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Immunopathogenesis and Susceptibility to Cutaneous Leishmaniasis among Iraqi Patients

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Abstract

Leishmaniasisa parasitic disease that affects humans and animals, is mainly transmitted from one host to another through the bite of an infected phlebotomus sand fly vector.Theaim of this study is to evaluate the mechanism of immune-pathogenesis of leishmaniasis, and to determine a possible role for genetic polymorphism in selected gene on the susceptibility to leishmaniasis among Iraqi patients.The present study enrolled a total of 116 subjects in a case-control study design (58 patients with Cutaneous Leishmaniasis and 58 apparently healthy subjects of serving as a control group) for a period from May 2020 to February 2021,all patients 58 in this study were referred and diagnosed clinically in the dermatology outpatient clinic in the Babylon hospital, Iraq. Blood samples were taken from all subjects in the study forepidemiologic,immunological, and genetic study.The resultsofImmunological analysis results show median levels of serum MIFin patients with Cutaneous Leishmaniasis were higher than the median levels of control groups, 5.89 (2.64) pg/ml versus 3.09 (0.98) pg/ml, the difference was highly significant ($P < 0.001$). Median levels of serum Granzymes Bin patients with Cutaneous Leishmaniasis were higher than the median levels of control groups, 31.22 (16.01) pg/ml versus 15.98 (5.35) pg/ml, the difference was highly significant ($P < 0.001$). Median serum level of CAMP LL37 in patients with Cutaneous Leishmaniasis was 8.39 (6.19) pg/ml, while that of control groups was 4.83 (1.04) pg/ml.Hence serum level of CAMP LL37 was significantly higher in patients with Cutaneous Leishmaniasis than control group ($P < 0.001$).PCR-RFLP for TOLLIP polymorphism Hardy Weinberg equation was applied to node genotypes, CC, CT and TT, distribution within the control group and results are showed the homozygous wild genotype CC was encountered in 37 out of 50 control subjects; the heterozygous CT genotype was seen in 9 out of 50 control subjects and the homozygous mutant TT genotype was seen in 4 out of 50 control subjects, . Individuals homozygous for rs3750920 T allele had three times risks of developing CL compared to individuals homozygous for the rs3750920 C allele ($P =< 0.001$; OR, 2.62 [95% CI 1.48 - 4.63]). The current study showed that polymorphism is associated with CL we foundcommon genetic variants of TOLLIP gene (rs3750920) that are associated with cutaneous leishmaniasis susceptibility, these genetic markers were associated with an increased risk for developing CL in a case-population study in Iraq and it is the first study deals with the genetic polymorphism of TOLLIP gene in endemic areas in Iraq.

Keywords*:CL,CAMP-LL-37,Granzyme B,MIF,Vit D,TOLLIP gene,SNIP.*

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Introduction

Leishmaniasis is a vector-borne disease caused by a protozoan of the genus Leishmania[1].

