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Study Of Comparison Between Traditional Methods And Molecular Diagnosis Of Cutaneous Leishmaniasis In Al- Muthanna Province

A Thesis

Submitted to the Council of the College of Science Muthanna University in Partial Fulfillment of the Requirements for the Degree of Master Science in Biology/ Microbiology

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بسم الله الرَّحْمَنِ الرَّحِيمِ

نَحْنُ نَقُصُّ عَلَيْكَ نَبَأَهُمْ بِالْحَقِّ ۚ إِنَّهُمْ فِتْيَةٌ آمَنُوا

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الكهف : جزء من الآية 13

Declaration

I certify that the thesis entitled (Study Of Comparison Between Traditional Methods And Molecular Diagnosis Of Cutaneous Leishmaniasis In Al- Muthanna Province) by (Jamila Obeid Mezher) has been prepared under our supervision at the Science college-Muthanna University in partial fulfillment of the requirements for the degree of Master of Science in Biology/Microbiology.

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Recommendations of Head department In view of the available recommendations , I forward this thesis debate by the examining committee .

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We certify, as an examining committee, that we have read the thesis entitled " **Study Of Comparison Between Traditional Methods And Molecular Diagnosis Of Cutaneous Leishmaniasis In Al-Muthanna Province** " and examined the student **"Jamila Obeid Mezher ",** in 15/3/2017, and we found that the thesis meets the standards for the degree of master of Science in Biology/Microbiology.

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DEDICATION

To

…The ideal in my life my mother

… My sister

…The most expensive persons My brothers

Nadhem & Baqer

I present my modest effort

Jamila

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Abstract

This study aimed to compared the sensitivities and specificities of PCR assays used for parasite identification with leishmanial culture and microscopic detection in order to validate these PCR techniques for the molecular diagnosis of cutaneous leishmaniasis .

Mean age of patients' group was 14.4±11.94 years with a range of (1-45 years) while the mean age of control group was 13.75±11.81 and a range of (2-46 years) . Regarding gender , patients group included 45 males (72.58%) and 17 females (27.42%) suggesting that the disease is more common in males with a male to female . Mean age of male patiens was 16.1+12.92 years , while mean age of female patients was 9.91+7.42 years .

The distribution of patients according to residency was as follows : 23 patients (37.15 %) from Al-Warkaa , 10 patients (16.13 %) from Al-Hillal , 9 patients (14.52 %) from Al-Khther , 8 patients (12.9 %) from Al-Salman , 4 patients (6.45 %) from Al-Mamlaha , 3 patients (4.84 %) from Center , 2 patients (3.23 %) from Al-Swir, one patient from Al-Majd (1.61%) , one patient from Al-Najmi (1.61%), one patient from Al-Draji (1.61%) .

Three methods were used to identify the presence of the parasite for purpose of comparison . These were Light Microscopic examination , culture and PCR .

The *Leishmania subtypes in* the present study were distributed as follows: *L. tropica* accounted for 69.35% while *L. major* accounted for 22.58% of cases .

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List of Abbreviations

A	Adenine
APS	Ammonium peroxodisulfate
ARF	Annual Rainfall
bp	Base pair
BLAST	Basic Local Alignment SearchTool
BM	Bone marrow
BSA	Bovine serum albumin
C	Cytosine
CA	Central Asia
CE	Capillary Electrophoriesis
CL	Cutaneous leishmaniasis
DAT	Direct agglutination test
DCL	Diffuse cutaneous leishmaniasis
ddH2O	Double distilled water
Dμ2 (δμ2)	Ddm Delta mu squared
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DMSO	Dimethyl-sulfoxid
Dps	Proportion of shared alleles
EDTA	Ethylenediamine-tetra acetic acid

fg	Femtogram
FP	Filter papers
Fis	In-breeding coefficient
Fst	F-statistic
G	Guanine
HCl	Hydrochloric acid
He	Expected heterozygosity
Ho	Observed heterozygosity
IMM	Infinite Allele Model
ITS	Internal transcribed spacer
K	Number of population
KZ	Khazakistan
Kbp	Kilo base-pair
KDNA	Kinetoplast DNA
MAJ	*L. major*
ME	Middle East indicating South West Aisa
MCL	Mucocutaneous leishmaniasis
MCMC	Markov chain Monte Carlo
MLEE	Multilocus enzyme electrophoresis
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA

NNN	Novy-MacNeal-Nicolle medium
NW	New World
NJ	Neighbor-joining tree
NWA	North West Africa
OIF	Oil immersion field
OW	Old World
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PAGE	Polyacrylamide gel electrophoresis
PKDL	Post kala-azar dermal leishmaniasis
Pg	Picogram
PS	Palestine
RFLP	Ristriction fragment length polymorphism
RNA	Ribonucleic acid
RR	Relative risk
rRNA	Ribosomal RNA
RT-PCR	Reverse-transcriptase PCR
SDS	Sodium dodecyl sulphate
SMM	Stepwise Mutation Model
sp.	Species
ssU RNA	Small sub-unit RNA

