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Polymorphism of *MIF* and *NRAMP1* Genes and their effect on TNF-α, MIF and IL-1β Level among Patients of Cutaneous Leishmaniasis

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بِسمِ اللهِ الرَّحمنِ الرَّحيمِ

«يَرفَعُ اللهُ الذينَ آمَنوا مِنكُم َوالذّينَ أُوتُوا العِلمَ دَرَجات و اللهُ بِما تَعمَلونَ خَبيُر »

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I certify that this thesis (**Polymorphism of *MIF* and *NRAMP1* Genes and their effect on TNF-α, MIF and IL-1β Level among Patients of Cutaneous Leishmaniasis**) was prepared under my supervision at the Department of Microbiology, College of Medicine/ University of Al-Qadisiya, in a partial fulfillment of the requirements of the degree of Doctorate of Philosophy of science in Medical Microbiology.

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Certificate of Examining Committee

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Dedication

To

The one who dedicated her life for me and taught me patience, loyalty, and honesty, who saved me with her Prayers

My first lover …"The pure spirit of my mother"

To

Whom he devoted his life for me and taught me patience and loyalty.He was my first teacher who brought me up and improved my education, "my father"

". Respect and Homage

To

Whom were the beacon of the way, "presence and absence" by their thoughts and bodies, "my brothers"

"Pride and gratitude"

To

Every one treated my hurt, exhausted my sadness and broadcast the ray of hope in my heart and supported me,

"Respect and appreciation"

I present my modest effort

Hasan

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Summary

Cutaneous Leishmaniasis is vector-borne disease transmitted by biting of the sandfly, it is a severe health problem in many countries and endemic in most regions of Iraq especially regions with poor populations.

This case-control study was arranged to investigate of *Macrophage Migration Inhibitory Factor and Natural Resistance-Associated Macrophage Protein1* genes polymorphism with susceptibility to Cutaneous Leishmaniasis infection in Iraqi population in Al-Muthanna province. Five ml blood samples obtained from Sixty patients with Cutaneous Leishmaniasis attending the outpatient clinic of the dermatology department in Al-Hussein Teaching Hospital and specialized Center of Allergy in Al-Muthanna have been recruited in the study and compared to 30 health control, there was 34 male and 26 female with male to female ratio 1.30 :1, their ages ranged between 1-80 years, 3ml blood samples were assessed for serum measurement of Cytokines using Enzyme Linked Immunosorbent Assay technique. Two ml blood sample was utilized for genomic DNA extraction from peripheral blood leukocytes for detection of single nucleotide polymorphism *of Natural Resistance-Associated Macrophage Protein 1* and *Macrophage Migration Inhibitory Factor* genes using Polymerase Chain Reaction–Restriction Fragment Length Polymorphism technique. The distribution analysis showed that the highest Cutaneous Leishmaniasis patients were included in this study their ages ranging between 11-20 years old.

The results showed significant differences in distribution of Cutaneous Leishmaniasis in rural regions was (71.7%) which was more than urban regions (28.3%). The mean concentration of Macrophage Migration Inhibitory Factor cytokine level decreases gradually according to variation in age of patients and control groups respectively. The mean concentration of Tumor Necrosis Factor Alpha and Interleukin 1 Beta were significantly higher in patients as compared to control groups, and increases gradually with increasing of age in patients and control groups. While serum human Interleukin 1 Beta, on the other hand, showed no significant difference with increasing

of age in control group.

There was no significant difference in the *Macrophage Migration Inhibitory Factor* genes (MIF G-173C) polymorphism between Cutaneous Leishmaniasis patients and control groups (P=0.234), as well as no association between MIF G-173C polymorphisms and Cutaneous Leishmaniasis, frequency of allele C was more in controls than the patients group (18.75% and 15%) respectively, when association between *Macrophage Migration Inhibitory Factor* genotype and serum Macrophage Migration Inhibitory Factor cytokine in patients groups were studded, there are less MIF secretion induced by C allele in patients groups comparison with G allele, however, this effect was not observed in control groups .

When *Natural Resistance-Associated Macrophage Protein 1* (RS17235409) Polymorphism studied, there was a significant difference in frequency of alleles and genotypes (RS17235409) between patients and controls. The results indicated that genetic variations of RS17235409 not associated with susceptibility to Cutaneous Leishmaniasis infection, and the frequency of allele A was more in controls than the patients (31.25% and 15%) respectively. However, when mutations occur in the *Natural Resistance-Associated Macrophage Protein 1* gene result in a non-functional or unstable protein and then leading to an increased proliferation of parasites in the macrophage, this a clearly recorded in present study was A allele associated with lower levels of Tumor Necrosis Factor Alpha and Interleukin 1 Beta in patients and control groups comparison to that absorbed in allele G.

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Abbreviations

Symbols	Meaning
ACL	Anthroponotic Cutaneous Leishmaniasis
AIDS	Acquired Immune Deficiency Syndrome
AluI	Arthrobacter Luteus
Avall	Anabaena Variabilis
CBC	Complete Blood Count
CCR5	C-C Chemokine Receptor Type 5
CD8	Cluster Of Differentiation 8
CDC	Centers For Disease Control
cDNA	Complementary DNA
CL	Cutaneous Leishmaniasis
COX-2	Cyclooxygenase 2
CXCL1	C-X-C Motif Chemokine Ligand 1
DAT	Direct Agglutination Test
DCL	Diffuse Cutaneous Leishmaniasis
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic Acid
DTH	Delayed Type Hypersensitivity
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
GP63	Glycoprotease
HBS	Hepatitis B
HCS	Hepatitis C
HIV	Human Immunodeficiency Virus
HRP	Horseradish Peroxidase
I-CAM-1	Intercellular Adhesion Molecule1
IFAT	Indirect Fluorescent Antibody Test
IFN-γ	Interferon-Gamma
IL	Interleukins

IM	Intramuscularly
IV	Intravenously
kDNA	Kinetoplast DNA
LPG	Lipophosphoglycan
LST	Leishmanin Skin Test
MCL	Mucocutaneous Leishmaniasis
MCP-1	Monocyte Chemoattractant Protein-1
MCP-1	Monocyte Chemoattractant Protein1
mDCs	Monocytes-Derived Dendritic Cells
MHC	Major Histocompatiblity Complex
MIF	Macrophage Migration Inhibitory Factor
NNN	Nicolle-Novy-Mcneal
NO	Nitric Oxide
NRAMP1	Natural Resistance-Associated Macrophage Protein 1
OD	Optical Density
PCR	Polymerase Chain Reaction
PKDL	Post Kala -Azar Dermal Leishmaniasis
PMNs	Polymorphonuclear Neutrophils
RFLP-PCR	Reaction-Restriction Fragment Length Polymorphism
ROS	Reactive Oxygen Species
rRNA	Ribosomal Ribonucleic Acid
SLC11	Solute Carrier 11
SPSS 23	Statistical Packages For Social Science
TB	Tris-Borate
TLR4	Toll-Like Receptor 4
TNF-α,	Tumor Necrosis Factor-Alpha
V-CAM-1	Vascular Cell Adhesion Molecule1
VL	Visceral Leishmaniasis
ZCL	Zoonotic Cutaneous Leishmaniasis

Chapter One

Introduction and Literatures Review

1. Introduction and Literatures Review

1.1. Introduction

Human leishmaniasis is a parasitic disease transmitted by sand flies, its characteristic by a spectrum of cutaneous , mucocutaneous and visceral diseases that depend largely on the species of the parasite involved and host immune response (Ahmed *et al.*, 2003, Blum *et al.*, 2004).

Cutaneous leishmaniasis is the most common form of leishmaniasis, about (1-1.5) million of cases every year and about (50 to 70%) of all cases in the world (Blum *et al.*, 2004, Tripathi *et al.*, 2007). Cutaneous leishmaniasis occur each year more than 90% of cases occur in five countries in the old world (Afghanistan, Algeria, Iran, Iraq and Saudi Arabia) and two countries in the new world including Brazil and Peru (Desjeux, 2001). *Leishmania major* and *Leishmania tropica* considered as common causes of CL in Iraq(Gonzalez, 2003)

Cutaneous leishmaniasis is more common in rural than urban areas, the incubation period is 2-8 weeks, the symptoms differ in the regions, according to the species of parasite and the immune patient response, CL begins as an erythematous papule increase in size produced a nodule, eventually ulcerates and crusts over, the border is raised and distinct, the ulcer is painless unless share with bacterial or fungal infection, the sores may change in size and stay over time, the clinical manifestations involve the nose, mouth and pharynx (Rozendaal, 1997, Control and Prevention, 2012).

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine produced by the pituitary gland and multiple cell types, including macrophages , dendritic cells and T-cells, upon releases MIF modulates the expression of several inflammatory molecules, such as TNF-α, nitric oxide and cyclooxygenase 2 (COX-2),these important MIF characteristics have prompted investigators to study its role in parasite infections, several reports have demonstrated that MIF plays either a protective or deleterious role in the immune response to different pathogens (de Dios Rosado and Rodriguez-Sosa,

2011). MIF secretion is tightly regulated by stress and immune stimuli, including endotoxins, inflammatory cytokines as IFN-γ, TNF-α and glucocorticoids (Bacher *et al.*, 1997, Bernhagen *et al.*, 1998, Sprong *et al.*, 2007). Once secreted, MIF exhibits a broad range of immune and inflammatory activities, including the induction of inflammatory cytokines such as TNF-α, IFN-γ, IL -1β, IL-12, IL-6 and IL-8 (Bozza *et al.*, 1999, Mitchell *et al.*, 2002).

Natural resistance-associated macrophage protein 1 (*NRAMP1*) gene is a member of the solute carrier family 11 (proton-coupled divalent metal ion transporter), member (A1) *SLC11A1* (Vidal *et al.*, 1995, Mulero *et al.*, 2002). In humans, *NRAMP1*gene is located in the chromosome region 2q35, containing 16 exons (Taype *et al.*, 2006). In a resting macrophage, the protein encoded by *NRAMP1* is assembled into the membrane of late endosome; while following phagocytosis it is relocated to the membrane of phagosome (Canonne-Hergaux *et al.*, 2002, Kim *et al.*, 2003). This protein transfers divalent metal ions across the phagosomal membrane and might be a critical factor for resistance to some microbial infections, *NRAMP1* influences a variety of antimicrobial responses of a macrophage, including induction of radical oxygen and nitric oxide intermediates, production and activation of various pro-inflammatory cytokines (TNF-α and IL-1β) and regulation of anti-inflammatory cytokine IL-10 (Blackwell *et al.*, 2000, Courville *et al.*, 2006). Due to the importance of *NRAMP1*, especially RS17235409 (rs17235409) polymorphisms in the control of intracellular infections (Fattahi-Dolatabadi *et al.*, 2016), the associations of the mentioned polymorphisms with susceptibility to CL infection were investigated in this study.

Aim of study:

This case-control study aims to investigate of *MIF and NRAMP1* genes polymorphism with susceptibility to CL infection in Iraqi patients in AL-Muthanna province through following objectives:

1- Study sample subjects, personal Information like age, gender and location.
2- Study of polymorphism in *MIF* and *NRAMP1* genes, and their association to genes variants susceptibility to CL infection.
3- Estimation of TNF-α, IL-1 β and MIF cytokine levels and their role in pathogenicity of CL infection.
4- Determination effect of polymorphism of *MIF* and *NRAMP1* genes on cytokines secretion.

1.2. Literature Review

1.2.1 Historical Highlight on Cutaneous Leishmaniasis

Al-Razi in his work Khulasat Al-Tajarib (1500AD), was the first who mentioned the lesion Baghdad, as (Extremely common in Baghdad lozenge, if the sore remains untreated, it will continue for many years) the people call it Baghdad lozenge, "Baghdad Button" More than 300 years later the disease was still very common among Baghdad children (Pringle, 1957).

Alexander Russell in 1756, made an important advance in the discovery of leishmaniasis after examining a Turkish patient, according to Russell, "After it is cicatrized, it leaves an ugly scar, which remains throughout life, and for many months has a livid colour, when they are not irritated, they seldom give much pain". Russell called this disease, "Aleppo boil" (Klaus and Frankenburg, 1999).

The first observer in human leishmaniasis was Gunnigham, who in 1885 described a peculiar parasitic organism in a section of Delhi Boil, in Calcutta , while Russian military surgeon, Borovsky (1891) gave an accurate description later of its morphology, in (1900) observed the parasite by William Leishman during his examination of a spleen smear prepared dead soldier duo to 'Dumdum fever' or Kala-azar contracted at Dum Dum city in India, and in (1903) this discovery was published in the British Medical Journal, Charles Donovan also recognized the parasite in other kala-azar patients and published his discovery a few weeks after Leishman ,after examining the parasite using Leishman's stain, these amastigotes were known as Leishman-Donovan bodies and officially, this species became known as, *L. donovani* ,the first author who adopted the modern name of *Leishmania tropica* for the parasite of oriental sore was Luhe (1906), (Roberts and McLeod, 1999, Paniker, 2013). Cutaneous leishmaniasis (Oriental sore) in old world is an old disease which has been given many local names as Baghdad boil, Aleppo boil, Delhi boil, little sister, kandahar sore, lahore sore and hashara (James *et al.*, 2011). The first demonstration of the parasite of CL in Baghdad was by (Wenyon, 1911), Adler and Theodor (1926) noted the relation of the disease to *Phlebotomus sergenti* , in 1930 they were able to infect a

human volunteer with Baghdad canine strain and produced a lesion identical to Baghdad boil (Sharquie *et al.*, 1988).

1.2.2 Epidemiology of Cutaneous Leishmaniasis

There are about 30 species of leishmania, and more than 10 species are medically important (Bates, 2007). Leishmaniasis affects around 12 million people and is endemic in 88 countries (72 of which are developing countries), putting an estimated 350 million people at risk, annual incidence is estimated at 500,000 new cases of visceral leishmaniasis, and as many as 1 – 1.5 million for cutaneous leishmaniasis (Desjeux, 2004). The leishmaniasis cause an estimated 70,000 deaths each year, and an estimated 2.4 million disability-adjusted life years (Reithinger and Dujardin, 2007). CL spread in Africa, Europe and Asia, as well as in countries Middle East (Syria, Lebanon, Jordan, Saudi Arabia and Iran), a 90% of CL cases occur in Afghanistan, Algeria, Brazil, Pakistan, Peru,Saudi Arabia, Syria and Iran (Müller *et al.*, 2009, Alavinia *et al.*, 2009), while 90% of VL cases occur in Bangladesh, north eastern India, Nepal, Sudan and north eastern Brazil (Desjeux, 2004, WHO, 2010).

The number of cases reported globally has increased over the period 1900, in part due to improved diagnosis, but also due to an increase in anti-leishmanial drug resistance and a lack of adequate vector or reservoir control tools (Reithinger and Dujardin, 2007). The distribution of leishmaniasis has been modified since the emergence of the HIV/AIDS pandemic, and co-infection of HIV/*Leishmania*, which can lead to uncommon clinical forms of the disease and resistance against current treatment, has been reported in 35 countries (Cruz *et al.*, 2006, Santos *et al.*, 2008).

Cutaneous Leishmaniasis in Iraq was declined during the anti-malaria control program and anti-malaria house spraying with DDT, but when this was discontinued in the mid-1960s, the incidence was returned, During the Gulf War (1991), cases number of CL were peaked with an incidence of 45/10,000 population in 2008 (AlSamarai and AlObaidi, 2009). Sharquie was confirmed a clear increase in incidence of CL in Iraq at the beginning (1980), the spread of the disease is a pandemic in the eighties which synchronous with Iraq-Iran war is due to the large influx of sensitive personnel in the

territories As well as the circumstances of the war, which made many places to be more suitable for settlement of Insect vector (Sharquie *et al.*, 1988).

The epidemiology of leishmaniasis depends on the characteristics of the parasite species, the local ecological characteristics of the transmission sites, the current and past exposure of the human population to the parasite and widely varying human behavior (Kamhawi, 2006).Cases of cutaneous leishmaniasis caused by *L. tropica* mostly occur in the suburbs of big cities (Baghdad, Mosul) among large conglomerations of people where the sanitary conditions are unsatisfactory, incidences caused by *L. major* are much more common, they appear primarily in rural areas, especially in the northern and southern provinces of the country (Alvar *et al.*, 2012).

Cutaneous leishmaniasis mostly present in central Iraq and the greater Baghdad area, but since the Gulf War the disease has extended to all provinces of Iraq (Awad and Jarallah, 2006, Alvar *et al.*, 2012). It is considered as a major health problem in 14 countries (Desjeux, 2004).The cutaneous leishmaniasis is one of the endemic diseases in Iraq, many studies were performed on both vector and reservoir in many parts of Iraq during the last century (Salman, 2017). Dependence on the available database of Iraqi CDC were total cases of CL in Iraq for the period (2008-2015) were 17001 cases, highest reported cases of CL was recorded in 2015 (4000 cases) in percent of 23.5%. The results were cleared that the percent of CL reported cases were increased from (5.9%) in the year 2013 to (23.5%) in the year 2015, the total of the incidence rate of CL cases for this period of years calculated as (45 per 100,000), where maximum reported incidence rate was observed in 2015 which was 10.5/ 100,000 (4000 cases) and minimum reported cases was 2.9/100000 (1005 cases) which observed in 2013, a total of CL cases was decreased as (1829-1250 cases) in (1989 – 2008), but it back to increase to (3113 cases) in 2010 (Al – Obaidi, 2013).

1.2.3 The classification of parasite

The classification of *Leishmania* was initially based on ecobiological criteria such as vectors, geographical distribution, tropism, antigenic properties and clinical manifestation (Bañuls *et al.*, 2007). Lanson and shaw (1987) have divided *Leishmania* spp. into two subgenera, *Viannia* and *Leishmania*, based on the site of their development in the gut of the sand fly, the viannia subgenus includes *L.(Viannia) brasiliensis* and related species that develop in the hindgut before migrating to the midgut and foregut (peripylaria) , species in the subgenus *Leishmania* such as *L. donovani*, occupy only the midgut and foregut (Suprapylaria), (Croan *et al.*, 1997). However, biochemical and molecular analysis showed that pathological and geographical criteria were often inadequate and thus other criteria such as the patterns of polymorphism exhibited by kinetoplastic DNA markers, proteins or antigens came to be used to classify *Leishmania* (Kolaczinski *et al*., 2004).

There are different species, morphologically indistinguishable but they can be differentiated by enzyme analysis and PCR technology, monoclonal antibody (Akilov *et al.*, 2007, Odiwuor *et al.*, 2011).

Kingdom: Protista

Subkingdom: Protozoa

Phylum: Sarcomastigophora

Subphylum: Mastigophora

Class: Zoomastigophora

Order: Kinetoplastida

Suborder:Trypanosomatina

Family: Trypanosomatidae

Genus: *Leishmania*

Subgenus : *Leishmania*

Subgenus : *Viannia*

Classification Scheme of *Leishmania* spp. (Odiwuor *et al.*, 2011)

1.2.4 Life cycle of parasite

There are two main life cycle stages, 1st amastigotes, the disease causing stage of the parasite, are intracellular, non-motile parasites that reside and replicate within the mature phagolysosome compartment of macrophages of the mammalian host that include; Rodents, Dogs,Gerbils,Hyraxes,Bats, Porcupines, Opossums,Sloths, Primates, Foxes and Anteaters (WHO, 2010), 2nd The promastigotes are flagellated forms that develop in the sandfly vector (Mougneau *et al.*, 2011).

When the sandfly takes a blood meal, amastigotes, which are found in macrophages in the skin of the host, are taken up by the fly, Parasites that are present within the liver or spleen are not accessible to the sandfly, the development of the parasite within the vector is triggered by an increase in pH and a decrease in temperature in the sandfly midgut (Bates, 2007), and takes between six and nine days, depending on the species (Kamhawi, 2006).