Leishmaniasis is a severe parasitic disease, viewed via the World Health Organization (WHO) to be one of the most necessary ignored diseases globally. Approximately 12 million people are infected with greater than twenty distinct pathogenic Leishmania species worldwide (i.e. tropical and subtropical areas ofAsia, the Middle East, sub-Saharan Africa, and South America. The disease is endemic in more than 98 countries and an estimated 350 million people are at risk.The 84% of global CL incidence in 2016 were reported in Afghanistan, Algeria, Brazil, Colombia, Iraq, Pakistan, Peru,theSyrian Arab Republic, Tunisia, and Yemen[2].Early identification of the infectious nature of the disease is dated back to more than 100 years ago and the pioneer who made that recognition was the Russian physician "Piotr FokichBorovsky" who successfully isolated the parasite from Asian cutaneous lesions[3].Cutaneous leishmaniasis is an endemic disease in Iraq, mainly in central and southern regions[4].Immunity to leishmaniasis is mainly initiated by way of innate immune cells followed by cell-mediated immune response[5].Granule-secreted enzymes (Granzymes) are a family of serine proteases discovered in the mid1980s and proposed to be key mediators of cytotoxic T lymphocyte (CTL) and natural killer (NK) granule exocytosis-mediated cell death.One likely explanation of how cytotoxic cells mediate inflammation and tissue injury in CL is that, after degranulation of cytotoxic cells, granzyme B and perforin are released into the extracellular space, inducing apoptosis of infected macrophages and bystander cells[6].Cathelicidins, are key players in the human host's immune defense. This class of pleiotropic peptides is an important mediator of innate immunity against microbial pathogens and provides first-line defense against infection by promoting rapid elimination of pathogens.The human cathelicidin is expressed by monocytes, macrophages as well as neutrophils. This AMP, LL37, able to create pores, hereby disrupting membranes. Although the exact mode of action is unknown, two models have been widely accepted being the "carpet" and "toroidal" model[7].Vitamin D indirectly stimulates the production of cathelicidin.This increased cathelicidin production was caused by vitamin D by activation of the CAMP signal pathway, thus limiting the leishmania infection.Vitamin-D derivativesinduce Cathelicidin-Mediated-Leishmania restriction in human primary macrophages:to investigate the role of cathelicidin in Leishmania restriction further, we increased expression of cathelicidin using Vitamin D derivatives[8].MIF was originally discovered (in 1966) as a lymphokine, derived from activated T-cells, that inhibited the random migration of macrophages and was shown to be involved in the mechanism of delayed-type hypersensitivity. The proinflammatory properties of MIF also make it a crucial mediator in the immune response against a wide variety of pathogens including parasites.MIF can also be harmful to the host, many pathogenic protozoans, including Leishmania, produce their own MIF cytokine. These secreted parasite-produced MIF are structurally similar to human MIF, bind the MIF receptor (CD74), and stimulate immune cells and epithelial cells to cause the release of cytokines such as TNF-α, IL-8, and IL12[9].Cutaneous leishmaniasis (CL) is usually manifested as a nodule which gradually develops to a self-healing lesion leaving a scar, but a polymorphism is seen in lesion characteristics, and diverse atypical forms are reported.The interaction of inflammatory and regulatory responses delimited by cell-mediated immune responses drives disease expression and may result in asymptomatic infection, self-healing, or chronic leishmaniasis[10].Pathogenesis follows a complex set of interactions between factors triggered by the host's innate and acquired immune responses, which are strongly influenced by some aspects such as the host's genetic background. Such polymorphisms in TLRs can influence the immune response

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(susceptibility or resistance) of an individual to a pathogenic infection principally by affecting the host-pathogen interactions[11].

Material and Patients

Blood samples were taken from all subjects in the study for immunological, genetic and epidemiologic study.

1.Patients:A hundred and sixteen subjects from different ages and both genders were enrolled in this case-control study, this study groups were classified as the following,including58 patients with Cutaneous Leishmaniasis (Baghdad boil) (case group)and 58 normal healthy individuals no CL (control group).All patients in this study were referred from different regions of Iraq and were diagnosed clinically by consultant dermatologist in dermatology out patients clinic of central general hospitals of Al- Hilla,Iraq from September-2020 to February-2021).

2. Samples Collection (Blood samples and serum samples for)**:**

Serological Markers: Serum for serological study (Human Granzymes B,Human Cathelicidin Antimicrobial Peptide(CAMP LL-37),Human Macrophage Migration Inhibitory factor(MIF) detected by enzyme linked immunosorbent assay,sandwichELISA Kit,Bioassay Technology Laboratory,China, Lot number(E0899Hu,E4039Hu and E0141Hurespectively). Parameters were measured according to instruction of the manufacturing company.

Genetic study: Blood for genetic study for TOLLIP polymorphism rs3750920 genotyping by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). Genomic DNA from blood samples were extracted by using **G-spin™ Total DNA Extraction Kit,**iNtRON,Koreaand done according to company instructions. The RFLP-PCR primer for detection of Toll-interacting protein (TOLLIP-rs3750920) gene polymorphism were designed by (de Araujo, *et al*. 2015). These primers were provided from (ScientificReseracher. Co. Ltd. Iraq)

3. Statistical Analysis:were summarized, presented and analyzed using statistical package for social science (SPSS version 25) and Microsoft Office Excel 2010. Numeric data were presented as mean, standard deviation, median and interquartile range (IQR) while nominal data were expressed as number and percentage. Chi square test was used to compare one numeric and one nominal data groups.Mann Whitney U test was used to compare median value between two non-parametric groups.Correlationcofficiant was estimated by spearman correlation and Pearson correlation.