T	Thymine
Taq	*Thermus aquaticus*
TBE	Tris borate EDTA
TE	Tris EDTA
TEMED NNNN-	Tetramethylene diamine
TM	Turkmenistan
U	Unit
U	Uracil
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UV	Ultra violet
UZ	Usbekistan
VL	Visceral leishmaniasis
WHO	World Health Organization

Chapter One
Introduction

1.1- Introduction

Leishmaniasis are infection of parasites attributable to a range of *Leishmania* parasites supported by a broad variety of vectors and reservoirs spread on all occupied continents and caused by more than 20 species of *Leishmania* and a in nature common severe infection , it includes cutaneous leishmaniasis , visceral leishmaniasis and mucocutaneous leishmaniasis (Herwaldt , 1999) . Adler and Theodor, (1957) showed that 350 million citizens are at hazard in 88 countries ,(66-22) of which are in the Old World and the New World respectively , and 72 in the emergent countries , with generally rate of (1–1.5) million cases of cutaneous leishmaniasis and 500,000 cases of visceral leishmaniasis .

Cutaneous leishmaniasis is attributable to *Leishmania braziliensis* complex and *Leishmania mexicana* in the New World and by *Leishmania tropica* , *Leishmania major* and *Leishmania aetiopica* complex in the Old World (Ashford ,1996 and 1999 and 2000) . It has been expected that 350 million people are at danger with 500,000 new visceral leishmaniasis cases each year , definite cases of visceral leishmaniasis have been reported from 66 countries , 90% of the world's visceral leishmaniasis load occurs on the East Africa (Sudan , Ethiopia and Kenya) , Indian sub continent (India , Bangladesh and Nepal) and Brazil (WHO1991, 1996 and 1998) . About 90% of cases occur in Iraq , Iran , Syria , Saudi Arabia , Algeria , Afghanistan , Peru , and Brazil (Al-Jawabreh *et al*., 2003).

Cutaneous leishmaniasis is a polymorphic disease , its symptoms ranging from asymptomatic infection to mild nature - limited cutaneous ulcer or to more delayed and common lesions (Al-Majali *et al*., 1997) . This scientific polymorphism may result from changeability either in the parasite pathogenic variety or in the host immune response . This heterogeneity is reflection to result in regular accumulation of different

mutations , sexual recombination , genetic replace and hybridization (Arda and Kamal , 1983) .

Finding of Cutaneous leishmaniasis is hazardous because of the high cost and major toxicity of recent treatment regimens (Vega-Lopez , 2003) . As well , for in cooperation epidemiological and clinical reasons , it is main to recognize the *Leishmania* species in each area , even though different species need various administrative methods , different *Leishmania species* can cause also appearing cutaneous lesions in the like ecological region , unfortunately, the traditional investigative techniques for Cutaneous leishmaniasis have some limits (Al-Rai , 2005) .

Microscopic examinations are inexpensive and rapid , but they have short compassion mostly in chronic lesions (Al-Rai , 2005) . At the same time as , cultures of *Leishmania* are more sensitive , they are vulnerable to microbiological corruption and difficult attributable to exacting growth supplies of special strains (Anis *et al*., 2001) . Also , some strains grow fine over than others *in vitro* and this detail causes careful growth of essential strains through culture in varied infections (Ashford , 1999) .

Molecular techniques , for example polymerase chain reaction (PCR) , permit strict recognition and description of parasites in isolates obtained from patients (Barker, 1989) . Molecular techniques designed for species resolve , PCR-RFLP is proved to be the most susceptible and specific practice (Ayala, 1998) . Skin biopsy and as well materials obtained by skin cut/exudates have been used for PCR in different studies (Ben-Ismail *et al*., 1997) .

1.2 Aims of the study :

The aim of this study was to isolate and identify the cutanoeus leishmaniasis and compare several methods of diagnosis of parasites .

Chapter Two
Literatures Review

2.1. History :

Leishmaniasis has been an earliest society state complicated in South-West Asia and the Arab World reported from instant Assyrians in Mesopotamia and immemorial as the pharaohs ruled in Egypt , it was described by Arab-Islamic scientists resembling Avicenna (Ibn-Sina , 980-1037 A.D.) who wrote a full chapter in his well-known book aristocratic Alkanoun Fi El Tebb calming the chance of mosquitos being concerned in the transmission of the infection , Al-Rhazi (850- 923 A.D.) before described cutaneous leishmaniasis as a disease common in Balk (Afghanistan) and Baghdad (Bray , 1987 ; Oumeish , 1999 , Morsy , 1996 ; Cox , 2002) .

Russell distinct cutaneous leishmaniasis in Aleppo-Syria in 1756 , the town of Jericho is well-known as an very old classic middle for cutaneous leishmaniasis , lively for at smallest amount the last 130 years as established by Robert Ruby's visit to Palestine who wrote in 1873 : " Then you would notice among people in Riha (Arabic name for Jericho) or in dealings with the Bedouins, that almost everyone in the valley had on his hands or face at least one large ugly scar "(Klaus and Frannken, 1999) . Two scientists , De Bermann , the French , (1910) and Hunt emueller , the German , (1914) , with the help of Master mann and Canaan , designated cases of leishmaniasis in Jericho where the later thought that he had made a new discovery and allowed himself to name it as *Plasmosoma jerichoense* .

