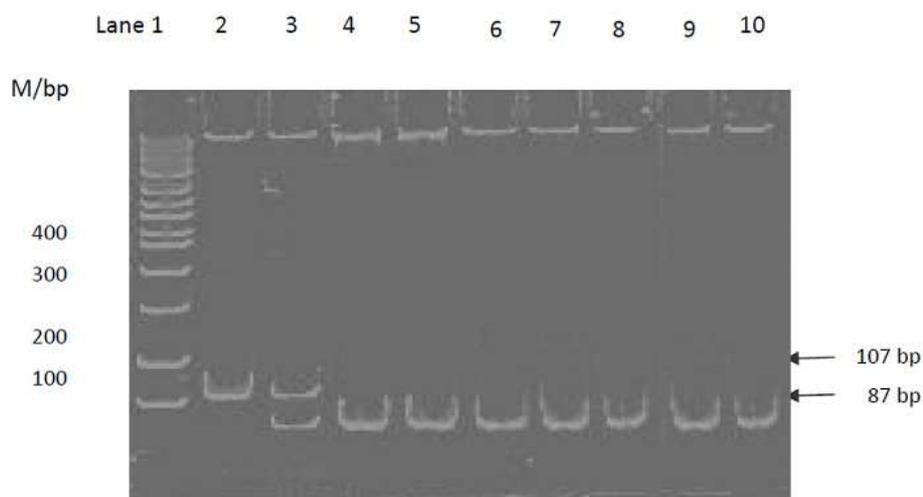
Fig. 2. Detection of TNF-α -308 polymorphism using PCR-RFLP method Legand: Lane 1: M: 100-bp DNA marker. Lane 2: undigested PCR products. Lanes 3, 4, 5, 6, 7, 8, 9 and 10: Pateints 1-8 after NcoI digestion. Patients 2, 3, 4, 5, 6 7 and 8 are homozygous for TNF1; patients 1 is heterozygous TNF1/TNF2
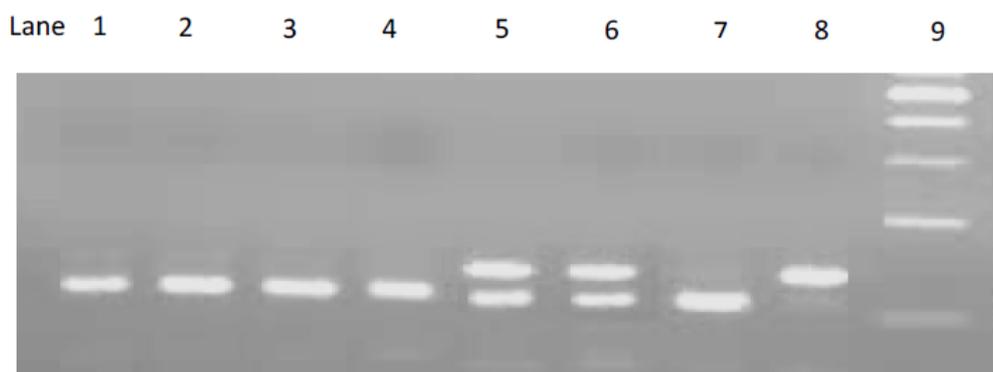

Fig. 3. Detection of TNF-α -857 C > polymorphism using PCR-RFLP method Legand: Lane 9: M: 100-bp DNA marker. Lanes 1,2, 3, 4 and 7 are homozygous wild type (C/C); Lanes 5 and 6 are heterozygous (C/T), while lane 8 is homozygous mutant (T/T) genotype

Table 1. Assessment of association of TNF-α-308 G>A polymorphism and brucellosis patients