Results and Discussion

This result was supported by the recommendation of theCutenousLeishmaniasis(CL)

Subjects Immunological Analysis Results

Serum macrophage migration inhibitory Factor (MIF) level in patients and control groups.

The comparison of serum MIF level between patientswith Cutaneous Leishmaniasis and control groups has been carried out and the results were demonstrated in table (3-5).Median levels of serum MIFin patients with Cutaneous Leishmaniasis were higher than in comparison

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the median levels of control groups, 5.89 (2.64) pg/ml versus 3.09 (0.98) pg/ml, the difference was highly significant (P < 0.001).

Table (3-5): Frequency distribution of patients with Cutaneous Leishmaniasis and control subjects according to level of Serum macrophage migration inhibitory Factor.

	Case – control comparison		
MIF (pg/ml)	**Patients n = 58**	**Control n = 58**	***P***
Range	4.03 – 23.19	1.52 – 3.99	**< 0.001 † HS**
Median (IQR)	5.89 (2.64)	3.09 (0.98)	

n: number of cases; IQR: inter-quartile range; †: Mann Whitney U test; HS: Highly significant at $P \leq 0.001$

In this current study was show highly significant(P < 0.001) between patients andcontrols.Machrophage migration inhibitory factor (MIF) is a proinflammatory cytokine which has an important role in the regulation of inflammatory and immune responses[12]. Previous reports have shown that MIF plays a critical role mediating host resistance to Leishmaniasis. Juttner *et al.* found *in vitro* that both recombinant murine or human MIF, at 1.5 and 2.5 µg/ml concentrations could activate macrophages to kill *Leishmania major*. MIF appears to be capable of killing *Leishmania* parasites by acting directly or indirectly with other cytokines, such as TNF-α and reactive products like nitric oxide[13].

Serum Granzymes B level in patients and control groups

The comparison of serum Granzymes B level between patientswith Cutaneous Leishmaniasis and control groups has been carried out and the results were demonstrated in table (3-6), Median levels of serum Granzymes Bin patients with Cutaneous Leishmaniasis were higher than in comparison the median levels of control groups, 31.22 (16.01) pg/ml versus15.98 (5.35) pg/ml, the difference was highly significant (P < 0.001).

Table (3-6): Serum *Granzymes B* level in patients with Cutaneous Leishmaniasis and controlsubjects.

	Case – control comparison		
***Granzymes* B (pg/ml)**	**Patients n = 58**	**Control n = 58**	***P***
Range	21.96 – 98.90	8.30 – 19.80	**< 0.001 † HS**
Median (IQR)	31.22 (16.01)	15.98 (5.35)	

n: number of cases; IQR: inter-quartile range; †: Mann Whitney U test; HS: Highly significant at $P \leq 0.001$

This current study was agree with past study, we first showed that in endemic foci of L. major infection subjects having a previous contact with the parasite showed the presence of Leishmania-specific cytotoxicity[14]. In the past studies, cytotoxicity observed in the coculture of infected macrophages with peripheral blood lymphocytes was mediated by granzyme B[15].

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Serum CAMP level in patients and control groups.

SerumCAMP LL37 levels were measured for all participants and results are shown in table (3-7).Median serum level of CAMP LL37 in patients with Cutaneous Leishmaniasis was 8.39 (6.19) pg/ml, while that of control groups was 4.83 (1.04) pg/ml.Hence serum level of CAMP LL37 was significantly higher in patients with Cutaneous Leishmaniasis than control group (P < 0.001).

Table (3-7): Serum CAMP LL37level in patients with Cutaneous Leishmaniasis and control subjects.

	Case – control comparison		
***CAMP LL37* (pg/ml)**	**Patients n = 58**	**Control n = 58**	***P***
Range	5.91 – 49.8	2.85 – 5.94	**< 0.001 † HS**
Median (IQR)	8.39 (6.19)	4.83 (1.04)	

n: number of cases; IQR: inter-quartile range; †: Mann Whitney U test; HS: Highly significant at $P \leq 0.001$.