The people of Jericho called it and still organize , 'Habat Riha'(Jericho button or boil) , Adler and Theodor in 1926 (1957) were the first to confirm that the *Phlebotomine* sand fly, *P. papatasi* , is the vector for cutaneous leishmaniasis in Jericho . They isolated *Leishmania* parasites , called *L. tropica* , from this sand fly species . This was recognized by Naggan *et al.* (1970) and Schlein *et al.* (1982) . Gunders *et al.* (1968) showed that in Jericho the reservoir host for *Leishmania*

parasite which he , then , called *L. major* was *Psammomys obesus* . incomplete doctors unceasing to see cutaneous leishmaniasis patients , e.g., in Jerusalem (Al-Quds) and Nablus (Arda , 1983 and Canaan , 1945).

2.2. The *Leishmania spp.* :

Cutaneous leishmaniasis is some of which may have derivative from even earlier editions from 1500 to 2500 BCE , Persian doctors , including Avicenna in the 10th century CE , provided detailed descriptions of what was called balkh uncomfortable (Le-Blancq and peters , 1986) . In 1756 , Alexander Russell , after examining a Turkish patient , provided one of the clinical descriptions of the disease , doctors in the Indian sub-continent would describe it as *kala-azar* (pronounced *kala -azar*, the Urdu, Hindi, and Hindustani phrase for "black fever", *kala* significance black and *azar* meaning fever or disease) (Lemrani and Nejjar , 2002) .

Evidence of the cutaneous form of the disease in Ecuador and Peru appears in pre-Inca ceramic showing skin lesions and malformed faces seeing posterior to the first century CE in the Americas , some 15th- and 16th-century copies from the Inca period and from Spanish-colonials reference "valley sickness", "Andean sickness", or "white leprosy", which are expected to be the cutaneous form , David Douglas Cunningham , Surgeon Officer of the British Indian army , may have seen it in 1885 without being able to relate it to the disease (Maynard , 1999) .

Study of etiology of "oriental sore", locally known as sart uncomfortable , and in 1898 available the first exact description of the causative agent , correctly described the parasite's relation to host tissues and correctly referred it to the protozoa , however , because his results were published in Russian in a journal with low transmission , his results were not globally recognized during his life time (Morsy , 1996) .

Leishman recognized definite creatures in smears taken from the spleen of a patient who had died from "dum-dum fever" and offered them to be trypanosomes , found for the first time in India , a few months later in 1901 , Captain Charles Donovan (1863–1951) confirmed the definition of what became known as Leishman-Donovan bodies in smears taken from people in Madras in southern India , but it was Ronald Ross who proposed that Leishman-Donovan bodies were the intracellular stages of a new parasite , which he named *Leishmania donovani* (Mostashari *et al.*, 2003) . Charles Donovan was first suggested that relation with the disease *kala-azar* , and was decisively confirmed by Charles Bentley's discovery of *L. donovani* in patients with *kala-azar* (Oumeish , 1999) .

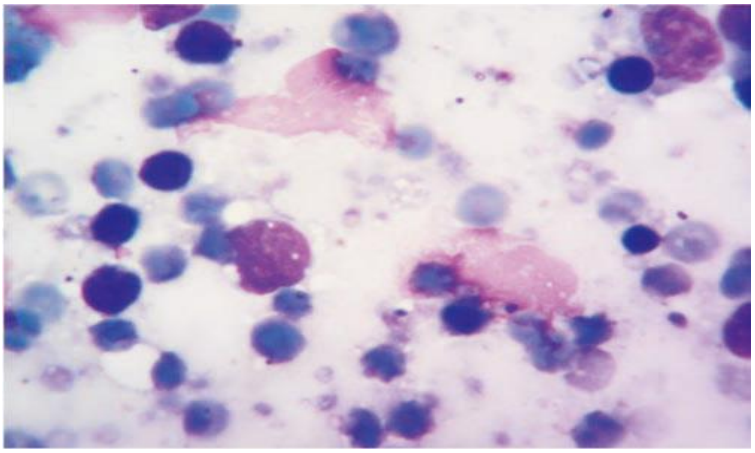

Figure 2- 1: *Leishmania Spp.* amastigotes in collected samples of the liver tissue from *Mays libycus* , Zarqan County, Fars Province , South of Iran , 2012 (1000x). (Al-Jawabreh *et al* ., 2004)

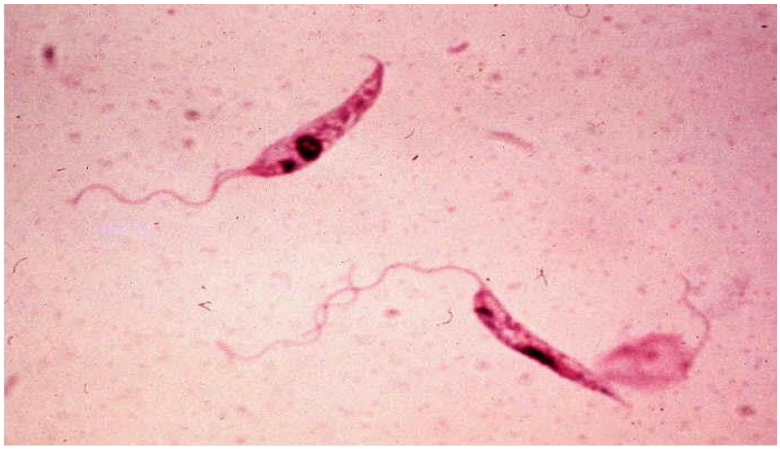

Figure2- 2. Promastigotes : flagellated motile forms found in the vector and in Culture (1000x). (Paetkau *et al.*, 1995) .