There are at least five developmental stages, which occur within the sandfly vector each with morphological and functional variations that occur to maximise survival in the sandfly (Gossage *et al.*, 2003, Vermeersch *et al.*, 2009). Firstly, the amastigote differentiates into weakly motile procyclic promastigotes that are relatively resistant to sandfly digestive enzymes. These replicate in the blood meal, which is separated from the midgut by a peritrophic matrix (Kamhawi, 2006). After a few days, replication of the parasites is reduced and they differentiate into strongly motile nectomonad promastigotes, which accumulate at the anterior end of the peritrophic matrix, and whose function is to break out of the PM in a process that is facilitated by a parasite secretory chitinase (Bates, 2007). Nectomonads migrate towards the anterior thoracic midgut with some of them attaching themselves to the microvilli of the midgut epithelium until they reach the stomodeal valve at the junction between the foregut and midgut, it is the ability of these nectomonads to attach to the epithelium cells and avoid expulsion during defecation that allows the infection to persist beyond the bloodmeal phase (McConville *et al.*, 2007). At the stomeadal valve, the nectomonads differentiate into replicating leptomonad forms, which secrete promastigote secretory gel , a parasite derived product that is important for establishing infection (Bates, 2007). Some

leptomonads attach themselves to the surface of the stomodeal valve and differentiate into haptomonad promastigotes, and others differentiate into metacyclic promastigotes, the mammalian infective form, these highly motile metacyclic promastigotes have a small body with a long flagellum and are resistant to complement mediated lysis, allowing their survival within the mammalian host while they differentiate into amastigotes (Alrajhi, 2011).

Metacyclic promastigotes are injected into the skin of the mammalian host by the sandfly Once transmitted, the metacyclic promastigote infects local tissue and are phagocytosed either directly by macrophages or by neutrophils, the first cells to be recruited to the site of infection , which undergo apoptosis after 2-4 days and are subsequently phagocytosed by macrophages (Laskay *et al.*, 2008). Immediately after phagocytosis, the metacyclic promastigotes are located within macrophage derived phagosomes that fuse with endocytic organelles resulting in the formation of an acidic compartment known as the parasitophorous vacuole (Ponte-Sucre, 2003). Within the parasitophorous vacuole the metacyclic promastigotes differentiate into replicative amastigotes that periodically escape from the host cell to reinfect other phagocytic cells (macrophages or dendritic cells) or non-phagocytic cells (fibroblasts), (McConville *et al.*, 2007)

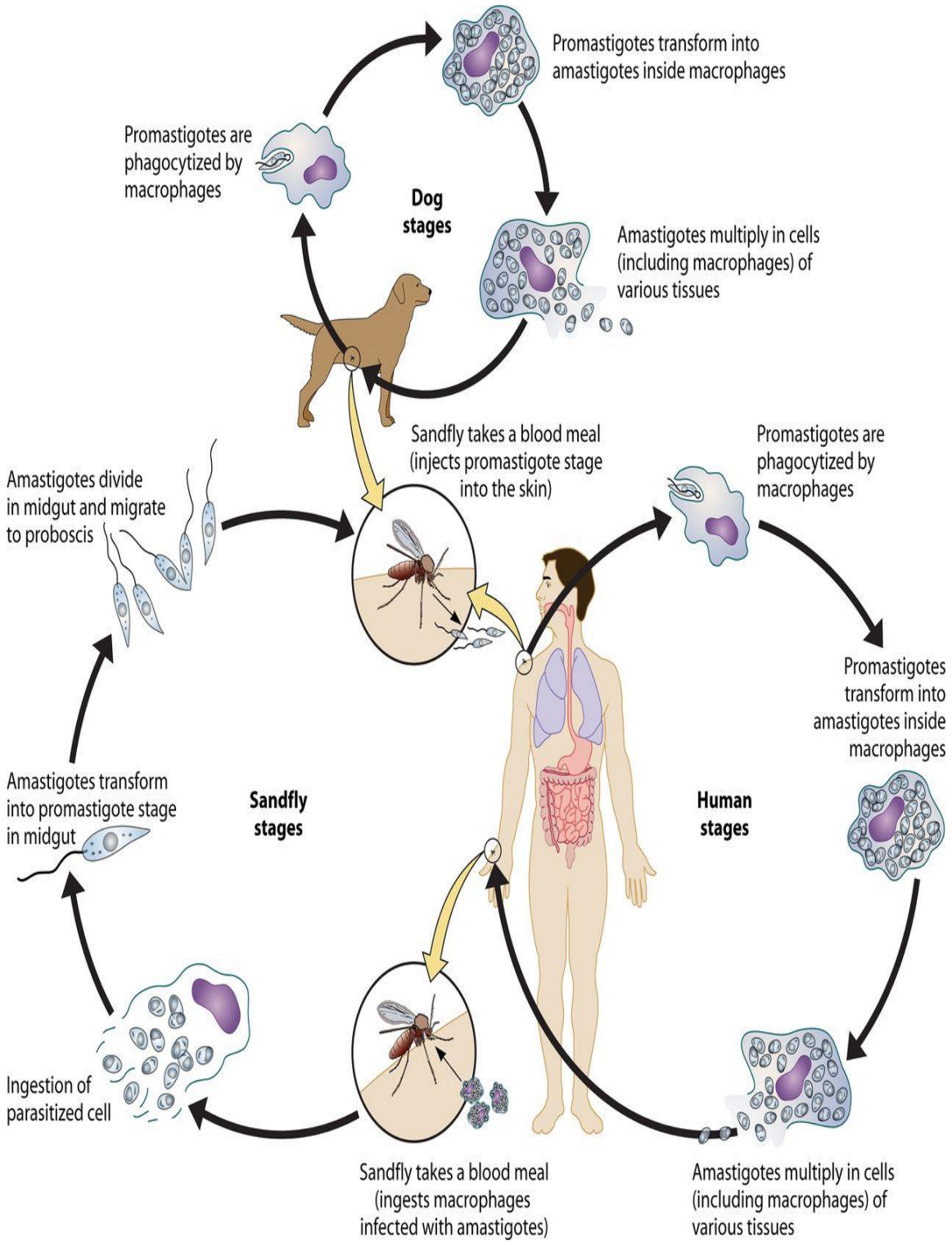

Figure (1-1): Life cycle of *Leishmania* parasites have a dimorphic life cycle alternating between the vector and the mammalian host by (Akilov *et al.*, 2007).

1.2.5 The Vector

Vectors of leishmaniasis are sand flies, small blood sucking insect in the order *Diptera,* family *pyschodidae,* sub family *phlebotominae,* genus, *phlebotomus*, there are approximately 700 species of phlebotominae sand flies divided among 6 genera, of which only two, i.e. *Phlebotomus* in the old world and *Lutzomyia* in the new world (NW) are medically importance (Homsi and Makdisi, 2010, Sharma and Singh, 2008). The *Phlebotomus* has a life span 30 days at the most and its fly a short distances only (90-150 meters), the activity period of adult sand flies is typically seasonal, because seasonal phenomena are also very sensitive to variations in temperature, phenological observations may provide high resolution of ongoing climate changes in addition to geographical dispersion parameters It is active during the first hours of the night and very often in a hot weather until morning, as they fly at night to avoid heat (Campino *et al.*, 2006).

Sand flies are 2-3 mm varicolored insect, differentiated by pointed velvet wings, arched back and big dark eyes, they are abundant all year round the tropical areas and during the summer in temperate zones, 35 of 600 species have proved to be vectors of human leishmaniasis (Kamhawi, 2006). The haematophagous sand fly female's feed all through the night but especially at dusk, however they can also bite during the day when disturbed in their resting places, female sandflies require blood to obtain the necessary proteins for egg development (Sharma and Singh, 2008).

The insect sucking the leishmanial parasite with the during blood meal from an infected host, many species of sandfly are unable to support the growth of *Leishmania*m (Peckova, 2005). The main factors that influence the ability of a given species of sandfly to act as a vector are, first, resistance by the parasite to the action of digestive enzymes produced in the midgut of the fly; secondly, the presence on the inner surface of the gut of the vector of the appropriate lipophosphoglycan-binding sites that match the lipophosphoglycan on the surface of the promastigotes, enabling them to attach to the inner surface of the sandfly gut; and, thirdly, completion of the development of the parasite to produce metacyclic promastigotes, the only form of the

parasite that can survive in the vertebrate host (Kamhawi, 2006, Wasserberg *et al*., 2003).

The main insect vector for transmission of *L. major* is the sand fly species *Phlebotomus papatasi* (El-Badry *et al.,* 2008). USA military medical army were conducting their study in Iraq on sand fly during April-September 2003, approximately 65,000 sand flies were collected, about half of, which fond that female, taxonomic analysis indicated that the most common species in the *Phlebotomus* genus were *P. papatasi, P. alexandri* and *P. sergenti*, all of which can be vectors of leishmanial parasites, approximately 24000 females *Phlebotomus* sand flies, in pools of one to 15 flies, had tested for infection by using polymerase chain reaction (PCR) technique (Control and Prevention, 2003).

1.2.6 Transmission of *Cutaneous Leishmania*

Transmission of *Leishmania* occurs between female phlebotomine sandflies and mammalian hosts, about 30 species of phlebotomine sandflies (family Psychodidae, subfamily *Phlebotominae*) are proven vectors (Desjeux, 1996, Bates, 2007, Alavinia *et al.,* 2009).Worldwide, vector-borne transmission is the most common mode of transmission, transmission can occur when infected sand flies are crushed into the skin wounds or mucous membrane (Singh, 2006).

There are other ways of transmission of the parasite , such as transmition from man to man or animal to man by direct inoculation of amastigotes, Person-to-person transmission also sometimes occur by direct contact (Paniker, 2013).

1.2.7 Virulence Factors *Cutaneous Leishmania*

Leishmania parasite has many virulence factors, which has play an important role in their pathogencity. A number of molecules has been implicated in parasite virulence like the glycolipid lipophosphoglycan (LPG) and the surface protease (gp63) glycoprotease or Leishmanolysin which protect *Leishmania* from immune response and are ability in cleave complement and facilitates parasite uptakes to inhibit host cell lysosome activity (Beverley and Turco, 1998).

A. Lipophosphoglycan (LPG):

An important determinant of *Leishmania* is infectivity the surface macromolecule lipophosphoglycan (LPG). This is the major macromolecule on the surface of *Leishmania* LPG consist of a polymer of units attached by a polysaccharide, disaccharide repeat units core to a novel lipid anchor. LPG has been implicated in many steps required for establishment of macrophage infection and for the survival in insect vector (Beverley and Turco, 1998).

B. Glycoproteinase (GP63) or Leishmanolysin:

The major surface glycoprotein of *Leishmania* promastigote of L. major is a Zn metalloprotase of (63kp), which is encoded by family of 7 genes Gp63 has the potential to cleave immunglobulins, complement factors and lysosomal proteins on other hand gp63 also helps the parasite to enter in the host cells and for it survival (Chaudhuri and Chang, 1988).

1.2.8 Pathogenesis of Cutaneous Leishmaniasis

When the promastigotes of *L. major* or *L. tropica* introduced into the skin of the person at the time an infected sand fly is sucking a blood meal, the parasite are engulfed by local white cells in neutrophlilic leukocyte phagocytosis is usually successful, but in macrophages the parasites round up to the *Leishmania* form, multiply and on escape from rupturing macrophages, while neutrophils or polymorphonuclear neutrophils (PMNs), the first cells to migrate to the site of infection, or injured tissues function as a primary effecter, or phagocytic cells, phagocytosing *Leishmania* (Sacks *et al.*, 1995). Phagocytosed neutrophils start secreting the chemokines like IL-8 essential to bring the more neutrophils at the site of infection, two to three days later the second wave cells monocytes / macrophages enters the site of infection, after infection with CL, the chemokines of *Leishmania* parasite the functional homologues of IL-8 are found in high level in the skin (Müller *et al.*, 2001).

Leishmania promastigotes have a dense covering of glycocalyx which is attached to the plasma membrane with the help of GPI (glycophosphoinositol), Lipophosphoglycan (LPG) is an important molecule which promotes the

infectivity of the parasite in mammalian host, it is a long phosphoglycan molecule having repeated sugar residues, glycan side chains and a capping oligosaccharide, it shows a great variability in its structure which helps in immune evasion, another important surface glycoprotein is zinc metalloproteinase(GP-63) which acts as a virulence factor (Gomez *et al.*, 2009). As mechanism of parasitism, it's beneficial to *Leishmania* to escape from other cells of the immune system, for example, the leukocytes before establishing the infection completely and neutrophils were found to be beneficial for parasite survival in the infected tissue, it has been shown that *Leishmania* promastigotes can induce the migration of human PMNs by releasing a factor (Leishmania Chemotactic Factor LCF), which potent chemotactic activity on neutrophils, but not other leukocytes, such as monocytes, or Natural killer cells (Van Zandbergen *et al.*, 2002)

The outcome of infection in each type of leishmaniasis depends on the complex interaction of virulence factors and host immunologic responses, CL caused by *L. tropica* and *L. major* are indistinguishable on clinical bases as both erupt in the same way, the size of the lesion ranging from a few millimeters to 4 centimeter or more (Cruz *et al.*, 2006)

The parasite leishmaniasis also capable of infecting fibroblasts, which may serve as a reservoir of infection repeated (Hallé *et al.*, 2009), note that the parasites undergoing of incubation for a period of weeks or months before they show all the skin develops at the site of the bting and become red color after that ulcerate and possibly get injury secondary bacteria in many species, for example, *L. major* often heal the lesion spontaneously with scarring atrophic (David and Craft, 2009), and is formed leaching in disease leishmaniasis in the skin site of the bite , and contains the macrophages in the first place , as well as the cells of lymphocyte cells and plasma (Douba *et al.*, 1997).

1.2.9 Clinical forms

Despite their similarities, pathogenic species of *Leishmania* cause different forms of the disease including: cutaneous leishmaniasis, Mucocutaneous Leishmaniasis, Diffuse Cutaneous Leishmaniasis and Visceral Leishmaniasis (Berman, 2005). Furthermore, possibly due to the interactions between the vector, the parasite, the host and the environment, basic clinical manifestations within the different forms of the disease vary by endemic region (Organization, 1990, Berman, 2005, Alrajhi, 2011).

1.2.9.1 Cutaneous Leishmaniasis (CL)

Infection with any of several species can produce cutaneous leishmaniasis (Nezhad *et al.*, 2012) According to the causative parasite species, CL can be classified into Old World and New World CL, the former is primarily due to *L.major* (known as rural or zoonotic CL-ZCL), *L.tropica* (urban or anthroponotic - ACL), and *L. (L) aethiopica* but also due to *L.infantum* and *L. donovani*. New world CL is caused by *L. mexicana*, *L. (L)amazonenesis*, *L. braziliensis*, *L. (V) panamensis*, *L. (V) peruviana*, and *L. (V)guyanensis* and also *L. chagasi* (Organization, 1990, Murray *et al.*, 2005, Alimoradi *et al.*, 2009, Nezhad *et al.*, 2012). CL due to *L. major* tends to heal spontaneously and without systemic complication or dissemination to other sites, individuals with history of CL are usually protected against future infection (Organization, 2002, Khamesipour *et al.*, 2005). In infection with *L. major* and *L. tropica*, an erythematous papule at the site of sandfly bite is normally the initial stage of the lesion later developing into painlessnodule which typically ulcerates in1-3 months (Dowlati, 1996, Murray *et al.*, 2005).

1.2.9.2 Mucocutaneous (Mucosal) Leishmaniasis (MCL)

Mucocutaneous Leishmaniasis is a potentially life threatening, degenerative inflammatory form of leishmaniasis of the nasal and oral mucous membranes, extending to the pharynx, the appearance of the disease resembles leprosy and it is associated with the same stigma, diagnosis and treatment is difficult (Evans,

1993). In the Old World MCL is rarely seen and is usually due to *L. tropica* and *L. aethiopica* (Organization, 1990, Kharfi *et al.*, 2003). However, cases associated with other species have also been reported (Zijlstra and El-Hassan, 2001, Yaghoobi Ershadi *et al.*, 2003, Guddo *et al.*, 2005). In Sudan, MCL is rarely observed as an aftermath of VL (unlike PKDL) (Zijlstra *et al.*, 2003).

Mucocutaneous Leishmaniasis in the New World is primarily due to *L. braziliensis*, *L. panamensis* and *L.guyanensis* (Organization, 1990, Weigle and Saravia, 1996). About 1-10% of CL patients in the New World develop mucosal manifestation within 1-5 years of healing (Murray *et al.*, 2005). CL in the New World is associated with the risk of developing mucosal infection if the treatment is delayed due to the causing parasite genus not being rapidly identified (Peyron-Raison *et al.*, 1996).Mucosal leishmaniasis begins with erythema and ulcerations at the noes, proceeding to nasal septum perforation and destructive inflammatory lesions, the latter can obstruct the pharynx or larynx and produce remarkable disfigurement(Murray *et al.*, 2005).

1.2.9.3 Diffuse Cutaneous Leishmaniasis (DCL)

Diffuse Cutaneous Leishmaniasis is a rare form of the disease caused by various species, in the Old World the primary causative agent is *L. aethiopica*, and in the New World the *L. Mexicana* complex and specifically *L. amazonensis* (Organization, 1990, CORBETT *et al.*, 2005). In DCL patients, the absence of delayed type hypersensitivity (DTH) is associated with widespread plaques, papules or multiple nodules especially on the face or the limbs and could resemble leprosy, a DTH normally is observed after cure, the disease does not heal spontaneously and tends to recur (Organization, 1990, Silveira *et al.*, 2005).

1.2.9.4 Visceral Leishmaniasis (VL)/Kala-azar

Kala-azar is primarily caused by members of the *L. donovani* complex (*L. donovan*i in Sudan and India, *L. infantum* in other parts of the Old World and *L. chagasi* in the New World), some cases of VL in humans and dogs are due to *L. tropica* (Sacks *et al.*, 1995, Lemrani *et al.*, 2002, Mohebali *et al.*, 2005,

ALBORZI *et al.*, 2006). Because of its associated complications and severity, VL has more significant public health consequences than other forms of leishmaniasis, children are especially susceptible, the spleen, the liver, the mucosa of the small intestine, the bone marrow, lymph nodes and other lymphoid tissues are heavily parasitized (Organization, 1990, Murray *et al.*, 2005). Symptoms include fever, weight loss, splenomegaly, hepatomegaly, lymphadenopathy, cachexia, nausea and it is typically fatal if not appropriately treated (Zijlstra and El-Hassan, 2001, Seaman *et al.*, 1996)

1.2.9.5 Post Kala -azar Dermal Leishmaniasis (PKDL)

Post Kala -azar Dermal Leishmaniasis is the dermal complication developed in VL patients after clinical cure and is characterized by a macular, maculopapular, and nodular rash which normally starts around the mouth (Zijlstra *et al.*, 2003).The rash is typically the only complication in PKDL. Chronic PKDL is the source of considerable morbidity, a PKDL is mainly seen in Sudan and India (about 50% of the Sudanese and 5-10% of Indian VL patients) and is restricted to infection with *L. donovani* (Zijlstra and El-Hassan, 2001, Musa and Khalil, 2015) (Zijlstra *et al.*, 2003)PKDL follows within 6 months of VL cure in Sudan and 2 years or longer in India, while the Indian PKDL needs treatment, the Sudanese form tends to self heal within 1 year post onset, chronic cases, however, need treatment and are hard to cure (Zijlstra and El-Hassan, 2001, Musa and Khalil, 2015).PKDL is believed to play a role in the transmission of kala azar by providing the human reservoir in the skin of the PKDL patient despite recovery from the symptoms of the visceral disease (Zijlstra *et al.*, 2003).

1.2.10 Immunology of Cutaneous Leishmaniasis

1.2.10.1 Humoral Immunity in Cutaneous Leishmaniasis

The demonstration of antibody in cutaneous leishmaniasis has been more problematic than that of cellular response (Ajdary *et al*., 2000). The humoral immune response in cutaneous leishmaniasis of the Old world that causes from *L. tropica and L.major* shall be weak in spite of the possibility of formation of antibodies quality (Ashford, 1996). the antibodies has been observed a minor role in determining the course of cutaneous leishmaniasis **(Alexander *et al.*, 1999).**

1.2.10.2 Cellular Immunity in Cutaneous Leishmaniasis

Leishmaniasis, like leprosy, is a spectrum of diseases ranging from self - healing cutaneous lesions to severe, non-healing disseminated cutaneous, mucocutaneous, or visceral infections, immunity in leishmaniasis is generally considered to be predominantly cell-mediated (Roitt *et al.*, 2001, Postigo, 2010).*Leishmania* parasites escape from the humoral immune response by hiding as an amastigote inside the phagolysosomes of the host macrophage, since the intracellular parasite is not exposed to the host's humoral response, circulating antibodies have no effect on the infection and may even be harmful (Ravichandran and Lorenz, 2007). Initiations of infection start when infected female of sand fly inoculate contains live, apoptotic or dead promastigotes and salivary components which play critical roles in shaping the host’s immune response (van Zandbergen *et al.*, 2006, Mougneau *et al.*, 2011). Following the injection of metacyclic promastigotes into the skin, they interact with multiple cell types (neutrophils, macrophages, dendritic cells, keratinocytes, and langerhans cells) where they then transform into intracellular amastigotes (Mougneau *et al.*, 2011). In particular, phagocytosis of parasites by macrophages induces the release of multiple chemoattractant factors such as MCP-1 and CXCL1, leading to recruitment of monocytes and neutrophils (Racoosin and Beverley, 1997). Both *in vitro* and *in vivo* studies have shown that neutrophils influence the outcome of *Leishmania* infection through several

ways including intracellular killing after phagocytosis, extracellular killing through the release of neutrophil extracellular traps (NETS),and through cooperation with macrophages (John and Hunter, 2008, Mougneau *et al.*, 2011).