In past study showed that the LL-37 level in the control group was high than diabetes and diabetic foot groups, diabetic foot was less than diabetes mellitus and control groups and there was significant difference between the study groups ($p<0.05$) can be causein don't wound healing in diabetic foot patients[16] and that agree withLL-37 and its precursor, hCAP18, are found in different tissue and cell types, playing an importantrole in innate immunity against diverse pathogens.

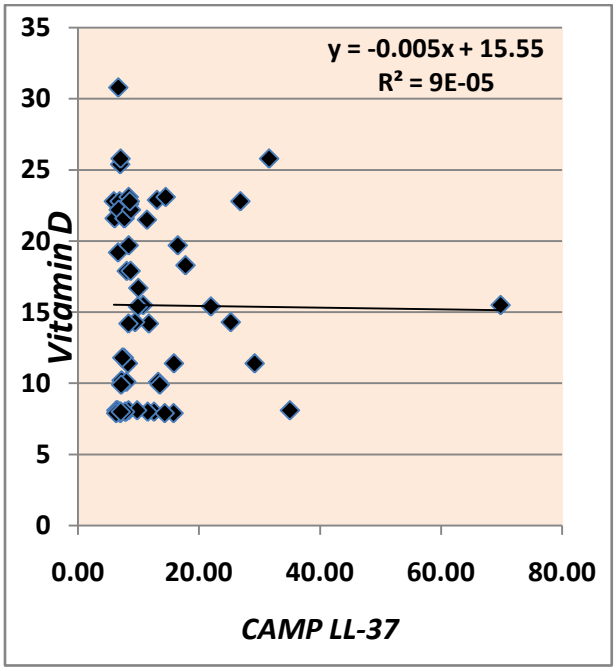

Figure (3-14): Correlations between Serum CAMP LL37 and Viatamin D in Patients with Cutaneous Leishmaniasis.

Serum CAMP LL37 level was not significantly correlated to Vitamin D of patients with Cutaneous Leishmaniasis , as shown in figure (3-14).

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Vitamin D plays an essential role in bone development.Several reports have shown associations between vitamin D deficiency and the incidence as well as the severity of chronic inflammatory diseases[17].Low vitamin D status has been shown to be a risk factor for infectious diseases[18].Crauwels et al. observed increased expression of LL-37 in skin biopsies from cutaneous leishmaniasis. They demonstrated that recombinant LL-37 reduced Leishmania parasite viability in a dose-dependent manner[19].

Molecular analysis:Genotypic and Alleles Analysis of *TOLLIP gene (rs3750920)polymorphism.*

Hardy Weinberg equation was applied to node genotypes, CC, CT and TT, distribution within the control group and results are shown in table (3-9). The homozygous wild genotype CC was encountered in 37 out of 50 control subjects; the heterozygous CT genotype was seen in 9 out of 50 control subjects and the homozygous mutant TT genotype was seen in 4 out of 50 control subjects, table (3-10),the observed distribution of control subjects according to NOD2 genotypes was significantly different from the expected one (P = 0.010), as shown in table (3-10).

Table (3-9): Hardy Weinberg equation

Genotypes	Observed	Expected	χ^2	*P* ¥
Homozygote reference CC	41	41.2	0.214	0.855 NS
Heterozygote CT	8	8.9		
Homozygote variant TT	9	8.2		

¥: Chi-square test; S: significant at $P \leq 0.05$

Table(3-10): *NOD*2 Genotype frequency distribution in patients with IBD and control group

Genotype	Cases *n* = 58	Control *n* = 58	χ^2	*P* ¥
CC	27	41	7.254	0.027 S
CT	12	8		
TT	19	9		

n: number of cases; ¥: Chi-square test; S: significant at $P \leq 0.05$.