2.3.The Life Cycle of *Leishmania* :

Leishmania is a protozoon going to the order of Kinetoplastida and to the family of Trypanosomatidae , the genus *Leishmania* includes more than 20 different species , it exists in two forms : the non-motile intracellular amastigote (3-5 micrometer in diameter) living in macrophages of the mammalian host , and the motile extracellular promastigote (15-30 micrometer in length , plus the flagellum) living in the intestinal tract of the sand fly vector (Dujardin *et al.*, 1995).

The amastigotes are able to survive inside the macrophages and to multiply within the acidic phago lysosomes of these host cells (Belli *et al.*, 1998) . After infection by a bite of a sand fly, pro-mastigotes enter macrophages , transform into amastigotes within (12-24h) and continue multiplication until the host cell dies , the released amastigotes infect other macrophages and the infection spreads , the parasite contains two unusual organelles , the nucleus and the kinetoplast , the kinetoplast is found in all protozoa of the order kinetoplastidae (*Leishmania* , *Trypanosoma* , *Plastocrithidia , Crithidia , Endotrypanum , Herpetomonas , Leptomonas , Phytomonas and Wallaceina*) , it is a rod-shaped mitochondrial structure consisting of a DNA network with two types of DNA: about 10,000 mini circles of approximately 2 kilo base pairs (kb) and 25-250 maxi circles of

approximately 30 kb each , these together constitute the mitochondrial genome (Singh , 2006) .

Dujardin *et al*., (1995) showed that , during its life cycle , *Leishmania* is interchanging between two major forms : as extracellular pro mastigotes in the gut of the sand fly and as a mastigotes inside the macrophages of the mammalian host , when the sand fly feeds , pro mastigotes are injected into the skin and are surrounded by host mono nuclear phagocytes , where they convert into a mastigotes and multiply within the phagolysosomal part until the phagocytic cells are damaged , releasing the parasites to enter extra cells and repeat the cycle (Figure2- 3) .