In addition, the uptake of promastigotes by neutrophils inhibits cellular apoptotic signals thereby prolonging their lifespan (Aga *et al.*, 2002). These "long-lived" neutrophils become transiently unable to kill *Leishmania* and acts to dissemination of parasites to other cells, particularly macrophages (Aga *et al.*, 2002, van Zandbergen *et al.*, 2004). Although neutrophils are the most abundant cells at the infection site during the first few hours to days, the pattern, and magnitude of monocyte influx may be more important in shaping the outcome of infection. About 1 week after infection, monocytes invade the infection site (León *et al.*, 2007), where they differentiatein to monocytes-derived dendritic cells (mDCs) that take up parasites (Mougneau *et al.*, 2011). Via a TLR-9-dependent pathway, mDCs play a vital role in the production of IL-12 and Type 1 IFNs, leading to activation of Natural Killer (NK) cells, the production of Interferon gamma (IFN-γ), where these cells are the primary source of the IFN-γ that responsible for macrophage activation and prevent the spread of the parasite and its growth (Liese *et al.*, 2008). Macrophage produce IL-1 which activate T-helper cells were mediate activation of macrophages to produce NO, resulting in killing or control *L. major* parasites and the secretion of TNF- α by macrophages is sufficient to mediate production of NO and killing of *L. major* parasites (Belosevic *et al.*, 1988). The T-helper cells I ($T\text{-}h_1$) produce "interferon- gamma" (IFN-γ) which enhances the phagocytic actions of the macrophages and "interlukine -2" (II-2) which mediated the activities and aggregation of macrophages ,while the ($T\text{-}h_2$) produce IL-4 ,IL -5 ,IL- 6 ,IL-10 and IL-13. Lymphokines affect macrophages and enhance the process of parasite destruction inside the cells (Basu and Ray, 2005)

In general, DCs are essential for the initiation and regulation of anti-*Leishmania* adaptive immunity (Kane and Mosser, 2000).

The tissue resident macrophages are the definitive host cells for parasite survival and replication. In addition, classical activation of infected macrophages by IFN-γ and tumor necrosis factor (TNF) stimulates the production of inducible nitric oxide (NO), an enzyme that catalyzes l-arginine to generate nitric oxide (Liew *et al.*, 1990). NO is a powerful cytostatic and cytotoxic molecule and plays a major role in killing many intracellular parasites, including *Leishmania,* thus, in leishmaniasis, macrophages play a dual role; they represent an important cell population responsible for killing of the parasites and also the major site of parasite replication

After phagocytosis, macrophages present the parasite antigen in context of Major Histocompatiblity Complex (MHC) class II molecules to T-cells (Antoine *et al.*, 1999). In CL infection amastigote interfere with the up regulation of MHC class II by IFN-γ on the transcriptional level and in recurrent infection by CL, cytotoxic T-cells CD8 interact with MHC class I (Kedzierski, 2010)

1.2.10.3 The role of Cytokines in pathogenicity of Cutaneous Leishmaniasis

Cytokines are small proteins, signaling molecules, approximately (8-80kDa) that usually act in an autocrine or paracrine manner. They are extremely potent, acting at Picomolar and sometime even Femtomolar levels 1 picogram (10^{-12}g), was able to protect one million cells from ten million virus particles in tissue culture experiment. Cytokines function as part of a larger inter-related system of proteins and signaling cascades, the cytokine network, direct cell-cell interaction and local production if soluble mediators, control communication between cells of the immune systems. Cell – cell interaction involves a number of different classes of molecules like Major Histocompatibility Complex (MHC), accessory molecules, integrins, co-stimulatory molecules and membrane forms of cytokines (Al-Obaidi, 2007). In leishmanial infection, after engulfment of parasites by macrophage, macrophage produces cytokine IL-1 to activate T-cell. The T-cell which, subpopulation to Th-1 and Th-2 cells which

produce different kinds of cytokines, Th-1 produce IL-2, IFN-γ and TNF-α, IFN-γ activate macrophage to produce (NO) by activation of enzyme like nitric oxide synthase for elimination of intracellular parasites (Locksley *et al.*, 1987). TNF-*α* produced by mononuclear phagocyte, fibroblast, B and T cell, macrophages participate in production of TNF- α, T cell induce macrophages to produce nitric oxide(NO), which cause control or killing parasites, TNF- α that secretion by macrophages also mediate in secretion of nitric oxide as well as activation of macrophages and parasite killing (Belosevic *et al.*, 1988).

IL-2 is mainly produced by helper T-cells that stimulate both helper and Cytotoxic T-cells to grow(Alexander and Russell, 1992). Th-2 cells secrete IL-4, IL-5 and IL-10, Th-2 immune response limits the action of Th-1 via IL-10 and IL-4, which deactivate macrophage, helping intracellular parasite, growth and disease progression (Liew *et al.*, 1997). (Birnbaum and Craft, 2011).

IL-1β is primarily produced by activated macrophages as well several cells include the mononuclear endothelial, keratinocytes, astrocytes, synovial cells, glial cells, osteoblasts, neutrophils , and numerous other cells, there are variant agent mediate stimulation of IL-1β production, like endotoxins, microorganisms, antigens as well as other cytokines (Awasthi *et al.*, 2004). IL-1β is well known that cytokines are considered to play a key role in the inflammation process, it is important proinflammatory cytokine that on the one hand mediate in secretion of nitric oxide as well as activate monocytes, macropages, and neutrophils, and on the other hand induce Th1 and Th17 adaptive cellular responses (Kim and Amar, 2006, Netea *et al.*, 2010).

1.2.11 The role of MIF in pathogenicity of Cutaneous Leishmaniasis

Macrophage migration inhibitory factor (MIF) is considered to be one of the first cytokines to be discovered, a MIF described an activity elaborated by activated lymphocytes that arrested the random movement of monocytes/macrophage in the late 1950s (Leng and Bucala, 2006). MIF was originally described in 1966 as a T-lymphocyte-derived ‘activity’ that inhibited

the random migration of macrophages (Baugh and Donnelly, 2003). In 1991, a search for new regulators of inflammation led to the re-discovery of MIF as a molecule released, similar to a hormone, by cells of the anterior pituitary gland after exposure to the lipopolysaccharide toxin (Calandra and Roger, 2003).

MIF is produced and stored in the pituitary gland and released systemically upon physiological stress , it constitutes about 0.05% of the total protein amount in the pituitary gland and it is stored in three different cell types, either alone or together with adrenocorticotropic-releasing hormone or thyroid-stimulating hormone, a Macrophages also store large quantities of MIF, similar to cells of the pituitary gland and T cells, and they play an important role in the local secretion of MIF during the innate immune response (Denkinger *et al.*, 2004).

Macrophage migration inhibitory factor is produced and secreted primarily by immune cells, such as lymphocytes, dendritic, neutrophils and pituitary cells (Bacher *et al.*, 1997, Baugh and Bucala, 2002). MIF secretion is tightly regulated by stress and immune stimuli, including endotoxins, inflammatory cytokines as IFN-γ, TNF-α and glucocorticoids (Bacher *et al.*, 1997, Bernhagen *et al.*, 1998, Sprong *et al.*, 2007). Once secreted, MIF exhibits a broad range of immune and inflammatory activities, including the induction of inflammatory cytokines such as TNF-α, IFN-γ, IL\-1β, IL-12, IL-6 and IL-8, among others (Calandra *et al.*, 1994, Calandra *et al.*, 1995, Bozza *et al.*, 1999, Mitchell *et al.*, 2002).

MIF favors the expression of the Toll-like receptor 4 (TLR4) gene, which encodes the signal-transducing element of the lipopolysaccharide (LPS) receptor complex (Calandra *et al.*, 1998, Calandra and Roger, 2003). Through the activation of transcription factors of the ETS family (Roger *et al.*, 2005). In addition, MIF counter-regulates the immunosuppressive effects of glucocorticoids (Petrovsky *et al.*, 2003). it sustains macrophage proinflammatory functions by inhibiting p53

(Hudson *et al.*, 1999). MIF also promotes the migration and recruitment of immune cells inducing the expression of chemokines a monocyte chemoattractant protein (MCP-1), and adhesion molecules as intercellular adhesion molecule (I-CAM-1) and vascular cell adhesion molecule as V-CAM-1 (Hou *et al.*, 2001, Ferro *et al.*, 2008)

Previous studies have shown that MIF cytokin plays a critical role in host resistance to Leishmaniasis, were 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* (Jüttner *et al.*, 1998). In the mammalian host, *Leishmania sp*. is an obligatory intracellular parasite infecting mainly macrophages, parasite killing requires macrophage activation with ensuing NO and ROS production (Kaye and Scott, 2011). Addition of MIF to macrophage cell cultures results in increased *L. major* elimination , though the MIF concentration required to reduce *L. major* burden in macrophages is high 1 μg/mL (Jüttner *et al.*, 1998).

1.2.12 Association between MIF-173G>C polymorphism and susceptibility to cutaneous leishmaniasis

Finally cDNA was cloned in 1989 in human, MIF genomic localization to chromosome 22q11 later mapped, studies using pure recombinant human MIF and specific neutralizing antibodies have shown MIF to be a potent pro-inflammatory cytokine, and a key modulator of immune and inflammatory responses (Donn and Ray, 2004). The human MIF gene is a small gene less than 1 kb, *MIF* gene is short, composed of 3 exons of 205, 173, and 183 bp and 2 introns of 189 and 95 bp (Donn and Ray, 2004, Renner *et al.*, 2005),figure(2-2). In the human MIF genome, D-dopachrometautomerase (DDT) is the only gene with striking homology to MIF, both DDT and MIF genes are located in close proximity to each other on chromosome 22. In mammalian MIF show 90% homology (Ma *et al.*, 2013).

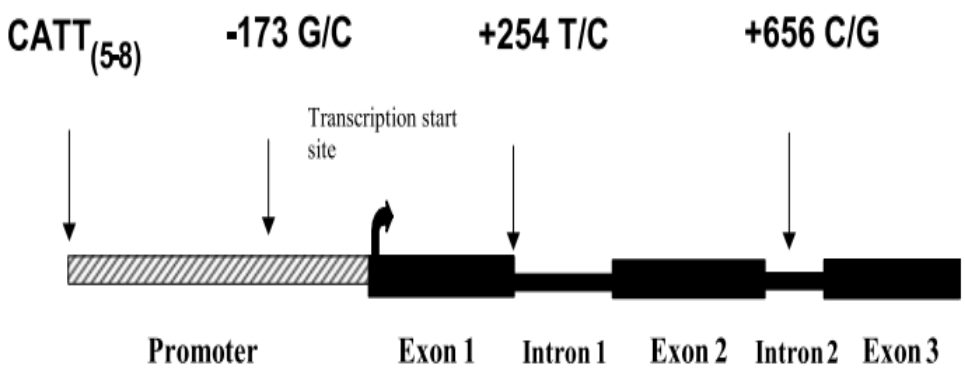

Figure (1-2): Structure of the human macrophage migration inhibitory factor *(MIF)* gene. The 3 exons, 2 introns, Arrows indicate the positions of the 3 single-nucleotide polymorphisms and of the CATT tetranucleotide microsatellite (Donn and Ray, 2004)

Several polymorphisms of *NRAMP1* gene have been evaluated in clinical studies, the majority of previous investigations have focused on the association between *NRAMP1* gene and diseases such as Crohn's disease (Gazouli *et al.*, 2008) ,lung tuberculosis(Medapati *et al.*, 2017), chronic periodontitis (Kadkhodazadeh *et al.*, 2016), leprosy(Brochado *et al.*, 2016), rheumatoid arthritis (Yang *et al.*, 2000), visceral leishmaniasis (Ortiz-Flores *et al.*, 2015), and Cutaneous leshmaniasis (Sophie *et al.*, 2017) For Leishmaniasis, a study by Satoskar e*t al* ,showed that *MIF* gene-deficient mice ,were highly susceptible to cutaneous Leishmania major infection, developing severe skin lesions late after infection exhibiting greater parasite burdens than wild-type mice (Satoskar *et al.*, 2001).

The polymorphisms within the promoter region of cytokines (e.g., TNF, IL-1, IL-4, and IL-10) and of cytokine or chemokine receptors (e.g., IL-7R, IFN-gR, IL-12R, and CCR5) have been associated with mostly enhanced, but sometimes also reduced predisposition to inflammatory and infectious diseases, by analogy with other cytokines and given the role of MIF in the control of inflammation and innate immune responses to microbial invasion, it was reasonable to postulate that mutations in the human *MIF* gene would

predispose affected hosts to altered susceptibility to or severity of inflammatory or infectious diseases, indeed, loss-of-function MIF mutations may affect the capacity of the host to mount inflammatory and innate immune responses, alternatively, gain of-function *MIF* mutations may predispose the host to more severe inflammatory and immune reactions (Renner *et al.*, 2005)

Previous study in brazil (2013), refer to found associated between MIF G-173C polymorphism and cutaneous leishmaniasis , were suggest that the C allele is associated with lower levels of MIF, and this decreased production may be associated with leishmaniasis susceptibility (Covas *et al.*, 2013).

1.2.13 The role of NRAMP1 in pathogenicity of Cutaneous Leishmaniasis

Homologs of natural resistance-associated macrophage protein (NRAMP) or solute carrier 11 (SLC11), conserved in eukaryotes and bacteria, form a family of proton-coupled transporters that maintain divalent metal including ($Me2^+$, $Mn2^+$, $Fe2^+$, $Co2^+$, and $Cd2^+$) homeostasis (Mackenzie and Hediger, 2004, Courville *et al.*, 2006). In a resting macrophage, the protein encoded by *NRAMP1* is assembled into the membrane of late endosome, while following phagocytosis it is relocated to the membrane of phagosome (Canonne-Hergaux *et al.*, 2002, Kim *et al.*, 2003). It defends against various intracellular pathogens by transfers divalent metal ions across the phagosomal membrane and might be a critical factor for resistance to some microbial infections including *Salmonella typhimurim*, *Leishmania sp* and *Mycobacterium bovis* (Vidal *et al.*, 1995, Blackwell *et al.*, 2003). *NRAMP1*influences a variety of antimicrobial responses of a macrophage, including induction of radical oxygen and nitric oxide intermediates, production and activation of various pro-inflammatory cytokines (TNF-α and interleukin-1β) and regulation of anti-inflammatory cytokine IL-10 as well as nitric oxide synthase (NOS), major histocompatibility complex (MHC) class II molecules, nitric oxide (NO) release, L-arginine flux, oxidative burst, and tumouricidal as well as antimicrobial activity (Blackwell *et al.*, 2000, Courville *et al.*, 2006).

NRAMP2 also known as *(SLC11A2*) expresses specifically at the apical membrane of epithelial cells or ubiquitously in recycling endosomes, plays an important role in regulating body iron levels and transports abroad range of divalent metal ions $Cu2^{+}$, $Mn2^{+}$, $Co2^{+}$, and $Pb2^{+}$ (Touret *et al.*, 2003). Recently, it was reported that Nramp are cellular receptors for Sindbis virus in both insect and mammalian hosts (Rose *et al.*, 2011), but this issue was elusive according to other report (Stiles and Kielian, 2011)

1.2.14 Association between *NRAMP1* (RS17235409) Polymorphism and susceptibility to Cutaneous Leishmaniasis

There are two *NRAMP1* genes that are associated with diseases in vertebrates, a *NRAMP1* (also called *SLC11A1*), in Human *NRAMP1*gene is located in the chromosome region 2q35, containing 16 exons (Taype *et al.*, 2006). *NRAMP1* protein encoded by *NRAMP1*(SLC11A1) gene which located in the chromosome region 2q35, and mutations in the *NRAMP1* gene result in a non-functional or unstable protein, resulting in an increased proliferation of parasites in the macrophage (Fritsche *et al.*, 2008). There is a correlation between *NRAMP1* mutations and susceptibility to a cluster of antigenetically unrelated intracellular pathogens including HIV21, *Mycobacterium leprae*, *M. tuberculosis* and *Leishmania* (Blackwell *et al.*, 2000, Abel *et al.*, 1998). Nucleotide analysis of SLC11A1 gene in inbred mice strains revealed a single non-synonymous amino acid substitution of glycine to aspartic acid (Gly169Asp), mice with this mutation were unable to produce a functional protein which made them susceptible to intracellular parasites (Malo *et al.*, 1994). Macrophages from mice with a mutant NRAMP1 protein have deficiencies in antigen presentation, while the presence of a NRAMP1 protein improves the function of macrophages in susceptible mice to mycobacterium (Anggraini *et al.*, 2010).

Among all the known NRAMP1 polymorphisms, a number of variants are located in the coding regions, some of which are missense mutations while

others are silent substitutions, the missense mutations are an aspartic acid-to-asparagine change at codon 543 in exon 15 (RS17235409) and a single-base substitution resulting in an alanine-to-valine change at codon 318 in exon 9 (A318V polymorphism), these missense mutations are important because they may affect the function of NRAMP1 protein (Mohamed *et al.*, 2004, Nugraha and Anggraini, 2011). Host genetic factors might play an important role in development of the clinical manifestations, especially in CL (Peacock *et al.*, 2007).

Genetic analysis and sequencing have identified multiple genetic polymorphisms within the human homologue SLC11A1 , however, these genetic variations when studied with respect to susceptibility to intracellular Leishmania protozoa reveal an inconsistent pattern across different regions of the world (Nugraha and Anggraini, 2011). Some studies indicated that genetic variations of *NRAMP* 1(RS17235409) might be associated with susceptibility to CL infection (Fattahi-Dolatabadi *et al.*, 2016), while other study shows that genetic variations in the candidate gene SLC11A1 do not affect susceptibility to cutaneous leishmaniasis in the sample population from Pakistan (Sophie *et al.*, 2017).Therefore, the current study was designed to determine and analyze the genetic variation(s) in SLC11A1 (RS17235409) and investigate if this polymorphism is associated with cutaneous leishmaniasis.

1.2.15 Treatment of Leishmaniasis

Cutaneous Leishmaniasis is considered as a self-healing disease, rapid treatment remains important to avoid unattractive scars and parasite dissemination (i.e. nodular lymphangitis and MCL), at present, no single optimal treatment exists for CL (Blum *et al.*, 2004, Berman, 2005).

The objective of treatment of CL is to improve the cosmetic effect of scar and to shorten the duration of the disease, there are many factors affect the treatment of CL such as clinical presentation, duration of disease, number, size and site of lesion and presence of secondary infection, a Pentavalent antimony,

stibogluconate (Pentostam) remains the drug of choice the treatment of all types of Leishmaniasis, it is thought to work by inhibition of adenosine triphosphate synthesis (ATP) (Santos *et al.*, 2008, Van Thiel *et al.*, 2010). Pentostam dose per lesion is 0.2-0.4 ml (100 mg/ml) or 15-20 mg /kg/day for 15-20 times every other day,more or less depending on the lesion and its response to treatment (Croft and Yardley, 2002, Herwaldt and Magill, 2010). Pentostam can be given intramuscularly (IM) or intravenously (IV) depending on the progress and stage of the lesion (Santos *et al.*, 2008). Though Pentostam are effective against some forms of leishmaniasis, increasing levels of resistance have been reported (Ouellette *et al.*, 2004).

1.2.16 Prevention and Control

As described in leishmaniasis transmission is maintained in a complex biological system involving the human host, parasite, sandfly vector and in some situations an animal reservoir (Amóra *et al.*, 2009). Therefore, control is unlikely to be achieved by a single intervention, a combination of case management strategies, integrated vector control and, if relevant, animal reservoir control is required and should be tailored to each context (WHO, 2010).

Vector and reservoir control by removal the rubble and destruction of gerbil burrows by deep ploughing in order to eliminate both the reservoir and the breeding and resting sites of *P. papatasi* was effective in certain parts of the former USSR and more recently in Iran, Jordan, Saudi Arabia and Tunisia, but the method is expensive and its sustainability is low ((Cruz *et al.*, 2002, Amóra *et al.*, 2009, WHO, 2010).