Table (3-11): TOLLIP allele frequency in Cutaneous Leishmaniasis patients and control group

TOLLIP Allele	Patients *n* = 116	Control *n* = 116	*P*	OR	95%CI	EF	PF
T	50	26	< 0.001 ¥ HS	2.62	1.48 - 4.63	0.62	……
C	66	90		0.381	0.215 - 0.67	…….	0. 62

n: number of alleles; ¥: Chi-square test; OR: odds ratio; CI: confidence interval; EF: etiologic fraction; PF: preventive fraction; HS: highly significant at $P \leq 0.01$

The Toll-Like Receptors (TLRs) are a group of receptors that are present in the host immune system the cellular level and a reactivated by pathogen associated molecular patterns(PAMPs) and damage-associated molecular patterns(DAMPs)[20].In the table (3-9) the significant at $P \leq 0.05$ for Homozygote reference CC (41) , Heterozygote CT (8) and Homozygote variant TT (9) when using Chi-square test

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show significant difference when compared with healthy individuals and Homozygote variant TT show significant at $P \leq 0.05$ that mean this genotype has mutant type. Individuals homozygous for rs3750920 T allele had three times risks of developing CL compared to individuals homozygous for the rs3750920 C allele (P =< 0.001 ; OR, 2.62 [95% CI 1.48 - 4.63]).The results of genotypes and alleles frequencies of rs3750920 is shown in table (3-10) and table (3-11). The T and C alleles of the rs3750920 was more common in patients with CL than in healthy individuals (P = < 0.001); odds ratio [OR], 2.62, 0.381 [95%confidence interval (CI) 1.48 - 4.63, 0.215 - 0.67] respectively.The CC,CT and TT genotypic frequencies were determined in patients with acute and chronic cutaneous leishmaniasis, and compared to those of control individuals. The current study showed that polymorphism is associated with CL we foundcommon genetic variants of TOLLIP gene (rs3750920) that are associated with cutaneous leishmaniasis susceptibility, these genetic markers were associated with an increased risk for developing CL in a case-population study in Iraq and it is the first study deals with the genetic polymorphism of TOLLIP gene in endemic areas in Iraq.The PCR-RFLP patterns for detection of the genotypes of the rs3750920 is shown in table (3-10) as mentioned elsewhere[21]. The genotype detail of SNPs rs3750920 was found 27 for C/C, 12 for C/T and 19 for T/T for 58 patients and compare with 58 healthy individuals which the genotype detail of SNPs rs3750920 was found 41 for C/C, 8 for C/T and 9 for T/T were statistically significant between them and the genetic model was within acceptable Hardy Weinberg equilibrium (p-value ≤ 0.05) Table (3-9).The polymorphism rs3750920 of the TOLLIP gene present in the intron and exon 4 was typed by PCR-RFLP using the restriction enzyme Msp I for rs3750920 (New England Biolabs) .The SNPs –19C/T(rs3750920) and 19TT(rs3750920) ,located in the TOLLIP gene of patient groups ,seem to influence the expression of the gene when compared with The SNPs –8C/T(rs3750920) and 9TT(rs3750920) ,located in the TOLLIP gene of healthy controls. Comparison of genotypic and allelic frequency between patients with CL and healthy subjects did show difference. These polymorphism do predict susceptibility to ,or protection against the development of CL in Iraqi patients.The comparison of alleles also showed that T allele is associated with susceptibility (the risk allele T), statistically in the table (3-11) show significant association and there were an excess of genotypes of the rs3750920 T allele (TT(19) + CT (12) in the patients with CL group compared to 9 and 8 respectively in the control group. was found using the multiplicative mode (T) 50 for patients with compare with controls (T) 26 and statistically significant (p-value $P \leq 0.01$ at 95% CI) and association with OR = 2.62 association as a risk factor for CL.Thisstudy agree with[22].

Conclusion

The present study showed that median levels of serum MIFin patients with cutaneous leishmaniasis were higher than the median levels of control groups.Median levels of serum Granzymes Bin patients with cutaneous leishmaniasis were higher than the median levels of control groups.Median serum level of CAMP LL37 was significantly higher in patients with Cutaneous Leishmaniasis than control group.The polymorphism (rs3750920) of TOLLIP gene is independently associated with an increased risk of developing CL. Do serious steps in order to prevent Leishmania distribution by controlling vector, keeping reservoirs hosts away from human residence, proper treating of infected persons.Recommendations of this work areUsing a sensitive molecular technique for parasites diagnosis beside the classical clinical diagnostic measures for accurate results in obscure cases of leishmaniasis.

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