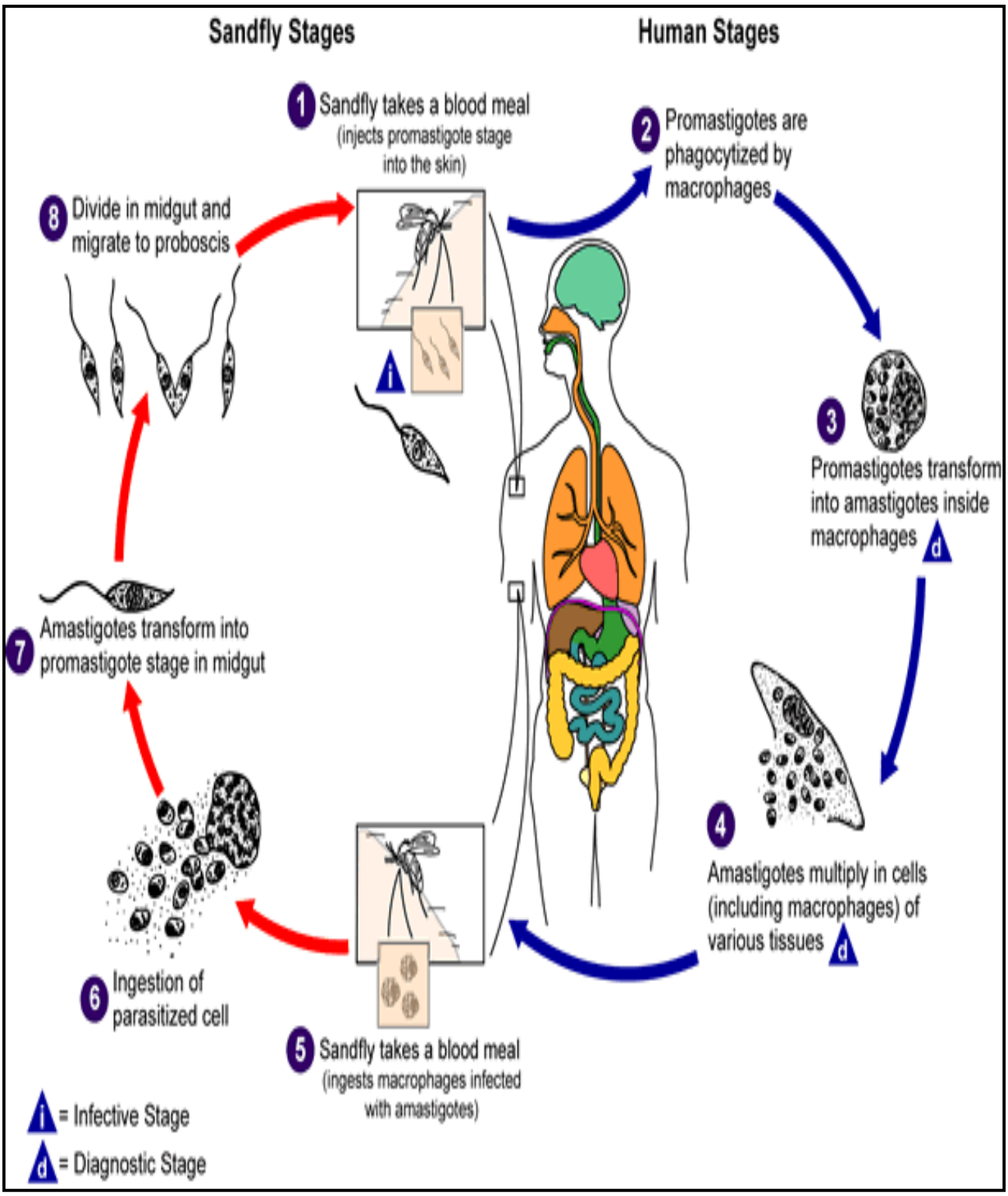

Figure 2- 3: Scheme of the life cycle of *Leishmania* (CDC, 2007) .

2.4 Taxonomy :

Rioux *et al*., (1990) classified the parasite as follows :

Kingdom: Protista

Subkingdom: Protozoa

Phylum : Sarcomastigophora

Subphylum: Mastigophora

Class: Zoomastigophora

Order: Kinetplastida

Family: Trypanosomatidae

Section : Salivaria

Genus: *Leishmania*

Species *: Leishmania tropica*
Leishmania major

2.5. Clinical Symptoms Of Cutaneous leishmaniasis:

In most patients , the skin lesion begins as a small erythematous papule about 2 to 6 weeks after inoculation , over the following month the papule slowly enlarges and a crust develops in its centre , with time , the crust falls away , exposing a shallow ulcer , if no treatment is given , the nodulous ulcer remains stable for 6 to 12 months before undergoing spontaneous resolution , usually a shallow depressed scar is left behind (Klaus , 1999) .

Cutaneous leishmaniasis caused by *Leishmania tropica* and *Leishmania major* are faint on clinical bases as both explode in the same way , the size of the lesion ranging from a few millimeters to 4 centimeter or more , the site and number of lesions(s) are an symptom of the type of cutaneous leishmaniasis , *L. major* usually presents as multiple lesions (≥3) and *L. tropica* is more often on the nose , it was shown that range is

more common in *L. major* infections (30%) than in *L. tropica* (19%) (Klaus , 1999 ; AL Jawabreh *et al*., 2004) .

Ten percent *L. tropica* upset the nose compared with 4% by *L. major* and the chin shows the same configuration , these sites are not the favored ones for either species , the preferred sites for *L. major* are cheeks , arms and legs which account for more than 70% of the cases , while the preferred sites for *L. tropica* are cheeks and arms forming over 50% , abnormal manifestations for *L. major* are the enlargement of regional lymph nodes which is found in about 10% of patients (Al-Jawabreh *et al*., 2004) .

At times , the infection spreads deeply into subcutaneous tissue and muscle (Al-Gindan *et al*., 1989; Vardy *et al*., 1993) . Hyperesthesia or anesthesia around the lesion was reported (Satti *et al*., 1989) . Another unusual clinical presentation of *L. major* cutaneous leishmaniasis is a sporotrichoid pattern , in which subcutaneous nodules develop along lymphatics during the course of the infections (Kubba *et al*., 1987) . Other rare varieties include a hyperkeratotic type , in which a thick adherent scale develops over an otherwise unremarkable lesion , and a chronic form in which the skin lesions remain active for two years or more , cutaneous leishmaniasis , generally, is adversely affected by HIV, the adverse effects are shown by diffuse widespread eruption of lesions over the body which may reach up to hundreds , a rare form of *L. tropica* cutaneous leishmaniasis is known as *Leishmania recidivans* , or lupoid leishmaniasis (Gillis *et al*, 1995).

This is a late manifestation of an *L. tropica* infection that comes years after the infection has resolved , it presents as boggy papules in or around the scars of primary lesions , the papules transform slowly into a spreading *Leishmania recidivans* (Momeni *et al*.,1995; Klaus *et al*., 1999) . There are few reports about *L. tropica* causing visceral leishmaniasis (Kala-azar)

in India (Sacks *et al*.,1995) and canine VL in Morocco (Lemrani and Nejjar , 2002) .