As a role of thumb, control measures are the result of breaking one or more elements in the life cycle. There is no single method that can be used for all situations and one method may be successful in one place but not in another, in addition, cost effectiveness has to be considered before adopting a certain method, moreover, control measures should always be revised and evaluated, some measures target the reservoir by eliminating the rodents, by destruction of

the animals' food sources, and /or ploughing burrows as in Jordan and Tunisia (Ashford, 1996, Klaus and Frankenburg, 1999).

The sand fly vector has continuously been the target for control measures, this included the destruction of breeding sites by removing garbage and debris left near houses, and by covering cracks in buildings, in addition, spraying of residual insecticide inside houses and outside under windows were used (Schlein *et al.*, 2001, Amóra *et al.*, 2009). Impregnated bed nets with various insecticides such as Deltamethrin were applied as control measure with significant reduction in CL incidence rate (Alten *et al.*, 2003).

The human host was also a mean for control either by early case detection and treatment are the most important control measures, allowing the patients to be treated 2-3 weeks after the appearance of the lesion, as is the policy in Jericho, to allow immunity to develop or by leishmaniazation as in Iran) (Khamesipour *et al.*, 2005). Further, there are attempts to develop *Leishmania* vaccine, but no definite results have been obtained yet (Valenzuela *et al.*, 2001). Several vaccines have been tested, but the only vaccine with proven efficacy is live *Leishmania major*, however, the vaccine causes significant lesions in some patients, and the use of this vaccine has been discontinued, the best prevention remains avoidance of the sandfly (Murray *et al.*, 2005).

1.2.17 Diagnosis

1.2.17.1 Classical methods for direct identification:

1.2.17.1.1 Clinical Features

In areas where leishmaniasis is endemic , the diagnosis may be made on clinical grounds only, which requires considerable familiarity with the disease,in endemic areas any painless boil of more than one month duration is regarded as cutaneous leishmaniasis (Al Hamdi *et al.*, 2010).

1.2.17.1.2 Parasitological Identification

The margin of the lesion is aseptically punctured with a hypodermic needle and syringe containing a small amount of saline and the aspirate which is drawn up into the needle is examined microscopically and /or cultured (Oshaghi *et al.*, 2010).

1.2.17.1.2.1 Microscopy

Classically, the diagnosis of leishmaniasis relies on direct microscopical identification of amastigotes,its sensitivity is limited to about 60% ,no species-specific diagnosis can be achieved (Herwaldt, 1999). Smears from skin lesions, tissue biopsies or aspiration are used, microscopy is performed on Giemsa stained smears or sections of the named specimens, typically round to oval amastigotes are seen inside of macrophages ,sometimes they are also found extracellular (Bensoussan *et al.*, 2006). The densely stained (violet) rod-shaped kinetoplast should be always identified(Hepburn, 2000, Al-Aubaidi, 2012).

1.2.17.1.2.2 Culturing

Cultures are obtained from aspirates or biopsies from the above-named sources, cultures are usually grown in NNN (Nicolle-Novy-McNeal) and in Schneider's Drosophila medium), the NNN medium is a rabbit-blood agar having an overlay of locke solution with added antibiotics (Singh, 2006, Al-Aubaidi, 2012). The sensitivity of culturing is variable and depends on various factors, as for example the viability of collected parasites, the strain, the media (different requirements for different species), the presence of a super infection, and the expertise of the investigator (Shahbazi *et al.*, 2008).

1.2.17.1.3. Isolations in an experimental animals

As an alternating method, inoculation of the clinical sample that obtained from patients in to mouse or in to Hamster foot bad or nose may be used for cultivation of certain *Leishmania* parasite (Singh, 2006).

1.2.17.2. Serological diagnosis

1.2.17.2.1. Leishmanin Skin Test (LST)

Delayed hypersensitivity is an important feature of cutaneous forms of human leishmaniasis and can be measured by the leishmanin test, also known as the Montenegro reaction (Vega-López, 2003). The LST is widely used for the screening of the exposure to *Leishmania* parasites within endemic areas, analogous to the tuberculin test, leishmanial antigen (killed promastigotes) is applied intradermally and a delayed hypersensitivity reaction is measured after 48-72 h (Masmoudi *et al.*, 2013). A reaction is seen in people with previous contact to the antigen who have developed cellular immunity, conversion occurs after several weeks in CL, and in VL usually only after treatment and cure, present and past infections can not be differentiated (Vega-López, 2003).

1.2.17.2.2. Indirect Fluorescent Antibody Test (IFAT)

The Indirect fluorescent antibody test is one of the most sensitive tests available, the test is based on detecting antibodies, which are demonstrated in the very early stages of infection and are undetectable six to nine months after cure (Singh, 2006).

1.2.17.2.3. Direct Agglutination Test (DAT)

The direct agglutination test (DAT) is a highly specific and sensitive test, it is inexpensive and simple to perform making it ideal for both field and laboratory use (Tavares *et al.*, 2003). The method uses whole, stained promastigotes either as a suspension or in a freeze-dried form, the freeze-dried form is heat stable and facilitates the use of DAT in the field, however, the major disadvantage of DAT is the relative long incubation time of 18 h and the need for serial dilutions of serum (Schallig *et al.*, 2001, Gari-Toussaint *et al.*, 1994).

1.2.17. 2.4. Enzyme Linked Immunosorbent Assay(ELISA)

The Enzyme Linked Immunosorbent Assay (ELISA) is a valuable tool in the serodiagnosis of leishmaniasis (Zijlstra *et al.*, 2001). The test is useful for laboratory analysis as well as for field applications, however, the sensitivity and specificity of ELISA is greatly influenced by the antigen used (Marques *et al.*, 2006).

1.2.17.3 Molecular methods: Polymerase Chain Reactions (PCR).

Microscopy and culture have the limitations of low sensitivity and are time consuming (Faber *et al.*, 2003). The molecular diagnosis approach is capable of detecting nucleic acids unique to the parasite, it would address these limitations. A variety of nucleic acid detection methods targeting both DNA and RNA have been developed (Reithinger and Dujardin, 2007). Amongst these, the PCR has proved to be a highly sensitive and specific technique, the PCR assay can detect parasite DNA or RNA a few weeks a head of appearance of any clinical signs or symptoms (van der Meide *et al.*, 2008).

Various gene targets and nucleic acids can be used in PCR, the important gene targets are 18S-rRNA, small subunit rRNA (SSU rRNA), a repetitive genomic sequence of DNA, the miniexon (spliced ladder) gene repeat, the b-tubulin gene region, gp63 gene locus, internal transcribed spacer (ITS) regions; micro-satellite DNAs such as maxi- and minicircles of kinetoplast DNA (Singh, 2006). Different DNA sequences in the genome of leishmania that conserved and variable regions in kinetoplast DNA (kDNA) minicircles are being used by various workers (Santos-Gomes *et al.*, 2000, El Tai *et al.*, 2001, van der Meide *et al.*, 2008, Hajjaran *et al.*, 2011). In Iraq by using PCR technology they found that there are three species of *Leishmania* that cause of CL, which are *L. tropica*, *L. major* and *L. infantum* (Control and Prevention, 2003).

Chapter Two

Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 Instruments and Equipments: The necessary instruments and apparatuses used for preparing the appropriate experiments in this study. Table (2-1).

Table (2-1): Instruments and Equipments

NO	Instruments and Equipment	Company	Origin
1	Autoclave	Hirayama	Japan
2	Camera	Canon	Japan
3	Centrifuge	Kokusan	Japan
4	Deep freezer -C°20	Jermaks	Germany
5	Distillator	GFL	Germany
6	Electrophoresis	Biometra	USA
7	Eppendrof centrifuge(1.5 µL)	Hamburg	Germany
8	Incubator	Memmert	Germany
9	Water path	Memmert	Germany
10	Thermocycler PCR	Labnet	USA
11	pH – Meter	LKB	Sweden
12	Electronic balance	Sartorius	Germany
13	UV–transilluminater	Optima	Italy
14	Vortex	IKA	USA
15	Nano drop	Biometra	USA
16	ELISA System	BioTek	USA
17	Disposable syringe 5ml	Sterile EO.	China
18	Micropipettes 5-50, 0.5-10, 100-1000µl	Gillson Instruments	France

2.1.2 Chemicals

The chemicals that were used in the present study are mentioned in table (2-2).

Table (2-2): Chemical substances

No.	Chemicals	Manufacturing company	Origin
1	Agarose	BDH	UK
2	Ethidium bromide	Biotech	Canada
3	TB-Buffer	Biotech	Canada
4	Ethanol 95%	Scharlab S.L.	Spain
5	Loading dye	Geneaid	Taiwan
	50 bp DNA ladder	Intronbiotechnology	Korea

2.1.3. Enzyme–Linked Immunosorbent Assay kits.

2.1.3.1 Human Macrophage migration inhibitory factor (MIF) Enzyme Linked Immunosorbent

This ELISA kit is used for the quantitative determination of MIF concentration in serum were provided from Elabscience/ China, as following table (2-3)

Table (2-3): Human MIF ELISA kit with its Components and Remarks

No	Items	Specifications
1	Biotinylated Detection Ab Diluent	1vial 10ml
2	Concentrated Biotinylated Detection Ab	1vial 120µL
3	Concentrated HRP Conjugate	1vial 120µL
4	Concentrated Wash Buffer (25×)	1vial 30ml
5	HRP Conjugate Diluent	1vial 10ml
6	Micro ELISA Plate	8 wells ×12 strips
7	Plate Sealer	5pieces
8	Reference Standard	2 vials

9	Reference Standard and Sample Diluent	1vial 20ml
10	Stop Solution	1vial 10ml
11	Substrate Reagent	1vial 10ml

2.1.3.2 Human Interlukin-1 beta (IL-1β) Enzyme Linked Immunosorbent

This ELISA kit is used for the quantitative determination of IL-1β concentration serum were provided from PeproTech Company/Germany as following table (2-4)

Table (2-4): Human IL-1β ELISA kit with its Components and Remarks

No	Items	Specifications
1	Capture antibody.	1vial 11 µg
2	Detection antibody	1vial 11 µg
3	Avidin-HRP conjugate	1vial 18 µl
4	Human IL-1beta standard	1vial 1µg
5	Phosphate buffer saline	10xPBS
6	Tween-20	1vial 20ml
7	Boric Sulfuric Anodizing (BSA)	1.5 mg
8	Reference Standard and Sample Diluent	1vial 20ml
9	Stop Solution	1vial 10ml
10	Substrate Reagent	1vial 10ml

2.1.3.3 Human Tumor Necrosis Factor-alpha (TNF-α) Enzyme Linked Immunosorbent

This ELISA kit is used for the quantitative determination of TNF-α concentration in serum were provided from PeproTech Company/Germany , as following table (2-5)

Table (2-5): Human TNF-α ELISA kit with its Components and Remarks

No	Items	Specifications
1	Capture antibody.	1vial 11 µg
2	Detection antibody	1vial 11 µg
3	Avidin-HRP conjugate	1vial 18 µl
4	Human TNF-α standard	1vial 1µg
5	Phosphate buffer saline	10xPBS
6	Tween-20	1vial 20ml
7	Boric Sulfuric Anodizing (BSA)	1.5 mg
8	Reference Standard and Sample Diluent	1vial 20ml
9	Stop Solution	1vial 10ml
10	Substrate Reagent	1vial 10ml

2.1.4 Polymerase Chain Reaction kits

In table (2-6) chemical materials used in PCR work to this study with their companies and countries of origin are listed.

Table (2-6): PCR kits with their Remarks

No.	Kit	Company	Country
1	**Genomic DNA Extraction Kit**	**Geneaid**	**Taiwan**
	GT buffer		
	GB buffer		
	W1 buffer		
	Wash buffer		
	Elution buffer		
	GD column		
	Collection tube 2ml		
	Proteinase K 10mg/ml		
2	**AccuPower™ PCR PreMix**	**Bioneer**	**Korea**
	Taq DNA polymerase		
	dNTPs (dATP, dCTP, dGTP, dTTP)		
	Tris.HCl pH 9.0		
	KCl		
	$MgCl_2$		
	Loading dye		

2.1.5 Primers

The genes primers which are used in REFLP-PCR for detection of *MIF* gene (Makhija *et al.*, 2007) and *NRAMP1* gene (Fattahi-Dolatabadi *et al.*, 2016) and provided from Bioner Company , as following table.(2-7)

Table (2-7): The sequence of forward and reverse primers that were used in the present study

Primer type	Sequence	Size	Reference
MIF -173G/C-F	5'-ACTAAGAAAGAC CCGAGGC-3	366bp	(Makhija *et al.*, 2007)
MIF -173G/C-R	5'-GGGGCACGTTGGTGTTTAC-3		
NRAMP1(D 543 N) -F	5- GCATCTCCCCAATTCATGGT-3	244bp	(Fattahi-Dolatabadi *et al.*, 2016)
NRAMP1(D 543 N) -R	5- AACTGTCCCACTCTATCCTG-3		

2.1.6 Restriction Enzyme

Table (2-8): The Restriction Enzymes were used in RFLP-PCR Assay for digestion of *MIF* gene (Makhija *et al.*, 2007) and *NRAMP1* gene (Fattahi-Dolatabadi *et al.*, 2016), with their Company and Country of Origin

Restriction enzymes	Target gene	Company/Country
AluI	MIF	Biolabs/ U.K
AvaII	NRAMP1	Biolabs/ U.K

2.2 Methods

2.2.1 Collection of specimens

2.2.1.1 Patients Group

A total of 60 cases of Cutaneous Leishmaniasis were selected from endemic areas during the period between February 2017 to April 2017in the out-patients clinic of the dermatology department in Al-Hussein Teaching Hospital and specialized center of sensitivity in Al-Muthanna Province. Patients were clinically diagnosis as patients with cutaneous leishmaniasis based on Dermatologist diagnosis

2.2.1.2 Control Group

They included 32 apparently healthy people without any cutaneous lesions and not suffer from any disease as well as carry out diagnostic tests as (HIV, HBS, HCV and CBC test).

2.2.1.3 Blood Samples

After sterilizing the area with alcohol (70%), aspiration blood sample (5ml) was collected from cubital fossa vein from CL patients and control groups. Collected sample was transferred immediately into 2 tubes as follows:

A. Two milliliter of blood in 5 ml tube (EDTA tube) used for PCR technique to detect of *MIF* and *NRAMP1* gene polymorphism.

B. Three milliliter of blood in plain tube (serum tube), then the blood samples were centrifuged at (4700 RPM for 5 min) to obtain blood serum then frozen at -20 ºC for screening of TNF-α, IL-1β and MIF cytokines levels.

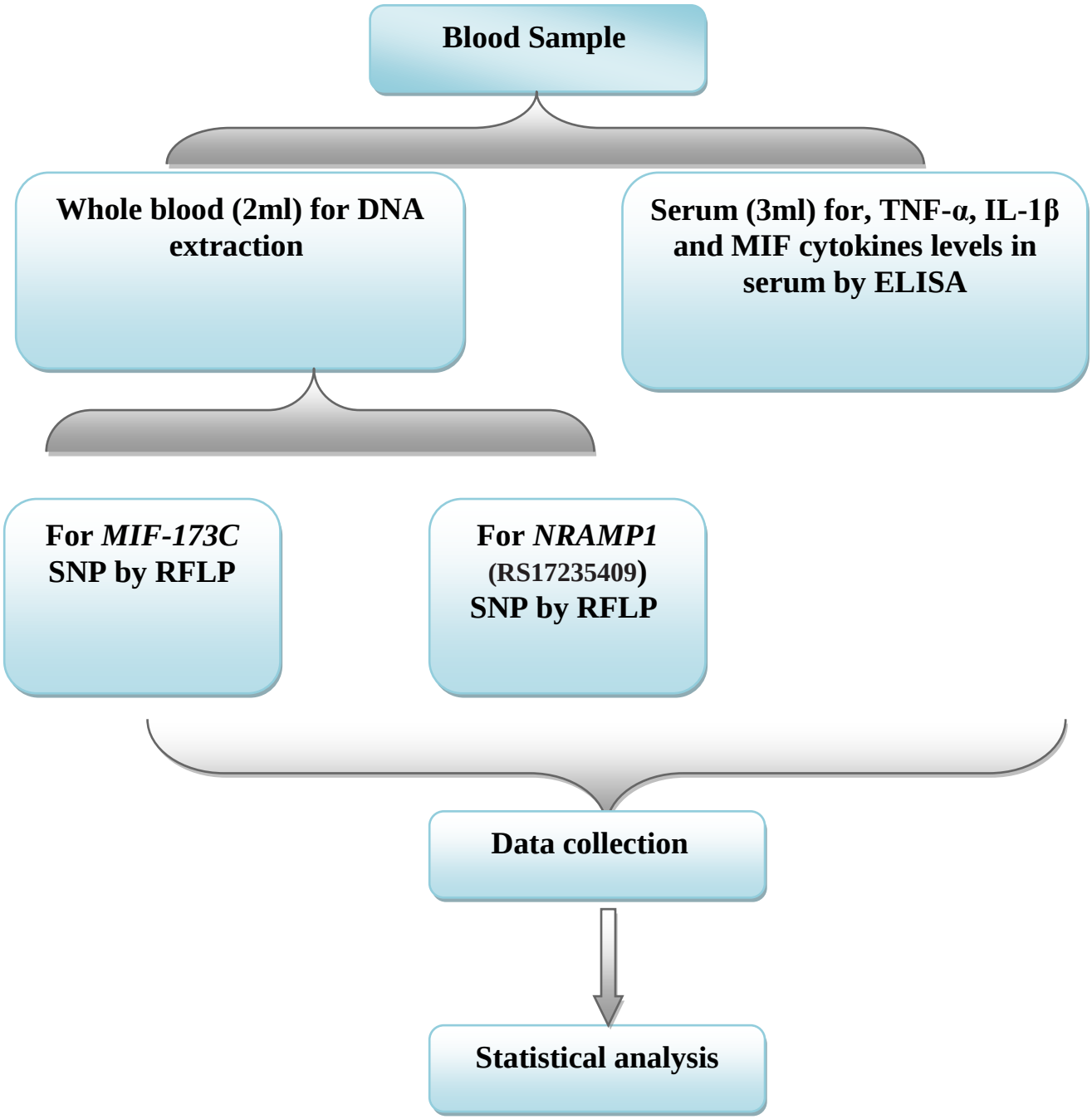

Figure (2-1): A Flow Chart Illustrating the Study Design

2.2.2 ELISA method

2.2.2.1MIF cytokine Assay

2.2.2.1.1 Preparation of Items and Solutions for ELISA Technique

1. **Wash Buffer**: A volume 30 ml of concentrated wash buffer diluted into 750 ml of wash Buffer with deionized.

2. **Standard working solution:** The standard was centrifuged at 10,000×g for 1 minute. and 1.0ml of Reference Standard &Sample Diluent was added, it leted stand for 10 minutes and it turned upside down for several times. After it dissolved fully, mixed thoroughly with a pipette. This reconstitution produces a stock solution of 50ng/ml. Then serial dilutions was marked as needed. The recommended dilution gradient is as follows: **50、 25、 12.5、 6.25、 3.125、 1.5625、 0.781、 0 ng/ml**.

Dilution method: Eppendorf tubes (7) taken, a volume 500 µL of Reference Standard & Sample Diluent added to each tube. Pipette 500uL of the 50ng/ml stock solution to one tube to produce a 25ng/ml stock solution. Pipette 500uL of the solution from former tube to the latter one in order according to this step. The illustration below is for reference. Note: the last tube is regarded as blank. Don't pipette solution to it from the former tube.