Model	Brucellosis patients (n = 69)	Healthy control (n = 112)	P value (Pearson)	Odd ratio (95% CI)
Codominant				
G/G	47 (68%)	74 (66%)	0.070	1.00
G/A	20 (29%)	37 (33%)		1.50(0.540-4.170)
A/A	2 (3%)	1 (1%)		0.00(0.000-NA)
Allelic effect				
G	0.83	0.83		
A	0.17	0.17	0.996	0.999 (0.572-1.747)
Overdominant				
G/G-A/A	49(71%)	75(67%)		1.00
G/A	29(29%)	37(33%)	0.318	1.690 (0.620-4.630)
Recessive				
G/G - A/G	67(97.1%)	111(99.1)		1.00
A/A	2(2.9%)	1(0.9%)	0.018	3.222 (1.008-5.702)
Dominant				
G/G	47(68.1%)	74(66.1%)		1.00
A/G - A/A	22(31.9%)	38(33.9)	0.777	0.912 (0.481-1.728)

Table 2. Assessment of association of TNF-α-857 C>T polymorphism and brucellosis patients

Model	Brucellosis patients (n = 69)	Healthy control (n = 112)	P value (Pearson)	Odd ratio (95% CI)
Codominant				
C/C	60(87%)	94(83.9%)	0.640	1.00
C/T	8(11.6%)	15(13.4%)		1.48(0.270-8.121)
T/T	1(1.4%)	3(2.7%)		4.13(0.140-125.650)
Allelic effect				
C	0.93	0.91		
T	0.07	0.09	0.482	0.755 (0.345-1.655)
Overdominant				
C/C -T/T	61(88.4%)	97(86.6%)		1.00
C/T	8(11.6%)	15(13.4%)	0.660	1.461 (0.270-8.000)
Recessive				
C/C – C/T	68(98.6%)	109(97.3%)		1.00
T/T	1(1.4%)	3(2.7%)	0.410	4.070 (0.130-123.580)
Dominant				
C/C	60(87%)	94(83.9%)		1.00
C/T – T/T	9(13%)	18(16.1)	0.443	1.82 (0.391-8.410)

Table 3. Results of test for deviation from hardy-weinberg equilibrium in control and infected cases of brucella

SNP	Controls	Cases
G308A of TNF-α	n11 = 74 (76.40)	n11 = 47 (47.09)
	n12 = 37 (32.21)	n12 = 20 (19.83)
	n22 = 1 (3.40)	n22 = 2 (2.09)
	fa1 = 0.83/+ 0.023	fa1 = 0.83/+ 0.032
	F = 0.14872	F = 0.00877
	p = 0.115515 (Pearson)	p = 0.941913 (Pearson)
	p = 0.078250 (Llr)	p = 0.941657 (Llr)
	p = 0.186914 (Exact)	p = 1.000000 (Exact)
C857T of TNF-α	n11 = 94 (91.98)	n11 = 60 (59.36)
	n12 = 15 (19.03)	n12 = 8 (9.28)
	n22 = 3 (0.98)	n22 = 1 (0.36)
	fa1 = 0.91 +/0.021	fa1 = 0.93 +/0.024
	F = 0.21182	F = 0.13750
	p = 0.024979 (Pearson)	p = 0.253387 (Pearson)
	p = 0.057079 (Llr)	p = 0.330758 (Llr)
	p = 0.051744 (Exact)	p = 0.295670 (Exact)

n11 (e): Genotype 11 (expected); n12 (e): Genotype 12 (expected); n22 (e): Genotype 22 (expected); f a1: Frequency of allele 1 +/standard deviation; F: Inbreeding coefficient; p (Pearson): Pearson's goodness-of-fit chisquare (degree of freedom = 1); p (Llr): Log likelihood ratio chisquare (degree of freedom = 1); p (Exact): Exact test

Discussion

Cytokines play a key role in the regulation of the immune response and the capacity of cytokine production differs among individuals and correlates with the polymorphism in the cytokine gene promoters (Ben-Ari *et al.*, 2003). Host resistance to Brucella has been mainly studied in mice and shown that cytokines control the immune response and influence the outcome of the disease (Baldwin and Parent, 2002). Although few studies in human, patients with acute brucellosis display a Th1-type response in contrast to those with chronic form who have diminished Th1 response in response to the Brucella specific antigens (Giambartolomei *et al.*, 2002). Polymorphisms affecting cytokines controlling the immune response against infectious diseases could be the potential markers of susceptibility and clinical outcome of

different infectious diseases in humans (Hajilooi *et al.*, 2006). In this study, we assessed the potential associations and their mode of penetrance between two polymorphisms in TNF-α gene promoter (-308 G/A and -857 C/T) and human brucellosis. We found no significant association for TNF-α-857 gene polymorphism under any of the five tested genetic models. Although, we demonstrated an increased risk for human brucellosis associated with homozygosity of TNF-α-308 A allele and this association is consistent with the recessive genetic model rather than other tested models.