Leishmaniases caused by obligate intracellular protozoan parasites of the genus *Leishmania* , order Kinetoplastida , are cutaneous leishmaniasis inically subdivided into three distinct entities : i) cutaneous leishmaniasis (cutaneous leishmaniasis) caused by *L. major* , *L. tropica* and infrequently *L. infantum* and *L. donovani* , in the Old World and *L. mexicanain* the New World ; ii) visceral leishmaniasis (VL) caused by assembly of the *L. donovani* complex and by *L. tropica* in the Old World and rarely *L. amazonensis* in the NW; and iii) mucocutaneous leishmaniasis (mcutaneous leishmaniasis) caused by *L. braziliensis* , *L. panamensis* , and *L. guyanensis* , in the New World , with reported cases by *L. donovani* , *L. major* , and *L. infantumin* the Old World (Morsy , 1996).

Sometimes a special form of cutaneous leishmaniasis is described as a fourth self-determining unit : diffuse cutaneous leishmaniasis caused by *L. aethiopicain* the OW and *L. amazonensisin* the NW , cutaneous leishmaniasis manifests itself starting from small erythematous papules through nodules and to ulcerative lesions , extraordinary clinical manifestations are sporotrichoid patterns , i.e., subcutaneous nodules developing along lymphatics, and hyperkeratosis, i.e., thick adherent scale , as well as leishmaniasis recidivans , also known as lupoid leishmaniasis (Sacks *et al*., 1995; Desjeux, 1996; Herwaldt, 1999; Saliba and Oumeish, 1999; Bulle *et al*., 2002; Ben Ami *et al*., 2002) .

In the Middle East , it was very difficult and even impossible to distinguish by the clinical picture whether cases were caused by *L. major* or *L. tropica* , visceral leishmaniasis (VL) , or Kala-azar , is associated with prolonged fever, splenomegaly , hepatomegaly , substantial weight loss , progressive anemia , pancytopenia , and hypergammaglobulinemia (Klaus and Frankenburg , 1999) . It can be impaired by serious bacterial

infections and is usually fatal if left untreated (Sundar and Rai, 2002) . A serious follow-up to Kala-azar is post-Kala azar dermal leishmaniasis (PKDL) which appears with in months or years of the cure of VL (Ashford , 2000) .

Mucocutaneous leishmaniasis is a severe form of cutaneous leishmaniasis , as it produces disfiguring lesions and mutilations of the face , nose and throat (Desjeux, 1999) . They usually appear in the mouth and nose where they erode underlying tissue and cartilage , if the lesions spread to the roof of the mouth and the larynx , they may prevent speech (Ashford , 2000) . Other symptoms include fever , weight loss , and anemia , there is always a considerable danger of bacteria infecting the already open sores , diffuse cutaneous leishmaniasis produces spread and chronic skin lesions resembling those of lepromatous leprosy , it is difficult to treat (Ashford , 2000) .

2.6. Epidemiology Of Leishmaniasis :

Improve epidemiological knowledge and assist policy making , the tripod of this activity is the laboratory , physicians/nurses and epidemiologist/statistician , leishmaniasis are parasitic infection caused by a range of *Leishmania* parasites supported by a wide range of vectors and reservoirs spread on all occupied continents (Ashford 1996 , 1999 and 2000) .

Leishmaniasis is widespread in 22 countries in the New World and in 66 nations in the Old World , human infections are found in 16 countries in Europe , including France , Italy , Greece , Malta , Spain and Portugal , leishmaniasis is also found in Mexico , Central America and South America—from northern Argentina

to southern Texas (not in Uruguay, Chile, or Canada) , southern Europe (not common in travelers to southern Europe) , Asia (not Southeast Asia) , the Middle East and Africa (particularly East and North Africa , with some

cases elsewhere) , the disease is not found in Australia or Oceana (Rose *et al*., 2004) .

In Southern Europe , where leishmaniasis is endemic , the incidence of visceral disease is increasing , often in association with HIV-1 infection , many such patients develop unusual cutaneous manifestations , in North America and Northern Europe cutaneous leishmaniasis is a disease seen in returning travelers , such as those conducting rural field studies , tourists and the military , unfortunately many of those infected are ignorant of the risks , take no personal protective measures and experience delays in diagnosis followed by inappropriate treatment upon their return , Mucocutaneous variant of the disease is also endemic in much of South America and is caused by *Leishmania braziliensis* , as with the other parasitic diseases , cutaneous leishmaniasis occurs with greater frequency in needy populations (Schoenian *et al*., 2001) .

Zoonotic cutaneous leishmaniasis is caused by *L.* major in most part of the Central Asia , Middle East and North Africa and transmission of infection is maintained in wild rodent/gerbil colonies , in Central Asia , anthroponotic cutaneous leishmaniasis is commonly caused by *L. tropica* , because it is transmitted anthroponotically (i.e. from human to human) by sand flies, the infection can spread rapidly in concentrated populations , particularly under poor
housing conditions i.e. overcrowding or lack of protection from blood sucking insects (Anis *et al*., 2001) .