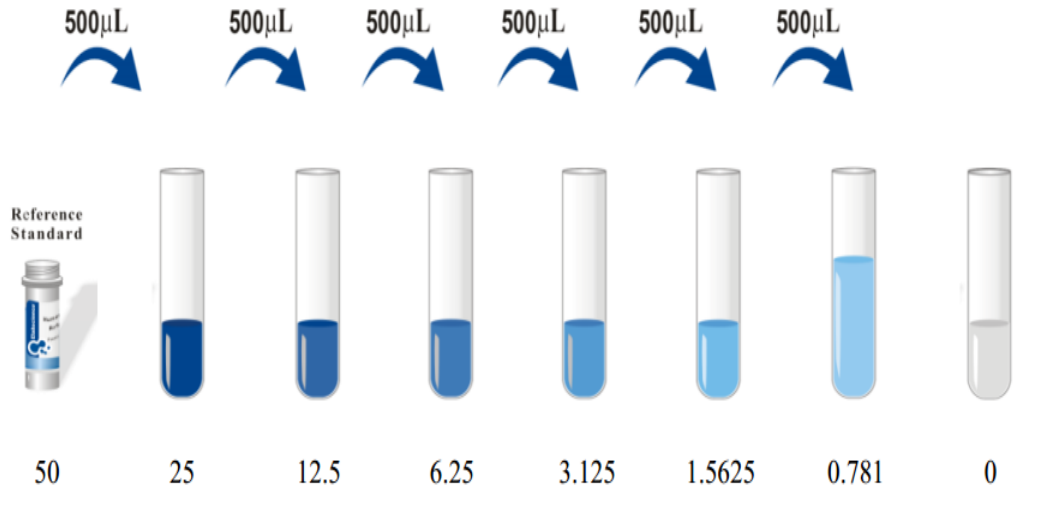

Figure (2-2). Dilution of standard (ng/ml)

3. **Biotinylated Detection Ab working solution:** The required amount Calculated before experiment (100µL/well). In actual preparation, should prepare 100-200µL more. The stock tube Centrifuged before use, the 100× concentrated Biotinylated Detection Ab diluted to 1×working solution by Biotinylated Detection Ab Diluent.
4. **Concentrated HRP Conjugate working solution:** The required amount Calculated before experiment (100µL/well). In actual preparation, should prepare 100-200µL more. The stock tube Centrifuged before use. The 100×Concentrated HRP Conjugate Diluted to 1×working solution by Concentrated HRP Conjugate Diluent.

2.2.2.1.2 Assay procedure

1. A volume 100µL of standard or sample was added to each well and incubate for 90mintues at 37℃
2. The liquid remove d, Biotinylated Detection Ab (100µ) added and incubate for 1 hour at 37℃
3. Aspirate and wash 3 times by using microplate washer.
4. A volume 100µL HRP Conjugate was added and incubate for 30 minutes at 37℃.
5. Aspirate and wash 5 times by using microplate washer.
6. Substrate Reagent (90µL) added and incubate for 15 minutes at 37℃.
7. Stop Solution (50µL) added.
8. Reading at 450nm immediately and the results was calculated, figure (2-1).

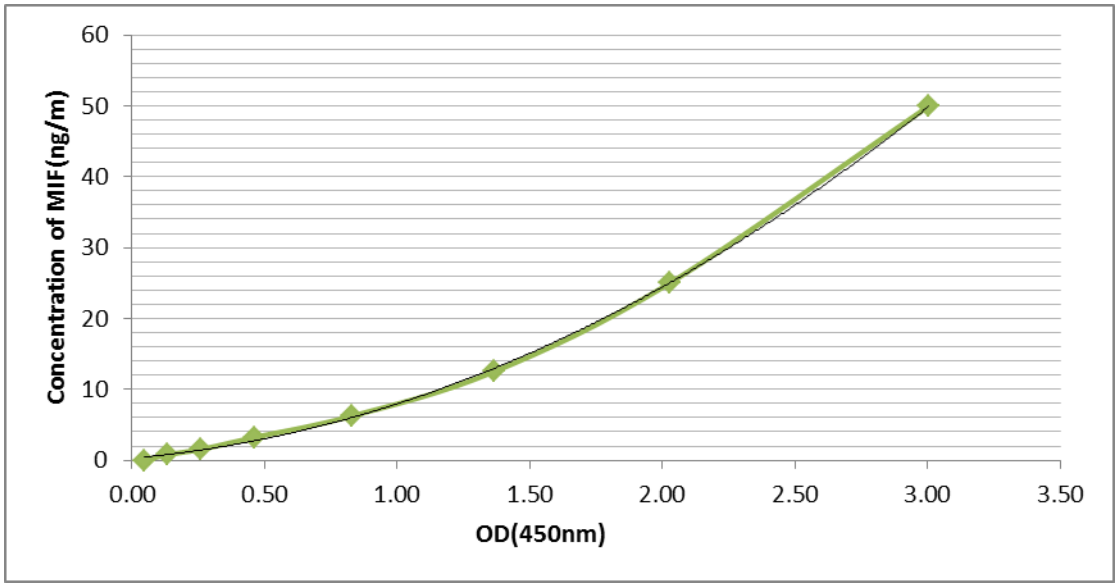

Figure (2-3): Standard Curve of MIF Concentration

2.2.2.2 IL-1 beta cytokine Assay

2.2.2.2.1 Preparation of Items and Solutions for ELISA Technique

1. Capture antibody. A volume (11 µg) of Goat anti –human IL-1beta + (0.5 mg) D-mannitol. Vial centrifuged and reconstituted in (110 µL) sterile water at concentration of 100mg/ml.

2. **Detection antibody**. A Volume (11 µg) of Biotilyated Goat anti-humaan IL-1beta + (0.5 mg D-manitol. Vial centrifuged and reconstituted in (110 µL) sterile water at concentration of 100mg/ml.

3. **Human IL-1beta standard**.a volume (1µg) of recombinant human IL-1beta + (2.2 mg) BSA+ (11 mg) D-mannitol. Vial centrifuged and reconstituted in (1ml) sterile water at concentration of 1µg/ml.

4. **Avidin-HRP conjugate**. A volume (18 ml) vial a liquated into two (9ml) and stored at frozen -20c.

5. **Phosphate buffer saline**. (10xPBS) diluted to (1x PBS) in sterile water.

6. Wash Buffer. Tween-20 (0.05%) diluted in PBS.

7. Diluent. Tween-20 (0.05%), BSA (0.1%) diluted in PBS.

8. Block Buffer. BSA (1%) diluted in PBS**.**

9. Plat preparation. Capture Ab diluted with PBS to concentration of (0.50 µg/ml).and (100 µL) added to each well in plate. Plat sealed and incubated

overnight. Wells aspirated to remove liquid and the plate washed 4 time by using (300 µL) of wash buffer. After last wish, plate inverted to remove residual buffer and blotted on paper towel. Block buffer (300 µL) added to each well and incubated for at 1 hour at room temperature. Plat aspirated and wished 4 time

2.2.2.2.2 Assay procedure

1. A volume 100µL of standard or sample was added to each well and incubate for 2 hours at room temperature.
2. The plat aspirated and wished 4 time by using microplate washer, Detection Ab (100µ) added and incubate for 2 hours at room temperature.
3. Aspirate and wash 4 times by using microplate washer.
4. A volume 100µL HRP Conjugate was added and incubate or 30 minutes at room temperature.
5. Aspirate and wash 4 times by using microplate washer.
6. Substrate Reagent (100µL) added and incubate for 15 minutes at at room temperature.
7. Stop Solution (50µL) added
8. Reading at 405nm immediately and the results was calculated, figure (2-4).

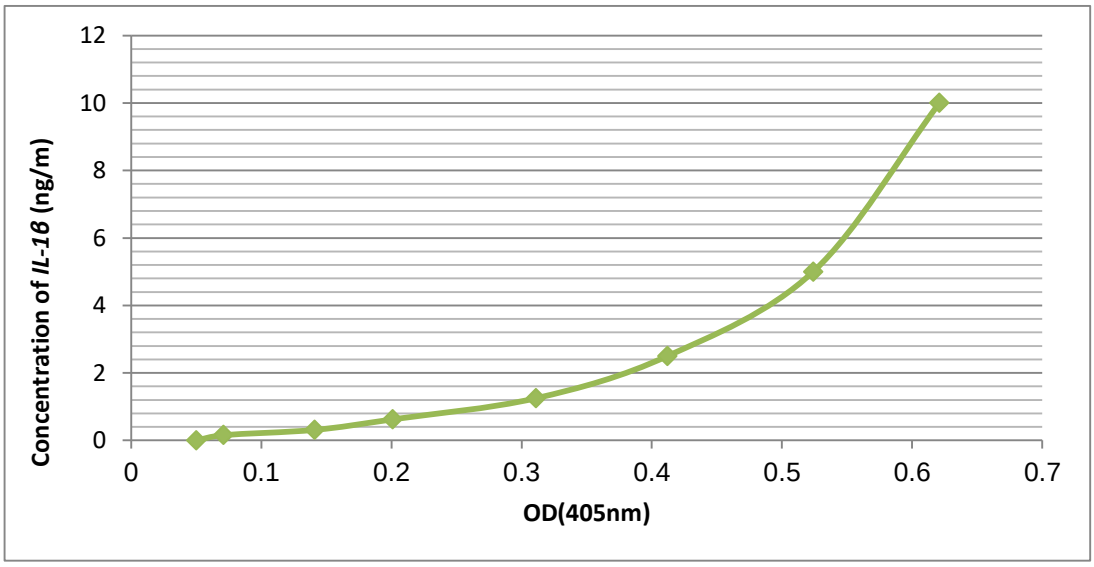

Figure (2-4): Standard Curve of IL-1β Concentration

2.2.2.3 TNF-α Cytokine Assay

2.2.4.1 Preparation of Items and Solutions for ELISA Technique

1**. Capture antibody.** A volume (21 mg) of Rbbit anti – humaan TNF-α + (0.5 mg) D-mannitol. Vial centrifuged and reconstituted in (210 µL) sterile water at concentration of 100mg/ml.

2. **Detection antibody**. A Volume (11mg) of Biotilyated Rabbit anti-humaan TNF-α + (0.5 mg D-manitol. Vial centrifuged and reconstituted in (110 µL) sterile water at concentration of 100mg/ml.

3. **Human TNF-α standard**. A volume (1µg) of recombinant human IL-1beta + (2.2 mg) BSA+ (11 mg) D-mannitol. Vial centrifuged and reconstituted in (1ml) sterile water at concentration of 1µg/ml.

4. **Avidin-HRP conjugate** .a volume (18 ml) vial a liquated into two (9ml) and stored at frozen -20c.

5. **Phosphate buffer saline** (PBS). (10xPBS) diluted to (1x PBS) in sterile water.

6. Wash Buffer. Tween-20 (0.05%) diluted in PBS.

7. Diluent. Tween-20 (0.05%), BSA (0.1%) diluted in PBS.

8. Block Buffer. BSA (1%) diluted in PBS**.**

9. Plat preparation. Capture Ab diluted with PBS to concentration of (0.50 µg/ml).and (100 µl) added to each well in plate. Plat sealed and incubated overnight.Wells aspirated to remove liquid and the plate washed4 time by using (300µl) of wash buffer.After last wish, plate inverted to remove residual buffer and blotted on paper towel.Block buffer (300µl) added to each well and incubated for at 1 hour at room temperature.plat aspirated and wished 4 time

2.2.4.2 Assay procedure

1. A volume 100µL of standard or sample was added to each well and incubate for 2 hours at room temperature.
2. The plat aspirated and wished 4 time by using microplate washer, Detection Ab (100µ) added and incubate for 2 hours at room temperature.
3. Aspirate and wash 4 times by using microplate washer.
4. A volume 100µL HRP Conjugate was added and incubate for 30 minutes at room temperature.
5. Aspirate and wash 4 times by using microplate washer.
6. Substrate Reagent (100µL) added and incubate for 15 minutes at at room temperature.
7. Stop Solution (50µL) added.
8. Reading at 405nm immediately and the results was calculated, figure (2-3).

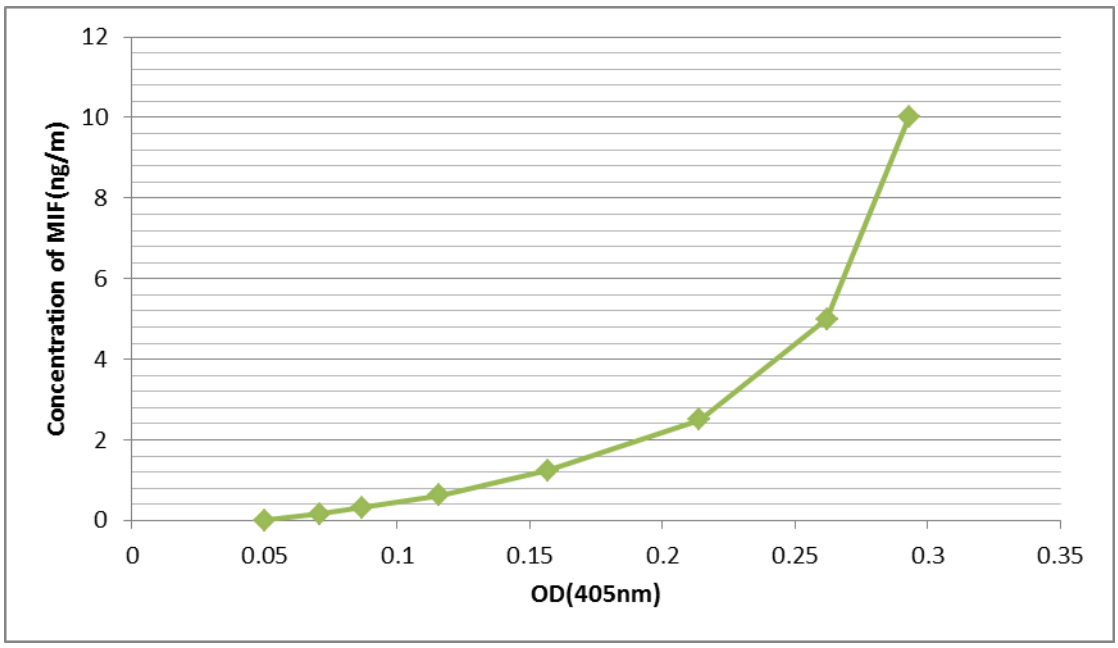

Figure (2-5): Standard Curve of TNF- α Concentration

2.2.3 Molecular Methods

2.2.3.1 Genomic DNA extraction

Genomic DNA from blood samples were extracted by using Accupower Genomic DNA extraction kit (Whole Blood) Geneaid Taiwan, and done according to company instruction as following steps:

1. A 200µl of whole blood was transferred to sterile 1.5ml microcentrifuge tube, and then added 20µl of proteinase K and mixed by vortex, then all tubes were incubated at 60℃ for 5 minutes.
2. After that, 200µl of GSB buffer was added to each tube and mixed by vortex to achieve maximum lysis efficiency, and then all tubes were incubated at 60℃ for 5 minutes.
3. A 200µl of absolute ethanol was added to mixture and mixed by vortex for 10 second, and then briefly spin down to get the drops clinging under the lid. The lysate was carefully transferred into GD Binding filter column that fitted in a 2 ml collection tube, and then closed the tubes and centrifuged at 14000 rpm for 1 minute.
4. Throughout lysate was discarded, and then 400µl Washing buffer 1 (W1) was added to each GD column, and centrifuged at 14000 rpm for 30 second.
5. Throughout washing buffer 1 was discarded, GD column was transferred to 2 ml collection tube, and then 600µl washing buffer 2 (W2) was added to each GD column, and centrifuged at 14000 rpm for 30 second.
6. Throughout washing buffer 2 was discarded, GD backed to 2 ml collection tube, and then the tubes were centrifuged once more at 14000 rpm for 3 minute to completely remove ethanol.
7. After that, GD column that containing genomic DNA was transferred to sterile 1.5ml microcentrifuge tube, and then added 100µl of pre -heated Elution buffer and left stand the tubes for 3 minutes at room temperature

until the buffer is completely absorbed into the glass filter of binding column tube.

8. Finally, all tubes were centrifuged at 14000 rpm for 30 second to elute DNA, and storage at -20℃ freezer.

2.2.3.2 PCR

The extracted genomic DNA from blood samples was checked by using nanodrop spectrophotometer (Biometra. USA), that measure concentration and purity of DNA through reading the absorbance in at (260 /280 nm).

PCR Mixture

PCR Mixture was provided by manufacturing company table (2-9).

Table (2-9): Mixture of PCR

Mixture solution	Volume	Concentration
Master mix	25µL PreMix (1X)	-
Forward primer	2.5µL (10pm/ µl)	25pm
Reverse primers	2.5µL (10pm/ µl)	25pm
Target DNA	5µL(20 ng/µL)	100 ng
Nuclease free water	15µL	-
Total volume	50µL	**-**

PCR Program

This program was listed in table (2-10) and (2-11).

Table (2-10) Amplification conditions of MIF gene, (Donn *et al.*, 2001)

Steps	Temperature	Time	No. of cycles
Initial denaturation	94 °C	5min	1 cycle
Denaturation	94 °C	30 sec	38 cycle
Annealing	59°C	30 sec	
Elongation	72 °C	50 sec	
Final elongation	72 °C	5min	1 cycle

Table (2-11): Amplification conditions of NRAMP1 gene, (Fattahi-Dolatabadi *et al.*, 2016)

Steps	Temperature	Time	No. of cycles
Initial denaturation	94 °C	4 min	1 cycle
Denaturation	94 °C	60 sec	38 cycle
Annealing	58°C	90 sec	
Elongation	72 °C	40 sec	
Final elongation	72 °C	10 min	1 cycle

Electrophoresis of PCR products

The PCR products were analyzed by agarose gel electrophoresis following steps:

1. Agarose gel was prepared by dissolving 1.5 g of agarose in 150ml of TBE buffer (1X) (pH 8) and left to cool to 50º C.
2. Then 4.5 µl of (10 mg / ml) of Ethidiumbromid was added and mixed well.
3. After fixing the comb on one end of the tray, poured off the agrose gently to avoid the formation of any bubbles, left it to solidify at room temperature, the comb was removed after hardening of agarose leaving wells and the electrophoresis chamber was filled with TBE buffer (1X), DNA sample (10 µl) of samples was loaded in the wells and one of the well was filled with DNA ladder (10 µl).
4. The electrophoresis ran at 80 volt/cm for 30 min.
5. UV- transilluminator was used to visualize DNA band and gel was photographed using digital camera (Sambrook and Russell, 2001).

2.2.3.3. RFLP

Polymerase chain reaction-restriction fragment length polymorphism (RFLP-PCR) master mix was prepared for detection MIF and NRAMP1 genes polymorphism in blood samples of cutaneous lieshmaniasis patients and healthy control by using restriction endonuclease (*AluI*) that digestion of the 366 bp PCR product of MIF gene and restriction endonuclease(*AvaII*) that digestion of the 244 bp PCR product of NRAMP1 gene this master mix done according to company instructions as following table (2-12).

Table (2-12): Mixture for genotype

Mixture solution	Volume
PCR product	10µL
Restriction enzyme buffer 10X	2µL
Restriction enzyme (10 unit)	1µL
Free nuclease water	7µL
Total volume	20µL

After that, this master mix placed in Exispin vortex centrifuge at 3000rpm for 2 minutes, then transferred into incubation at 37°C overnight. After that, RFLP-PCR product was analyzed by agarose gel electrophoresis (2.5%) and product analysis as following table (2-13).

Table (2-13): Restriction enzymes and DNA Fragments size

Restriction enzymes	Gene	Genotype	Fragment size
AluI	MIF	GG	268,98bp
		GC	268, 205.98,63bp
		CC	205.98, 63bp
AvaII	NRAMP1	GG	126, 79 and 39bp
		GA	126, 79, 39 and 205bp
		AA	205 and 39bp

2.2.4 Statistical analysis

Statistical analysis was conducted by using (SPSS 23). Determining the statistical differences among different groups and comparison of allelic and haplotype frequencies was made using the Pearson Chi-square test and odds ratio with (95%) confidence, while the hierarchical log-linear models were applied to compare the observed vs. the expected frequencies. Statistical differences among different interleukin groups determine by T- and Anova tests. Probability of ($P \leq 0.05$) was considered to be statistically significant (TEAM, 2010).

Chapter Three

Results

3. Results

3.1. Distribution of Cutaneous Leishmaniasis

The results presented in this chapter were based on the analysis of a random sample of 60 cases with an established diagnosis of Cutaneous leishmaniasi, according to gender, the results showed that the males were 34(56.7%) more frequent than females 26 (43.3%). There is no significant differences in gender distribution was observed between male and female (*P*=0.302), figure (3-1).