Our findings indicate no association between susceptibility of human brucellosis and TNF-α-857 C/T polymorphism. Although, TNF-α-857 T allele frequency was found to be higher in the healthy control (0.09) than in brucellosis cases (0.07). Nerveless, the proportion of genotypes carrying TNF-

α-857 T allele in healthy control group (0.16) either in heterozygous state (CT) or homozygous (TT) compared to their proportion in brucellosis cases (0.13) seem to have a protective effect against the disease. However, the difference was not statistically significant (P = 0.443, OR: 1.82, 95% CI: 0.391-8.410). Thus, our result suggests nonexclusive protective role of TNF-α-857 C/T polymorphism against human brucellosis in this study population.

Considering TNF-α-308G/A polymorphism, although, the TNF2 allele was found to be at similar frequency of (0.17) in both brucellosis patients and healthy control, our findings indicated an increased risk TNF-α-308 A allele for human brucellosis consistent with the recessive genetic model of penetrance (Odd Ratio: 3.222, 95% CI: 1.008-5.702, P = 0.018). Similar results were reported by Eskandari-Nasab and his colleagues who stated that the TNF-α-308 A allele or GA heterozygosity were associated with an increased risk of brucellosis (Eskandari-Nasab *et al.*, 2016). Furthermore, few other studies showed a significant association between the TNF-α-308 (A/A) genotype and brucellosis (Caballero *et al.*, 2000b; Davoudi *et al.*, 2006; Reza *et al.*, 2009). This finding is not consistent with the results reported by few studies in which TNF2 allele revealed no association with human brucellosis (Karaoglan *et al.*, 2009).

In this study, the relatively lower frequency of homozygozity for TNF2 in control group (0.009) may be either due to relatively smaller sample size (181) as according to TNF-α-308A allele frequency (0.17), homozygosity was expected at a frequency of 0.029 or may be due to a strong negative selective pressure for this allele as children who are homozygote for this allele may be more susceptible to several severe infectious diseases endemic in the region and most probably may die at younger age and rarely reach reproductive age (Mergani *et al.*, 2010). Further, the maintenance of high prevalence of the TNF-α-308A allele frequency in this study population (0.17) suggests a heterozygote advantage. Possibly the TNF2 allele in homozygote status leads to increased constitutive expression of TNF-α which could be harmfull to patients (Wilson *et al.*, 1997; Abraham and Kroeger, 1999). TNF-α might enhance disease either directly or through over-stimulation of the cytokine cascade (Clark *et al.*, 1989). However, in heterozygote, the expression of TNF-α may be more balanced than both homozygote TNF1 or TNF2 and may confer protective measures against several other infectious diseases. This explanation is supported by the observation in study in Tanzania where infants who were heterozygous for the TNF-α-308 polymorphism appeared to have fewer febrile episodes when they were free of malaria parasites (Stirnadel *et al.*, 1999).

Conclusion

Our findings indicate an increased risk of TNF-α-308A allele for human brucellosis consistent with the recessive genetic model of penetrance. Moreover, a protective role of TNF-α-857C/T polymorphism against human brucellosis in this study population could not be excluded.

Author's Contributions

Alaa B. Ismael: Contributed to conception and design, carrying out the PCR-RFLP with the coworkers and critically revised manuscript with its submission.

Adil Mergani: Contributed to design, acquisition, carrying out the PCR-RFLP with the coworkers, interpretation, data analysis and draft and revised manuscript.

Salama A-H Mostafa: Participate in laboratory experiments and manuscript revision.

Abdelmoneim M. Salim: Collection of samples, perform gel agarose analysis and manuscript revision**.**

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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