In India and Pakistan various endemic belts of simple cutaneous leishmaniasis has been found and the disease is usually caused by *L. tropica* and
man is the most common reservoir , the endemic areas in some Asian countries are shown in also endemic in various regions and causative parasite species is again *L. tropica* , various studies from Afghanistan have shown that the incidence of endemic but sporadic cutaneous leishmaniasis

has dramatically increased during decades of civil war , because of the associated deterioration of the infrastructure and migration , a study conducted in Afghan refugee camps (located in Afghanistan as well as in neighboring Pakistan) , the prevalence of acutaneous leishmaniasis lesions was found to be 1.7-2.7%, and prevalence of scars was 4.2- 4.7% , the analysis of putative risk factors for cutaneous leishmaniasis indicated that living in a stone house reduced the risk , whereas the presence of cows and dogs increased it , the mass return of *Leishmania*-infected refugees to urban areas in Afghanistan poses a particular risk , since housing is often poor and living conditions are crowded (Andresen *et al*., 1996) .

There cutaneous leishmaniasis ear and disturbing increase in the number of cases, e.g. cutaneous leishmaniasis in Brazil (1998: 21 800 cases; 1999: 30 550 cases; 2000: 35 000 cases) , cutaneous leishmaniasis in Kabul , Afghanistan (1994: 14200 cases, 1999: 200 000 cases), and cutaneous leishmaniasis in Aleppo, Syria (1998: 3900 cases; 1999: 4700 cases; 2000: 5900 cases) , this is related to economic development and to behavioral and environmental changes which increase exposure to sand fly vectors , e.g. new settlements , intrusion into primary forest , deforestation , massive migration from rural to urban areas , fast and unplanned urbanization , building of dams , new irrigation schemes (Lighthall and Gianini ,1992) .

Leishmaniasis is a reportable infection in Palestine and all neighboring countries like Jordan , Syria and Saudi Arabia , ministries of Health collect data

for various reasons like healing and control strategies (Ministry of Health , 2004) . Although the law mandate , underreporting is still believed to exist partially due to passive observation , incutaneous leishmaniasis uding only cases coming from cutaneous leishmaniasis inics and hospitals , previously before 1994 when the health authority was in the hands of the

Israeli military rule , there were attempts to progress the reporting system in Palestine (Jaber , 1987) .

In Palestine , two forms of leishmaniasis stay alive , one is the cutaneous leishmaniasis caused by *L. major* or *L. tropica* and the other is VL caused by *L. infantum* , leishmaniasis in common is reported in all Palestinian districts except Gaza strip with an representative incidence rate in West Bank of more than 10 per 100,000 in 2003 (Klaus *et al.*, 1994; Baneth *et al.*, 1998; Anders *et al.*, 2002; Al-Jawabreh *et al.*, 2004 , Jaffe *et al.*, 2004, Schoenian 2001; Ministry of Health , 2004) .

It is also seen in increasing numbers of military and civilian personnel deployed to Iraq , Kuwait and Afghanistan , occurring in several forms , the disease is generally recognized for its cutaneous form which causes nonfatal , disfiguring lesions , although epidemics of the potentially fatal visceral form cause thousands of deaths , annual incidence is estimated at 1-1.5 million cases of cutaneous leishmaniasis , 500,000 cases of VL , overall prevalence is 12 million people and the population at risk is 350 million (Lighthall and Gianini ,1992) .

Several definitions for surveillance have been proposed , that by Langmuir (1963) who stated that ”continued watchfulness over the distribution and trends of incidence through the systematic collection , consolidation , and evaluation of morbidity and mortality reports and other relevant data , together with distribution to those who need to know” is the most common , It is a continuous activity and not simply a study or a survey .

Cutaneous leishmaniasis caused by *L. major* or *L. tropica* is either endemic or epidemic (Ashford , 1999) . The endemic areas are usually identified by active or passive case reporting while the epidemic areas are usually identified by an early warning system , the distribution and epidemiology of both parasites is governed by several factors , these factors are population migration , urbanization , farming , malnutrition ,

climatic factors and global warming , notably biocutaneous leishmaniasis imatic and vegetation zones , and finally ambiguous rodent population fluctuations (Neoumine , 1996; Klaus *et al*., 1999, Ashford , 1999; Anis *et al*., 2001) .

Leishmania *major* and *L. tropica* are restricted to the Old World , mainly in the Mediterranean basin , East Africa , Indian subcontinent , and West and Central Asia . *L. major* cutaneous leishmaniasis is found in low lying arid and semiarid deserts (Klaus , 1999) . *L. tropical* cutaneous leishmaniasis , by contrast , is more common in urban areas and in villages in hilly rural areas (Klaus , 1999) . Examples for *L. major* foci are Jericho in Palestine (Jawabreh *et al*., 2001; Al-Jawabreh *et al*., 2003 and 2004) and Sidi-Bozaid in Tunisia (Ben Ismail *et al*.,1997) .

As reviewed by (Jacobson , 2003) , examples for urban *L. tropica* foci are Baghdad in Iraq , Aleppo in Syria , Kabul in Afghanistan and Sanliurfa in South-east Turkey . Within the past decade , the world's largest *L. tropica* focus was in Kabul (WHO , 2002) . Other smaller foci for *L. tropica* can be found in Shiraz in Iran , Mosul in Iraq , Ashkhabad in Turkmenistan , and Taza in Morocco.