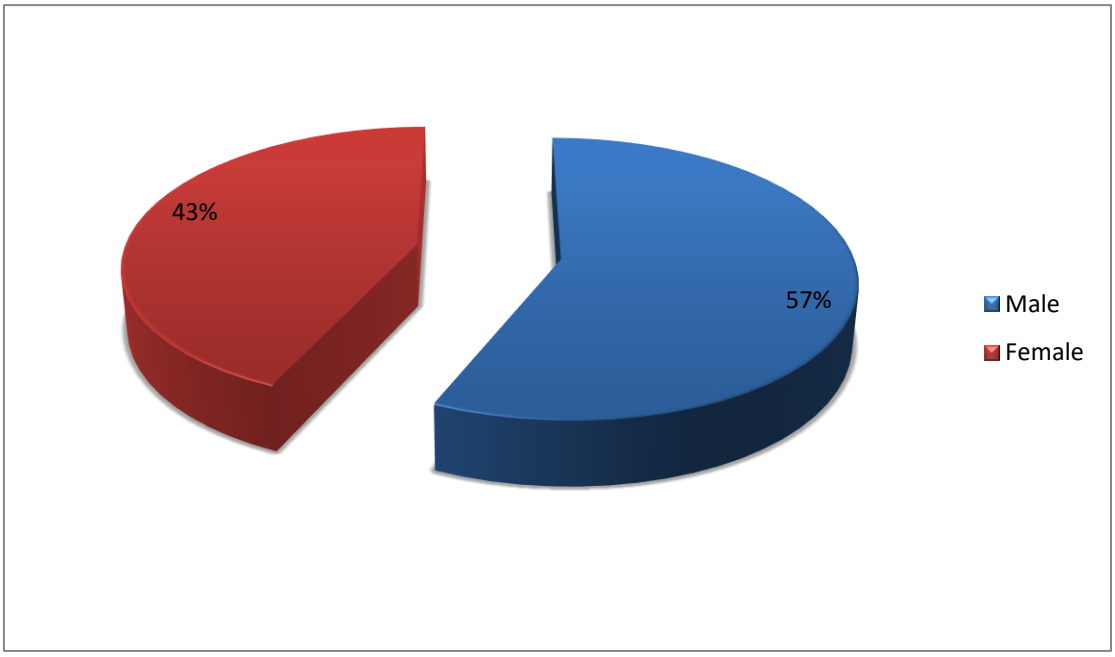

Figure (3-1): Distribution of CL patients according to gender

Cutaneous leishmaniasis patients were included in this study their ages ranging between 1-80 years old, 14 (23.3%) of them their ages ranging between 1-10 years old , 30 (50%) of them their ages ranging between 11-20 years, 12(20%) of them their ages ranging between 21-30 years and 4(6.7%) were ages ranging between 31-80 years old, (P <0.001). table(3-1).

Table (3-1): Distribution of CL patients according to age

Age group	N.	%	Male (34)		Female (26)		*P* value
			N	%	N	%	
1-10	14	23.3	8	13.3%	6	10.0%	*P*=0.593
11-20	30	50.0	16	26.7%	14	23.3%	*P*=0.715
21-30	12	20.0	8	13.3%	4	6.7%	*P*=0.248
30-80	4	6.7	2	3.3%	2	3.3%	*P*=1.000

The results showed that the distribution of CL in rural 43 (71.7%) was more than urban regions 17 (28.3%), there is a significant result with a P value (*P*=0.001), figure (3-2).

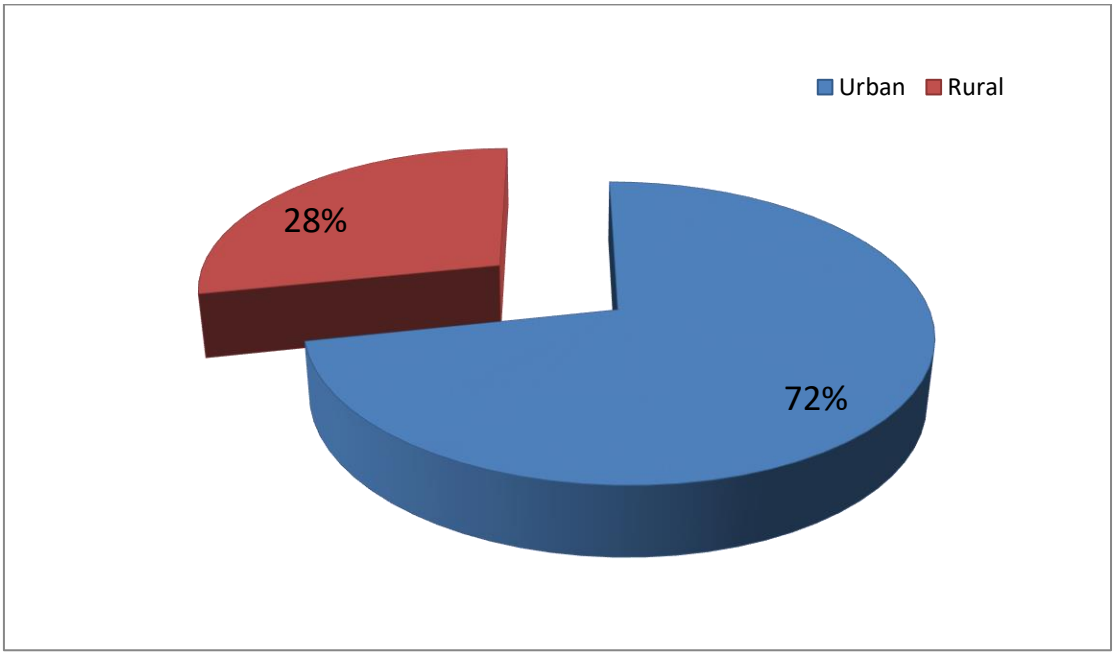

Figure (3-2): Distribution of CL according to geographic distribution

3.2. Molecular analysis

3.2.1 DNA Extraction

Genomic DNA from blood samples were extracted by using Accupower Genomic DNA extraction kit and checked by using Nanodrop spectrophotometer at (260 /280 nm).it was ranging between 1.8-2.2, with purity average equal 2, and DNA concentration mean was 20 ng/μL.

3.2.2 PCR-RFLP

3.2.2.1 DNA Amplification

3.2.2.1.1 Macrophage Migration Inhibitory Factor (*MIF*) Gene

The products of successful binding between the target DNA and specific primers for *MIF* gene were detected by gel electrophoresis and product size was 366bp for both patients and control group. Figure (3-3).

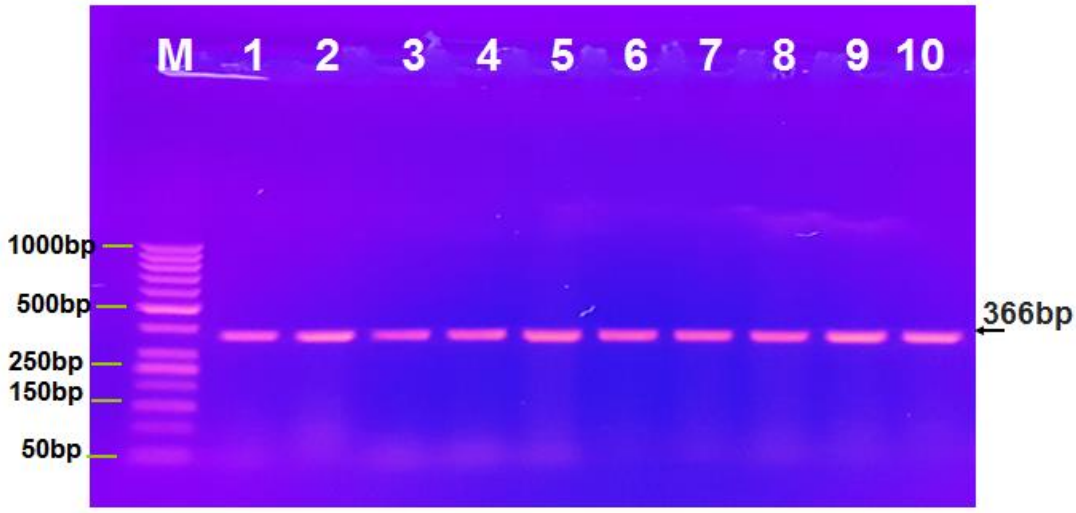

Figure (3-3): Agarose gel electrophoresis image that show the PCR product analysis of *MIF* gene from blood samples of cutaneous leshmaniasis patients and healthy control on 1.5% agarose gel at 80Vol/cm for 30 minutes

3.2.2.1.2 Natural resistance-associated macrophage protein 1*(NRAMP1)* gene

The products of successful binding between the target DNA and specific primers for *NRAMP1* gene were detected by gel electrophoresis and product size was 244bp for both patents and control group. Figure (3-4).

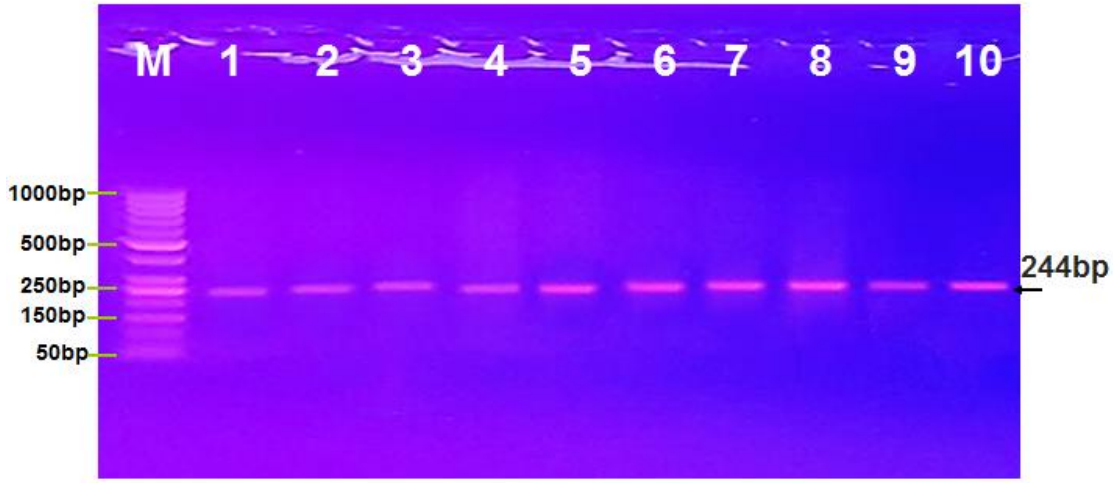

Figure (3-4): Agarose gel electrophoresis image that show the PCR product analysis of *NRAMP1 (RS17235409)* gene from blood samples of cutaneous leshmaniasis patients and healthy control on 1.5% agarose gel at 80Vol/cm for 30 minutes

3.2.2.2 Detection of Genes Polymorphism

3.2.2.2.1 Detection of MIF G-173C Polymorphism

The distribution of MIF G-173C polymorphism was detected by PCR-RFLP technique. At this locus there are three genotype; GG, GC and CC with band sizes 268/98 pb, 205/ 268/98/63 pb and 205/98 pb respectively, table (3-2), figure (3-5).

Table (3-2): Distribution of *MIF* -173 G>C **genotypes and alleles**

Variable Genotype	**Patients (n=60)**	**Control (n=32)**	**P value**	**Odd Ratio**	**95 % Confidence interval**		**Risk factor**
					Lower	Upper	
GG	44(73.30%)	20(62.5%)	*P*=0.282	1.650	0.660	4.125	1.203
GC	14 (23.3%)	12(37.5%)	*P*=0.151	0.507	0.200	1.289	0.773
CC	2 (3.3%)	0(0%)	-	-	-	-	1.552
Overall *P*	*P* = 0.234						
Allele Frequency							
G	102(0.85)	52(0.8125)	*P*=0.512	1.308	0.586	2.920	1.104
C	18 (0.15%)	12(0.1875)	P=0.512	0.765	0.343	1.707	0.906
Overall *P*	*P* = 0.512						

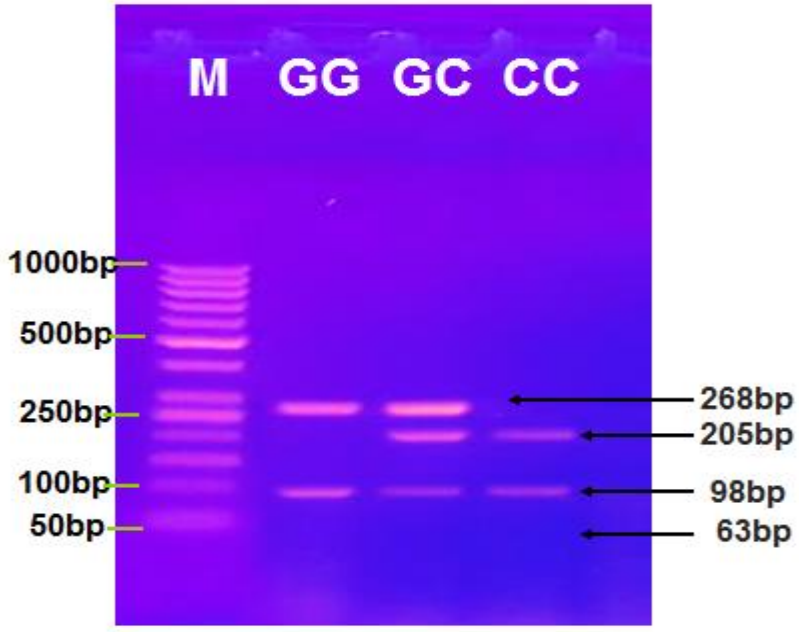

Figure (3-5): Agarose gel electrophoresis image that show the RFLP-PCR product analysis of *MIF* gene by using *AluI* restriction enzyme on 2.5% agarose gel at 80Vol/cm for 30 minutes

3.2.2.2.2 Detection of *NRAMP1* ((RS17235409) Polymorphism

Distribution of *NRAMP1* (RS17235409) Polymorphism was detected by PCR-RFLP technique, at this locus there are three genotype; GG, GA and AA with band sizes 126/79/39 pb, 205/ 126/79/39 pb and 205/39 pb respectively, table(4-3), figure (3-6).

Table (3-3): Frequency of alleles and genotypes of *NRAMP1* polymorphisms (RS17235409) in cutaneous leishmaniasis (CL) patients and the control group

Variable Genotype	Patients (n=60)	Control (n=32)	*P* value	Odd Ratio	95 % Confidence interval		Risk factor
					Lower	Upper	
GG	44(73.30%)	18(56.25%)	*p*=0.096	2.139	0.867	5.276	1.331
GA	14 (23.3%)	8(25%)	*p*=0.858	0.913	0.336	2.480	0.968
AA	2 (3.3%)	6(18.75%)	*p*=0.012*	0.149	0.028	0.790	0.362
Overall *P*	*p* = 0.036*						
Allele Frequency							
G	102(0.85)	44(0.6875)	*p*=0.01*	2.576	1.243	5.336	1.475
A	18 (0.15)	20(0.3125)	*p*=0.01*	0.388	0.187	0.804	0.678
Overall *P*	*p* = 0.01*						

*** represent a significant difference at 0.05 level.**

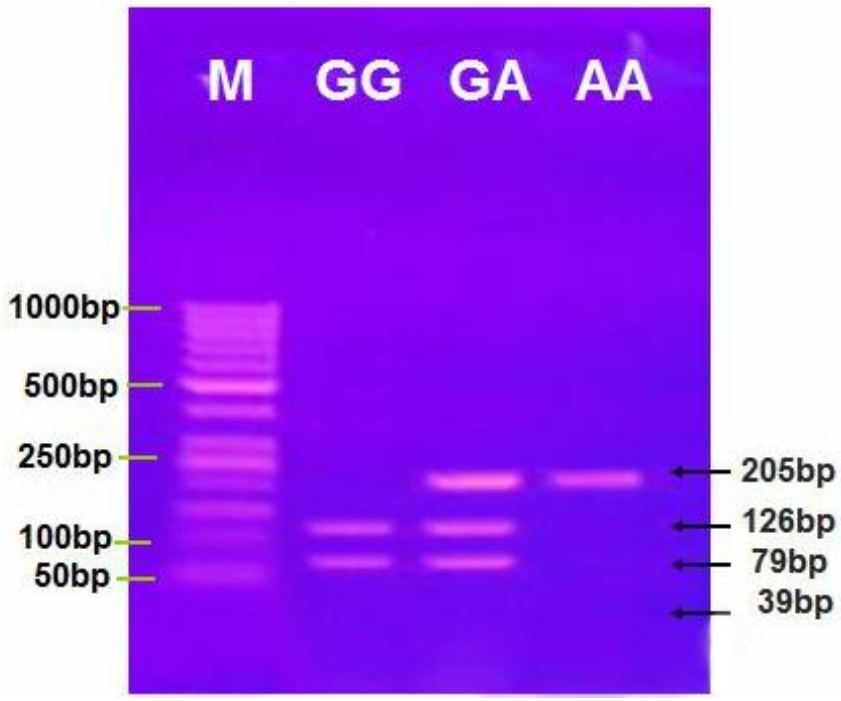

Figure (3-6): Agarose gel electrophoresis image that show the RFLP-PCR product analysis of *NRAMP1* (RS17235409) gene by using *AvaII* restriction enzyme on 2.5% agarose gel at 80Vol/cm for 30 minutes

3.3. Cytokines Concentration (MIF, IL-1β and TNF-α) in the Serum of CL Patients and control groups

4.3.1. MIF Concentration

The mean concentration of the cytokine MIF has no significant difference between patients and control subjects (8.159±0.826ng/ml) versus (8.418±1.33ng/ml) respectively (p=0.863). Figure (3-7).

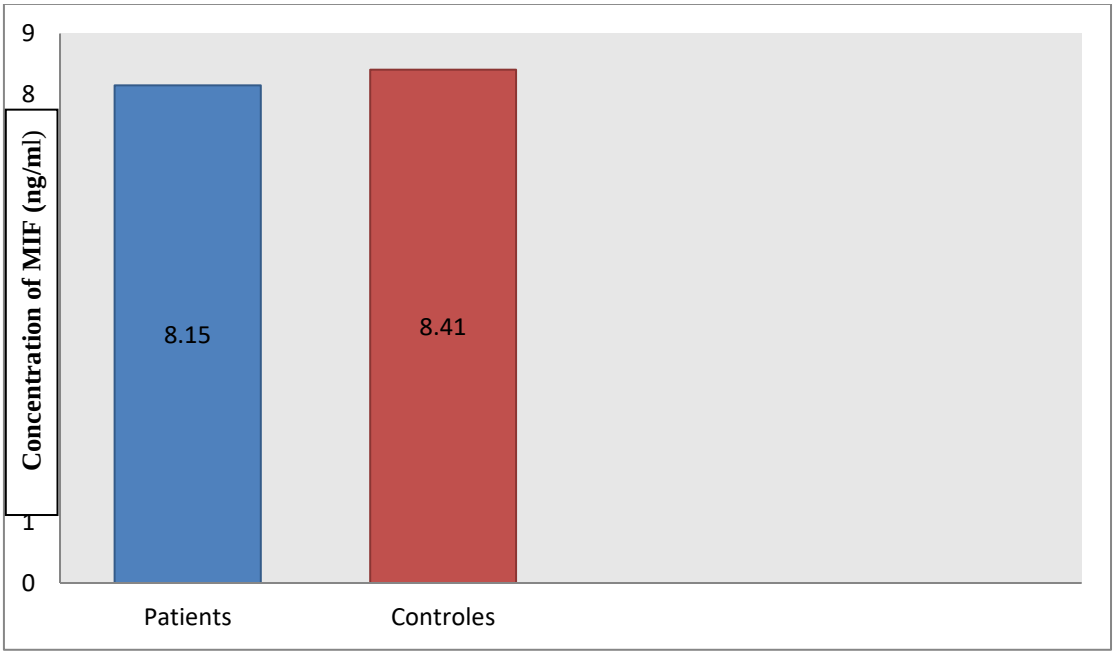

Figure (3-7): Comparison of mean concentration serum MIF cytokine between patients and control Group.

The mean concentration of MIF level was declining gradually according to variation in age were in patients and controls groups (P = 0.001 and P<0.001) respectively, figure (3-8).

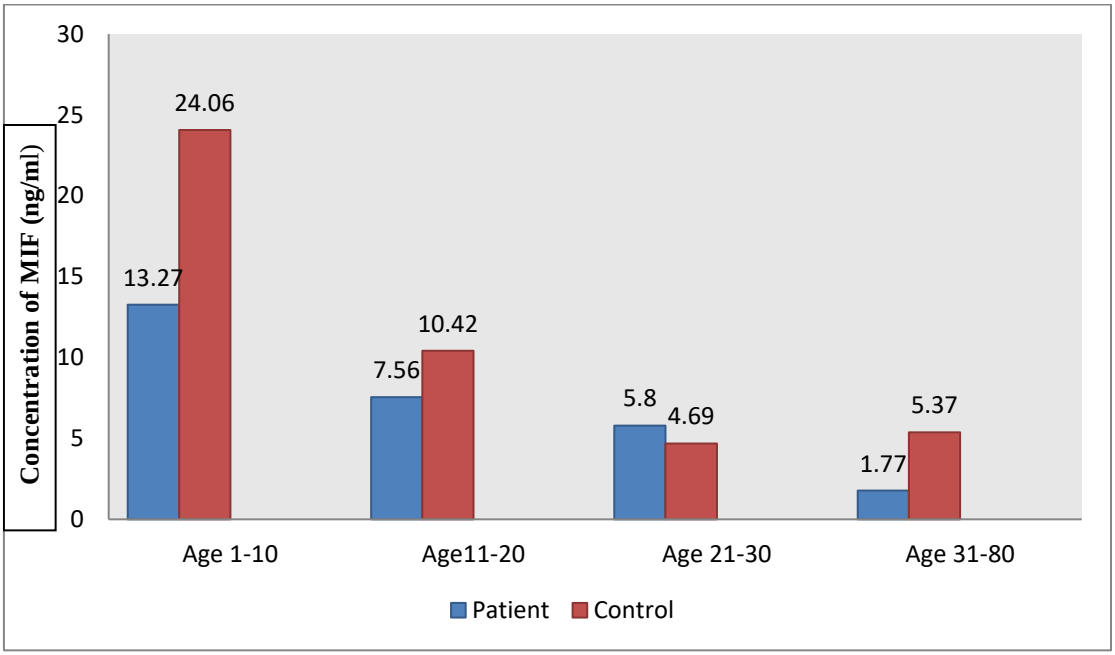

Figure (3-8): Association between age and serum MIF in patients and control groups.

Table (3-4) shows that the mean concentration concentration of MIF level according to gender in patients and control groups. The mean concentration of MIF was not significant difference between male and female in patients and control groups. The data showed that MIF mean concentration in male was (8.45±1.13ng/ml and 7.67±1.82ng/ml) in patients and control group respectively while the MIF mean concentration in female (7.77±1.22ng/ml and 9.36±1.98 ng/ml) in patients and control group respectively.

Table (3-4): Comparison of mean concentration serum MIF between male and female in patients and control

	Male	**Female**	***P* value**
Patients	8.45±1.13 ng/ml	7.77±1.22 ng/ml	P=0.594
Control	7.67±1.82 ng/ml	9.36±1.98 ng/ml	P=0.538
***P* value**	P=0.706	P=0.476	-

3.3.2. TNF- *α* Concentration

The mean TNF- *α* were significantly higher in patients as compared to control groups, (2.698±0.122ng/ml) versus (0.414±0.015ng/ml) respectively (p<0.001). Figure (3-9)

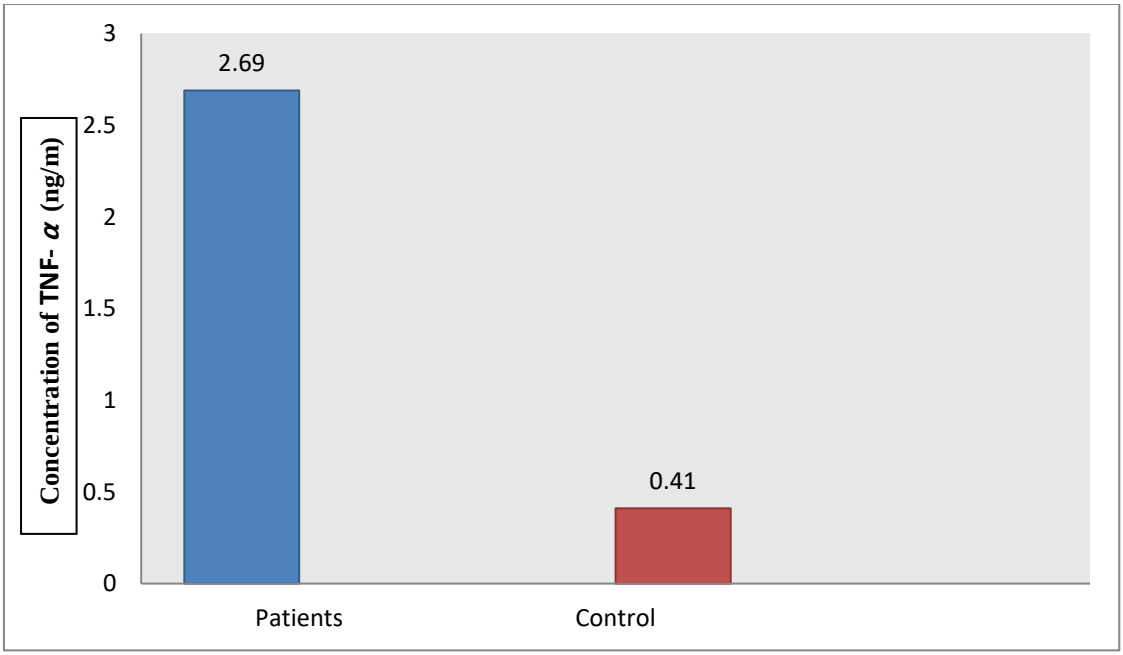

Figure (3-9): Comparison of mean concentration serum TNF- *α* cytokine between patients and control group.

According to the variation of age, It was found that the mean concentration of TNF- *α* increase gradually with increasing of age in patients and control groups, except for last age group 31-80 year, where no significant difference with other groups, table (3-5).

Table (3-5): Association between age groups and serum TNF- *α* in patients and control

Age groups	patients	control	P value
1-10	2.496+0.124 ng/ml	0.403+0.032 ng/ml	p<0.001
11-20	2.670+0.209 ng/ml	0.409+0.026 ng/ml	p<0.001
21-30	3.073+0.264 ng/ml	0.428+0.028 ng/ml	p<0.001
31-80	2.497+0.164 ng/ml	0.377+0.017 ng/ml	*P*=0.001

Results showed no statistically significant differences in mean concentration of TNF-α in male as compared to female patients and control groups (P = 0.155 and P=0.779) respectively, table (3-6). Since the mean concentration of TNF-α level in patients group in male was (2.530 ± 0.095ng/ml) and female was (2.919±0.250ng/ml), while control group was (0.410± 0.020ng/ml) in male and (0.419±0.025ng/ml) in female,

Table (3-6): Comparison of mean concentration serum TNF-α between male and female in patients and control

	Male	Female	P value
Patients	2.530 ± 0.095 ng/ml	2.919±0.250 ng/ml	0.155
Control	0.410± 0.020 ng/ml	0.419±0.025 ng/ml	0.779
P value	P<0.001	P<0.001	

4.3.3. IL-1β Concentration

The mean concentration of IL-1β interleukin has significant increase in patients group in comparison to control subjects (0.814± 0.054ng/ml) versus (0.482±0.020ng/ml) respectively (p<0.001). Figure (3-10).

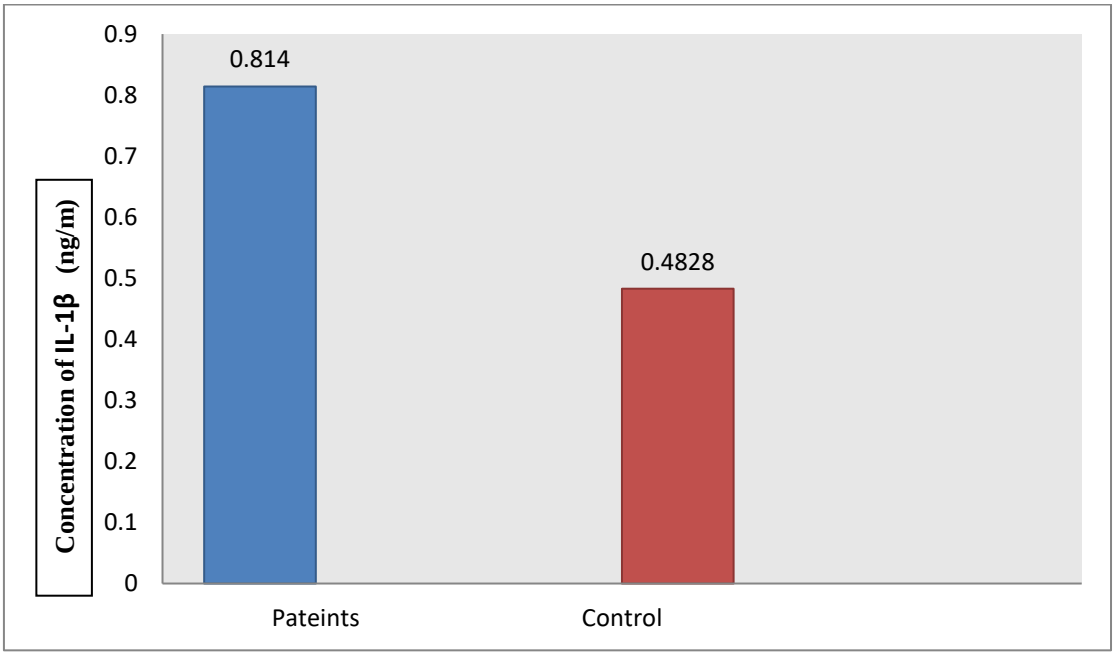

Figure (3-10): Comparison of concentration mean concentration of IL-1β cytokine between patients and control group.

The mean concentration of IL-1β level were increased continuously with increasing of age in patients ($P \leq 0.05$), figures (3-11).

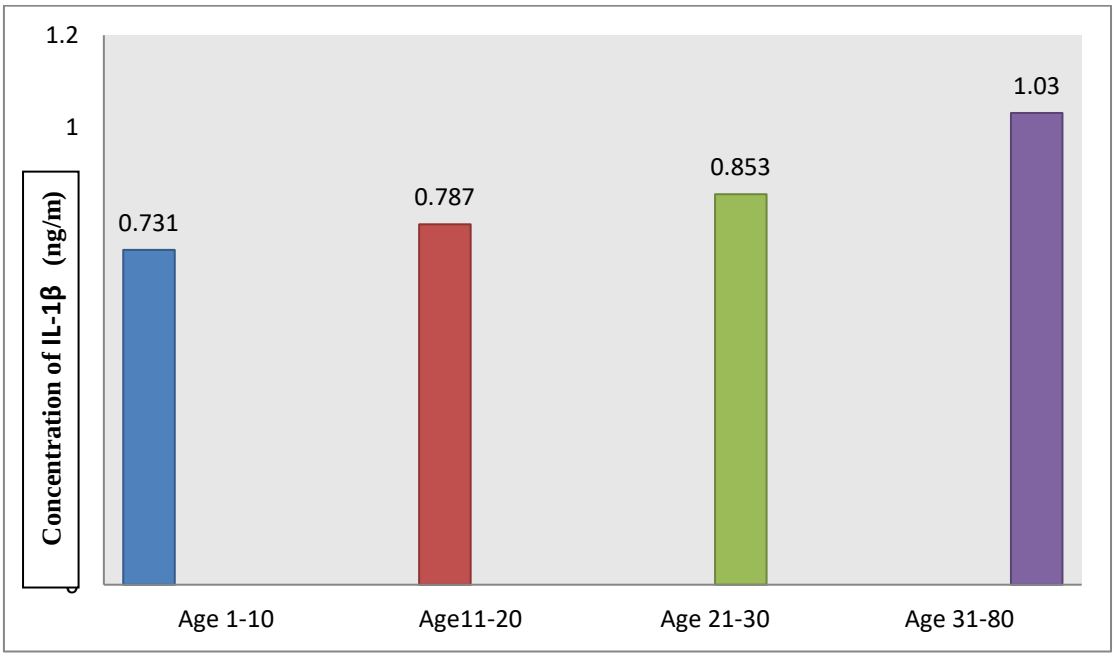

Figure (3-11): Association between Age and Serum IL-1β in Patients Groups.

There are no significant difference in mean concentration of IL-1β with increasing of age in control group (P=0.268), figure (3-12)

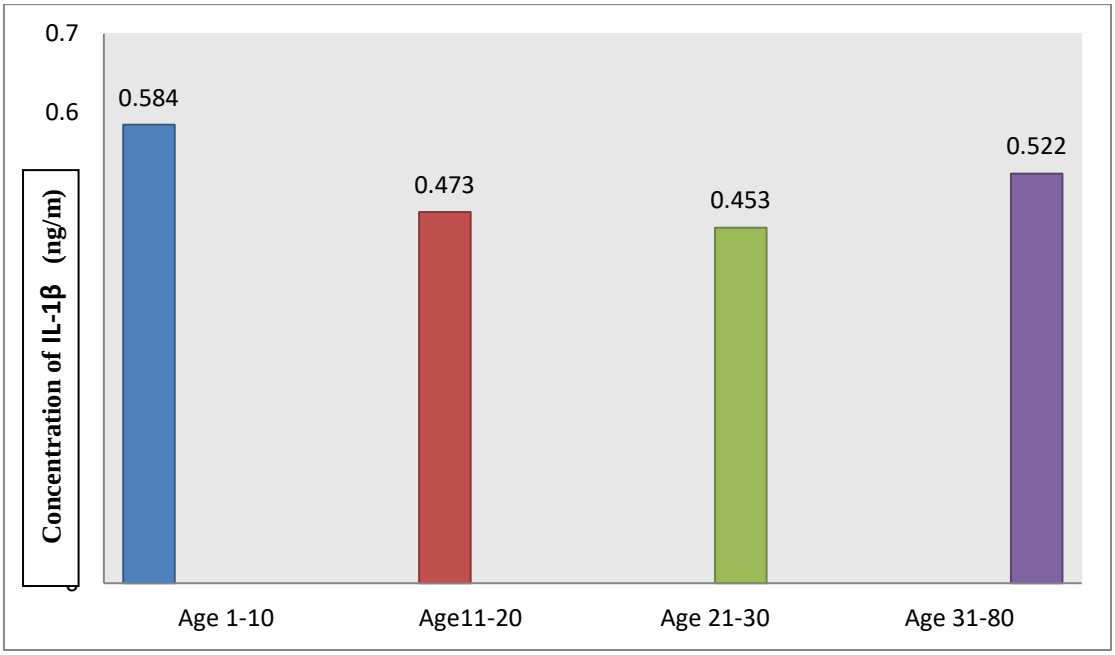

Figure (3-12): Association between age and serum IL-1β in control groups.

The mean concentration c of IL-1β between male and female in patients and control group. The mean concentration of IL-1β level has significant increase in female in comparison to that observed in male in patients group (P = 0.025), table (3-7). Since the mean concentration of IL-1β level in patients group were male (0.695±0.058ng/ml) and female was (0.935±0.091ng/ml). Results showed no statistically significant differences in mean concentration of IL-1β between male and female in control group (P =0.132), were mean concentration of IL-1β in male (0.454±0.024ng/ml) and female was (0.518±0.034ng/ml).

Table (3-7): Comparison of mean serum IL-1β between male and female in patients and control.

	Male	Female	P value
Patients	0.695±0.058 ng/ml	0.935±0.091 ng/ml	P = 0.025
Controls	0.454±0.024 ng/ml	0.518±0.034 ng/ml	P =0.132
P value	p<0.001	p<0.001	

3.4 Effect of gene polymorphism on cytokine secretion.

3.4.1 Association between MIF G-173C Polymorphism and MIF Cytokine.

The mean concentration of MIF cytokine concentration according to genotype in *MIF* gene table (3-8). It was found that the mean concentration of MIF cytokine level were decreased in *MIF* 173C allele (6.66 ± 0.96ng /ml) compression with *MIF* 173G allele (8.696±0.990ng/ml) in patients group (P≤0.05), while in control group on the other hand showed no significantly between genotype in *MIF* gene and mean concentration of MIF cytokine (P=0.698), the mean concentration of MIF cytokine was (98.618±1.776ng/ml) in *MIF* 173C allele and (9.756±2.487ng/ml) in *MIF* 173G allele.

Table (3-8): Mean concentration serum MIF cytokine according to the different genotype of *MIF* gene

Genotype	**Serum MIF Cytokines**		
	Patients	**Control**	*P* Value
GG	8.696±0.990 ng/ml	9.756±2.487 ng/ml	P=0.879
GC/CC	6.66 ± 0.96 ng/ml	8.618±1.776 ng/ml	P=0.658
P Value	P≤0.05	P=0.711	

3.4.2 Association between *NRAMP1* (RS17235409) Polymorphism and (TNF-α and IL-1β) Cytokine.

The mean concentration TNF-α cytokine concentration according to genotype in *NRAMP1* gene, figure (3-20). Result found the mean concentration of TNF-α cytokine level were decrease in (RS17235409) A allele in patients groups (2.527±0.104 ng/ml) compression with G allele (2.761±1.076ng/ml) in patients group (P≤ 0.05). In control group the mean concentration of TNF-α cytokine decrease in (RS17235409) A allele (0.402±0.0262ng/ml) comparison with observed in G allele (0.423±0.084 ng/ml) in control group (P≤0.05).

The mean concentration IL-1β cytokine concentration according to genotype in *NRAMP1* gene, table (3-9). Result found the mean concentration of IL-1β cytokine level were decrease in (RS17235409) A allele in patients groups (0.709±0.64ng/ml) compression with G allele (0.851±0.463ng/ml) in patients group (P≤0.05), In control group the mean concentration of IL-1β cytokine decrease in (RS17235409) A allele in control groups (0.468±0.028ng/ml) compression with observed in G allele (0.490±0.127ng/ml) in control group (P≤0.05).

Table (3-9): Mean concentration serum TNF-α and IL-1β cytokine according to the different genotype of *NRAMP1* gene

Genotype	TNF-α		P value	IL-1β		P value
	Patients	Control		Patients	Control	
GG	2.761±1.076 ng/ml	0.423±0.084 ng/ml	p<0.001	0.851±0.463 ng/ml	0.490±0.127 ng/ml	p<0.001
GA	2.755±0.439 ng/ml	0.411±0.096 ng/ml	p<0.001	0.736±0.278 ng/ml	0.511±0.115 ng/ml	p<0.001
AA	2.318±0.024 ng/ml	0.389±0.108 ng/ml	p<0.001	0.593±0.593 ng/ml	0.466±0.098 ng/ml	P=0.001
P value	P≤0.05	P≤0.05		P≤0.05	P≤0.05	

Chapter Four

Discussion

4. Discussion

4.1. Distribution of Cutaneous Leishmaniasis

4.1.1 Distribution of Cutaneous leishmaniasis according to gender

Cutaneous leishmaniasis is a vector-borne parasitic disease was endemic in many parts of the tropics, subtropics and Southern Europe, the World Health Organization estimate that 1.5 million cases of CL in the world, both *L. major* and *L. tropica* are common etiologic agents of CL in Iraq, CL is endemic in Iraq especially in rural area ,the disease is a self-limiting but it usually takes several months and even years for spontaneous recovery leaving an ugly scar (Control and Prevention, 2003, Postigo, 2010).

The results revealed that (56.7%) of patients were male and (43.3%) were female. This possibly, because males more exposure to infected vectors compared with the females, due to the high percent of males were working or sleeping in open areas (surfaces of houses) with less coverage of body (Arroub *et al.*, 2010).

Some studies have hypothesized that sex difference observed in some parasitic diseases can be attributed to hormonal effects , however controversy still exists regarding the role of sex hormones in immune response (Bailey and Lockwood, 2007). Behavioral factors that are making males more exposed to the sand flies in the environments are probably equally or more important (Stewart and Brieger, 2008)

Previous studies in Iraq were significantly male infectious with CL more than female, such as (Sarhan, 1998) in Baghdad, (Mahmood, 2006) in Tikrit, (Al-Mayale, 2004, Al-Jeboori, 2014) in Al-Qadisiya, (AlSamarai and AlObaidi, 2009) in AL-Haweja city which showed that males of CL patients more than females (57% and 43%) respectively,

and (Sharifi *et al.*, 1998) in Iran, While (Luz *et al.*, 2009) in Brazil showed that 71% of patients were males and that were more than the rate of males in the present study 56.6%.

While (AL-Zaidawi, 1997) and (AL-Atabi, 2014) in Tikrit and Wasit city respectively, who found that, the rate of infection in female was higher than in male, these differences may be explained due to this regions more exposed to insects bite than male patients, where that most of worker in the farms were females.

4.1.2 Distribution of Cutaneous leishmaniasis according to age

The age characteristic of patients who have CL, revealed that the highest frequency of CL patients among (11-20) years (50%), followed by the age group of 1-10 years (23.3%**),** then by age 21-30 years (20%) and the less frequency in the age 31-80 years (6.7%), the reason in that infections less frequency in those people with age more than 15 years (adults) might be because that adults develop resistance and immunity against CL due to their previous exposure to parasite (Kaye and Scott, 2011). These findings are comparable with previous studies in Iraq such as (Al-Mayale, 2004, Kashkool, 2009, Al-Jeboori, 2014) in Al-Qadisiyah province which showed most patients of CL were young people under 20 years and the age between 1-10 years , and showed that the highest rate of infections was in age under 20 years, in addition to (Rahi *et al.*, 2013). In Waist Province demonstrated results consistent with present study, were most of the patients of CL were under 12 years (71.8%), as well as other study in other country like (Khatami *et al.*, 2007) in Iran showed that young people under 20 years are the highest infected group.

However (AlSamarai and AlObaidi, 2009) in Alhaweja District in Kirkuk Province showed that incidence rate of CL infections was (57%) in patients over 20 years old, and the same as (Khalid *et al.*, 2012) in Pakistan were found that most patients were in the age group 25-35 years in percent equal to (51%). From 60 patients, were patients 11-20 years in male (26.7%) and female (23.3%), followed by patients (1-10) years old in male (13.3%) and female (10.0%), then by the patients 21-30 years in male (13.3%) and female (6.7%), in addition last group were the patients 31-80 years in male (3.3%) and female (3.3%) f, this finding was in agreement with that found by (Al-Obaidi, 2000) in Tikrit and (Hussian, 1993) in Baghdad and (AL-Gorban, 1996) in Door town.

4.1.3 Distribution of Cutaneous Leishmaniasis according to geographic distribution

The results showed that the incidence of CL in rural regions was 71.7% more than urban regions 28.3%, The higher percentage of the disease in rural regions, which is agricultural area and may due to exposure to vector and reservoir, this makes them more liable for exposure to causative agents in this endemic area, there are many factors that play an important role in the presence and distribution of cutaneous leishmaniasis lesions in rural areas, including the presence of animals, which is the reservoir for the disease , especially rodents, dogs (Fazaeli *et al.*, 2009, AlSamarai and AlObaidi, 2009). The results finding was agreement with (AL-Hucheimi, 2005, Alvar *et al.*, 2012) in Iraq. However a different result had been recorded by (Al-Mashhadany, 2002, AL-Hucheimi, 2014, Qader *et al.*, 2009, Al-Difaie, 2014, Najim *et al.*, AL-Atabi, 2014) who indicated that the disease was more common in rural regions in Iraq.

4.2 Detection of Genes Polymorphism

4.2.1 Detection of MIF G-173C Polymorphism

Investigates the association between cutaneous leishmaniasis and functionally active polymorphisms in the *MIF* gene. Frequencies of allele (173C Polymorphism) were measured in 60 patients of cutaneous leishmaniasis and 32 controls for this study. Distribution of MIF G-173C polymorphism was detected by PCR-RFLP technique, at this locus there are three genotype; GG, GC and CC with band sizes 268/98 pb,205/ 268/98/63 pb and 205/98 pb respectively, these results agree with previous study in United Kingdom (Makhija *et al*., 2007).

The homozygous wild genotype (GG) (OR= 1.650, 95%CI (0.660-4.125), P=0.282), wild allele G (OR=1.308, 95%CI (0.586.-2.920) P=0.512), mutant allele (C) (OR=0.765, 95%CI (0.343.-1.707) P=0.512) and heterozygous genotype (GC) (OR=0.507, 95%CI (0.200 -1.289) P=0.151). There was no significant difference in genotype distribution between cutaneous leishmaniasis patients and healthy controls. The results refers towards high frequency of GG genotype among patients when compared with healthy controls, in present study were G allele frequencies higher than C allele in both patients and control groups with statically significant ($p \leq 0.05$), and this result agreement with other studies from different countries on healthy persons such as United Kingdom (Donn and Ray, 2004) , United States (Baugh and Bucala, 2002) , Japanese (Nohara *et al.*, 2004), Spain (Amoli *et al.*, 2002) and Germany (Miterski *et al.*, 2004).

The GC genotype was detected in (23.3%) of the patients and in (37.5%) of the controls, moreover, frequency of allele C was more in controls than the patients.

The results showed that CC genotype frequency was showed in patient (3.3%), more than in healthy (0%) and this result agreement with (Covas *et al.*, 2013), which found that CC (13%) in patients more than (5%).

On the other hand, the result of present study found no significant in G allele in cutaneous leishmaniasis patients was higher than in healthy controls, where that no association between MIF G-173C polymorphisms and cutaneous leishmaniasis, however, other factors should be taken into account, such as variant in genetic makeup of their populations, or possibility other factor like the MIF-794CATT5-8 polymorphism and might be associated with different infecting *Leishmania* species, animal host species and vectors found in this area (Alam *et al.*, 2014). Therefore all this factors may be plays essential role in susceptibility to CL.

In Brazil previous study in (2013) reports on the association between *MIF* gene polymorphisms and cutaneous leishmaniasis (Covas *et al.*, 2013), which refer to found associated between MIF-173 C polymorphism and cutaneous leishmaniasis, were C allele was higher in patients with INS (OR= 1.79, p = 0.01).

other genetic studies have associated the MIF 173C allele with increased susceptibility to infections with other disease than cutaneous leshmaniasis, such as Chagas' disease (Torres *et al.*, 2009), tuberculosis (Gomez *et al.*, 2007, Sadki *et al.*, 2010, Li *et al.*, 2012) and severe malaria (Awandare *et al.*, 2009).

4.2.2 Detection of *NRAMP1* ((RS17235409) Polymorphism

Identification of polymorphisms of samples in the given population was thus carried out by restriction fragment length polymorphism analysis for the SNP (RS17235409) in exon 15, *NRAMP1* (RS17235409) Polymorphism was detected by PCR-RFLP technique, at this locus there are three genotype; GG, GA and CA with band sizes 126/79/39 pb,205/ 126/79/39 pb and 205/39 pb respectively , these results agree with previous study in Iran (Fattahi-Dolatabadi *et al.*, 2016).

The homozygous mutant genotype (AA) (OR= 0.149, 95%CI (0.028-0.790), P=0.012), the homozygous wild genotype (GG) (OR= 2.139, 95%CI (0.867- 5.276), P=0.096), wild allele G (OR=2.576, 95%CI (1.243.-5.336) P=0.01), mutant allele (A) (OR=0.388, 95%CI (0.187.-0.804) P=0.01) and heterozygous genotype (GA) (OR=0.913, 95%CI (0.336 -2.480) P=0.858).

The results showed that the effects of (RS17235409) polymorphisms on susceptibility to cutaneous leshmaniasis, were different in frequency of alleles and genotypes (RS17235409) between patients and controls with statistically significant, the finding result was consistent with (Fattahi-Dolatabadi *et al.*, 2016) also with other studies with other disease than cutaneous leshmaniasis includes chronic periodontitis (Kadkhodazadeh *et al.*, 2016) , visceral leishmaniasis (Ejghal *et al.*, 2014), tubereculosis (Pakasi *et al.*, 2012), and *bacillus Calmette-Guerin* (Decobert *et al.*, 2006) while (Ortiz-Flores *et al.*, 2015, Sophie *et al.*, 2017), which showed that no significant difference of alleles between cutaneous leishmaniasis patients and healthy control as well as other studies with other disease than cutaneous leshmaniasis (Gazouli *et al.*, 2008, Brochado *et al.*, 2016, Mehrotra *et al.*, 2011).

However, the results revealed that no association between (RS17235409) polymorphisms and cutaneous leishmaniasis, were the GA genotype was detected in (23.3%) of the patients, and in (25%) of the controls, moreover, frequency of allele A was more in controls than the patients. However, other factors should be taken into account like infecting *Leishmania* species are not the same in all cases. CL is highly endemic with Leishmania major responsible for mostly infections, as well as different vectors and animal host species in the areas of Iraq, in other hand a host Genetic factors play an essential role in host susceptibility, and possibility more SNPs other than (RS17235409) were included in efficient of NRAMP1.

Previous studies like (Samaranayake *et al.*, 2010) in Sri Lanka, showed a lack of association between the GA genotype and CL, and (Sophie *et al.*, 2017) in Pakistan, showed that no association between (RS17235409) polymorphisms and cutaneous leishmaniasis infection, were A allele was higher in patients with INS (OR= 2.447, p = 0.127).

, while (Fattahi-Dolatabadi *et al.*, 2016) in Iran showed that the GA genotype in patient more than control (23.4% and 11.1%) respectively, were found association between the GA genotype and CL infection with INS (OR= 2.455, p = 0.008).

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4.3. Cytokines Concentration (MIF, IL-1β and TNF-α) in the Serum of CL Patients and Control

4.3.1. MIF Concentration

The mean serum MIF concentration has no statistical difference between patients and control group. In Brazil (Covas *et al.*, 2013) found that MIF concentration in patients with cutaneous leshmaniasis more than control groups .

There was a significant correlation between age and serum MIF in patient and control groups, since MIF concentration higher in age group (1-10 years) and then decline continuously with increasing of age in adult.

There was no significant difference in mean serum MIF concentration between male and female in patients and control group. However, variants in MIF secretion mediated by environmental factor as stress were MIF is produced and stored in the pituitary gland and released systemically upon physiological stress, presence of endotoxin ,as well as the genetic factor (Nishino *et al.*, 1995, Bucala, 1996).

4.3.2. TNF- *α* Concentration

The mean concentration of TNF- *α* in patients has significant increase in patients group in comparison with control groupe. This increased expression may be due to an increased level of cellular activation or a relative increase in the number of cytokine-producing cells. The results were in agreement with (Al-Aubaidi, 2011) in Iraq and (Kocyigit *et al.*, 2002) in turkey, where they found circulating proinflammatory TNF- *α* cytokine levels were increased in patients with CL. Regarding to the result, it was found that there was significant correlation between age and mean level of TNF- *α* in serum of CL patients and control group, where increase of TNF- *α* concentration with increase age, except for last age

group 31-80 were less than other age groups. When mean serum TNF-α was studied in relation to gender frequency distribution, there was no significant difference in mean serum TNF-α between male and female in patients and control group .

4.3.3. IL-1β Concentration

The mean concentration of total IL-1β in all CL patients were significant increase in comparison to that observed in their control groups since, the result finding was in agreement with (Al-Aubaidi, 2011) (Kocyigit *et al.*, 2002) in Iraq and Turkey respectively, they found that serum levels of IL-1β increased in patients group in comparison to control group, as well as (Sodhi *et al.*, 1990) in India which found that IL-1β concentration in patients infected with Leishmaniaisis more than control groups .

Tee results revealed that that significant correlation between age and mean level of IL-1β in serum of CL patients, were increase of concentration with increase age. While there was no correlation between age and mean level of IL-1β in serum of control group.

In relation to gender, there was significant difference in mean serum IL-1β between male and female in CL patients group , while no significant in mean serum IL-1β was found between male and female in control group.

4.4 Effect of gene polymorphism on cytokine secretion.

4.4.1 Association between MIF G-173C Polymorphism and MIF Cytokine.

In regard to association between MIF genotype and MIF cytokine expression, data in present study found MIF-173C allele correlation with decrease level of MIF cytokine in CL patients group comparison with MIF 173G allele, this finding may hypothesize that environmental factor play important role in MIF secretion where MIF is produced and stored in the pituitary gland and released systemically upon physiological stress (Nishino *et al.*, 1995, Bucala, 1996).Moreover, possibility, additional polymorphisms mediate in MIF secretion, like the (MIF- 794CATT5-8) polymorphism, which may play a critical role in expression of *MIF* gene. The MIF-173C allele is correlation with less concentration of MIF cytokine, and this lower cytokine might be have effects on susceptibility of leishmaniasis (Oddo *et al.*, 2005).The results agreement with previous study on cutaneous leishmaniasis in Brazil which refer to found associated between MIF G-173C polymorphism and cutaneous leishmaniasis , were suggest that the C allele is associated with lower levels of MIF, and this decreased production may be associated with leishmaniasis susceptibility (Covas *et al.*, 2013).

4.4.2 Association between *NRAMP1* (RS17235409) Polymorphism and (TNF-α and IL-1β) Cytokine.

In a resting macrophage, *NRAMP1*gene encoded to protein, which assembled into the membrane of late endosome, were phagocytosis it is relocated to the membrane of phagosome (Canonne-Hergaux *et al.*, 2002, Kim *et al.*, 2003). *NRAMP1* protein transport divalent metal ions through the phagosomal membrane and might be essential factor for resistance to some microbial infections, *NRAMP1* induce a variant types of

antimicrobial responses of a macrophage, including induction of nitric oxide intermediates and radical oxygen, synthesis and activation of various pro-inflammatory cytokines such as (TNF-α and IL-1β) (Blackwell *et al.*, 2003, Courville *et al.*, 2006).

However, when mutations occur in the *NRAMP1* gene result in a non-functional or unstable protein and then leading to an increased proliferation of parasites in the macrophage might be reason by deficient antimicrobial responses that confer by *NRAMP1* protein (Fritsche *et al.*, 2008, Nugraha and Anggraini, 2011)**.** When *NRAMP1* gene studded, the result showed that allele A was able to induce less TNF-α secretion in patients and control group's compression with allele G.

On other hand when mean serum IL-1β was studied in relation to allele A, there was decrease in mean serum IL-1β comparison to that observed in allele G in patients and control group. were unstable NRAMP1protein because mutation result in less expression in (TNF-α and IL-1β) and resulting in increased susceptibility and proliferation of parasites in the macrophage**,** this result agreement with previous studies including (Fritsche *et al.*, 2008) in United Kingdom, which used murine macrophage cells stably transfected with a functional Nramp1 allele or Nramp1 non-functional, found that the production of and signaling by the anti-inflammatory cytokine TNF-α was significantly decreased in macrophages lacking functional Nramp1.

Conclusions and Recommendations

5.1. Conclusions

1. Distribution of infection in male was more than female.
2. Distribution of infection was highly in age ranging (11-20) year.
3. CL infectious in rural regions was more than urban regions.
4. No significant difference in MIF cytokine level between CL patients and control subjects**.**
5. Cytokines (TNF-α and IL-1β) plays an important role in the resolution of CL infection, their concentration in patients serum of all age groups were increased in comparison to that observed in control groups.
6. MIF G-173C polymorphisms did not affect susceptibility to CL in the population samples.
7. The MIF 173C allele is associated with lower levels of MIF cytokine, and this decreased production associated with leishmaniasis susceptibility.
8. The genetic variations of *NRAMP1* (RS17235409) gene don't associate with susceptibility to CL infection.
9. In polymorphisms of *NRAMP1* (RS17235409) gene, were A allele is associated with lower levels of (TNF-α and IL-1β) comparison to that observed in allele G, and this decreased production may be associated with susceptibility and proliferation of parasites in the macrophage**.**

5.2. Recommendations

1. Furthermore epidemiological study on distribution of cutaneous leishmaniasis in Al-Muthanna province.
2. Al-Muthanna province is endemic city and so is recommended to the healthy institution in this city to carry on wide surveillance to treat the patients especially those in villages which are belong to the districts and they are far from center of city, furthermore wide surveillance in spraying of insecticidal to kill the vectors and using insect repellent.
3. Avoidance of sand fly bites by using insecticides and window barriers, wearing thick cloths especially those were sleeping out-doors, and get rid of reservoir hosts such as rodents and stray dogs. Health programs about CL should be broadcasted through ordinary media.
4. Furthermore screening of cytokines levels in CL patients like IL-1, IL-TNF-α, 1L-4, IL-5, IL-6, IL-8 and IL-12 cytokines and its correlation with polymorphism.
5. More studies about *NRAMP1* polymorphisms are needed to investigate the effects of gene polymorphisms on the expression and function of the NRAMP1 protein, to approve the role of *NRAMP1* polymorphisms in susceptibility to CL infection.
6. Furthermore studies are needed to investigate the effects of *MIF* gene polymorphisms in susceptibility to CL infection by MIF G-173C and additional polymorphisms, such as the MIF 794CATT5-8 microsatellite polymorphism, which might play a role in the regulation of MIF gene expression.

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الخلاصة

داء الليشمانيه الجلدي هو مرض ينتقل عن طريق العض من ذبابة الرمل ، وهو مشكلة صحية حادة في العديد من البلدان ويتوطن في معظم مناطق العراق وخاصة المناطق ذات المجتمعات الفقيره. الهدف من الدراسه هو تحديد التغاير الوراثي في الجينات *Macrophage Migration Inhibitory Factor* و *Natural Resistance-Associated Macrophage Protein1* ومدى تاثيرها على الاصابه بداء اليشمانيه الجلديه في محافظة المثنى.

تم سحب حوالي ٥ مل من الدم ل(٦٠) مريض مصابا بداء الليشمانيه الجلديه خلال مراجعتهم لمستشفى الحسين التعلمي ومركز الحساسية التخصصي في المحافظة و(٣٠) شخص من االاصحاء كمجموعة سيطره ، كان عدد الذكور فيهم ٣٤ وعدد الاناث ٢٦ وبنسبه ذكور الى الاناث ١,٣٠:١ اعمارهم تتراوح بين ١-٨- سنه .حوالي ٣ مل من الدم تم استخدامها لقياس مستوى الحركيات الخلويه باستخدام تقنيه الإلايزا و٢ مل من الدم استخدمت لغرض استخلاص الماده الوراثيه لغرض تحديد التغاير الوراثي في الجينات باستخدام تقنيه تفاعل البلمره المتعدد.

بينت النتائج ان النسبه الاعلى من المرضى الخمجين باللليشمانية الجلدية تراوحت اعمارهم بين ١١-٢٠ سنه، كما بينت النتائج ان (٧١,٧%) من المصابين يسكنون المناطق الريفيه و (٢٨,٣%) منهم في المدن.

تم قياس معدل السايتوكين (Macrophage Migration Inhibitory Factor) في مصل مجاميع المرضى الخمجين باللشمانيه الجلدية والاصحاء حيث ان متوسط معدل تركيز السايتوكين قد انخفض باستمرار مع زياده اعمار المرضى و الاصحاء على التوالي.

اظهر تحليل السايتوكينات (Tumor Necrosis Factor Alpha and Interleukin 1 Beta) زياده معنويه في التركيز واضحه لدى المرضى مقارنه بالاصحاء .كما ان متوسط تركيز السايتوكينات (TNF-α and IL-1β) كان يزداد باستمرار مع زياده التدرج في العمر حيث لوحظ ان معدل التركيز في (TNF-α) يزداد تدريجيا في الفئات العمريه جميعها سواء كان في المرضى او الاصحاء باستثناء (IL-1β) حيث لوحظ ان الفئه العمريه فوق ال ٣٠ سنه سجلت انخفاض في مستوى تريكيز السايتوكين عن بقيه المجاميع العمريه، من جهه اخرى لم يسجل فرق معنوي في الفئات العمريه ضمن مجموعه السيطره بالنسبه للسايتوكين (IL-1β).

لم تسجل هناك فروق معنويه تدل على الاختلاف بالطرز الوراثيه في MIF G-173C بين المرضى والاصحاء ، كما لم يلاحظ اي ارتباط معنوي بين الاصابه بداء اليشمانيا الجلديه والطراز الوراثي MIF G-173C .

اظهرت الدراسه ان نسبة تواجد الاليل C في المرضى هي (١٨,٧٦%) اكثر مما تم تسجيله في مجموعه السيطره (15%) ، كما بينت النتائج ان هناك ارتباط معنوي وثيق بين التغاير الوراثي في جين *MIF* وتركيز السايتوكين MIF ، حيث لوحظ ان الحليل MIF 173C

يقلل من انتاج السايتوكين MIF مقارنه بالحليل MIF 173G ، هذا الارتباط المعنوي لم يسجل في مجموعه السيطره.

بينت النتائج ان هناك فروق معنويه تدل على التباين في (RS17235409) *NRAMP1* بين مجموعه المرضى والسيطره ، كما تشير النتائج انه لا يوجد ارتباط معنوي بين الاصابة بداء الليشمانية الجلديه و الطراز الوراثي RS17235409، حيث ظهر الحليل A بنسبه اعلى في مجموعه السيطره (٣١,٢٥%) اكثر مما هو عليه في المرضى (١٥%) ،كما لوحظ بان التغاير الوراثي الحاصل في RS17235409 ووجود الحليل A له ارتباط معنوي وثيق في انخفاض مستوى افراز السايتوكينات (TNF-α,IL-1β) في المرضى والاصحاء مقارنه مما هو عليه بوجود الحليل G .

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تحديد التغاير الوراثي في الجينات *NRAMP1* , *MIF* وتاثيرها على انتاج الحركيات الخلويه TNF-α, MIF و IL-1β في مرضى داء الليشمانية الجلدية

اطروحة مقدمة
الى مجلس كليه الطب / جامعة القادسية
وهي جزء من متطلبات نيل شهادة دكتوراة فلسفة في علم الاحياء المجهرية الطبية

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