2.7. Diagnosis :

The usual methods of Cutaneous leishmaniasis diagnosis have ranged from clinical depiction and epidemiological data , visualizing the amastigotes by microscopy of stained smears from skin touch specimens or biopsies to in-vitro culturing of the parasite , these conventional methods are however , limited in sensitivity need an practiced hand and do not distinguish between *Leishmania spp.* which differ in virulence and consequently may require different therapeutic regimes and control measures (Reed , 1996 ; Herwaldt , 1999) .

For all this and over the last decade diagnostic tests based on molecular biology techniques i.e PCR , were introduced and proved to be more sensitive and specific , the sensitivity of different diagnostic methods

was the theme of several studies , PCR protocols were given main concern over conventional methods , although few of these studies gave differing results , these discrepancies are due to factors like the different gold standards being used to describe a case of cutaneous leishmaniasis and sampling methods (Van Eys *et al.*, 1992 ; Wilson , 1995 ; Osman *et al.*, 1998) .

In the history of leishmaniasis , several methods were used for classification , description and recognition of the infecting parasites (El-Tai *et al.*, 2000) . These included simple methods such as geographical classification , e.g. Old World in opposition to New World and epidemiological and / or clinical criteria . More highly developed criteria began to emerge starting with EF serotyping (Schnur *et al.*, 1972 and 1977) , iso enzyme analysis (Miles *et al.*, 1980; Evans *et al.*, 1984; WHO, 1990; Andersen *et al.*, 1996) , and mono clonal antibodies (Noyes *et al.*, 1996) .

Then molecular techniques were introduced such as RFLP (Restriction Fragment Length Polymorphism) , kDNA and nuclear DNA/Southern hybridization (Jackson *et al.*,1984; Beverly *et al.*, 1987; Barker , 1989; Van Eys *et al.*, 1989, 1992; El-Tai *et al.*, 2001) , fingerprinting (Macedo *et al.*, 1992) , PCR fingerprinting with non-specific primers (Williams *et al.*, 1990; Tibayrenc *et al.*,1993 ; Pogue *et al.*, 1995 , 1995 ; Schoenian *et al.*,1996, El-Tai *et al.*, 2001) , molecular karyotyping (Lighthall and Gianini ,1992) , and PCR-SSCP (El-Tai *et al.*, 2001) .

However , most of these techniques lack discriminatory power or reproducibility and are not easy to compare when used in different laboratories ,

The clinical diagnosis of cutaneous leishmaniasis according to the case definition (WHO Recommended Surveillance Standards (1999) , is confirmed by demonstrating amastigotes by stained smear microscopy

and/or in-vitro culture . A mastigotes are intracellular parasites forms present in monocytes and macrophages , but they can also be found as extracellular parasites (Klaus *et al*., 1999 and Ashford , 2000) .

In *in-vitro* culture , the flagellated motile promastigotes which are longer than amastigotes , 10-15µm , are usually seen under light microscopy moving rapidly in a zig-zag motion , promastigotes are the form present in the sand-fly vector , the two species are two different parasites with each having a distinct transmission pattern , they cannot , however , be distinguished by morphology , neither microscopically as amastigotes in smears nor as promastigotes by *in-vitro* culture (Klaus *et al*., 1999 and Ashford , 2000) .

Other techniques were needed to diagnose and characterize these two along with other species of *Leishmania* , iso enzyme analysis formed the gold standard for species and strain typing (Le Blancq and Peters , 1986 ; Rioux *et al*., 1990) . It
showed that *L. tropica* is more polymorphic than *L. major* , serological techniques like enzyme-linked immunosorbant assay (ELISA) were valueless for diagnosis (Al-Jawabreh *et al*., 2003) , but others like EF serotyping were employed to serotype the two species from cultured promastigotes (Jaffe and Sarfstein 1987, Schnur *et al*. 1990) .

Molecular methods were used for a more sensitive diagnosis and genotyping , PCR techniques like permissively primed intergenic polymorphic-polymerase chain reaction (PPIP-PCR) (Eisenberger and Jaffe , 1999) and ITS1-PCR (El-Tai *et al*., 2001; Schoenian *et al*., 2003) were able to detect and to identify *Leishmania* parasites at species level . When these and other techniques, like single-strand conformation polymorphisms of the ribosomal internal transcribed spacer 1 (SSCP-ITS1) , were used for strain typing . *L. tropica* was proven to be a more variable species compared to *L. major* (Schönian *et al*., 2001) .

2.8. Multilocus Enzyme Electrophoresis (MLEE) :

Multilocus enzyme electrophoresis (MLEE) is considered the reference method for identification and cutaneous leishmaniasis assification of species and strains, and for studying variability within Leishmania (Rioux *et al*., 1986; Russell *et al*., 1999) . Promastigote mass cultures isolated from specimens are normally used for this analysis , in each run , a World Health Organization (WHO) reference strains is used , the techniques and the zymodeme nomencutaneous leishmaniasis ature adopted are those of Montpellier centre (Le-Blanq *et al*., 1986 ; Nimri *et al*., 2002) .

Multilocus enzyme electrophoresis has the advantage of backing a large data set and a well-managed reference laboratory (Rioux *et al*., 1986) . Multilocus enzyme electrophoresis has some disadvantages , it is expensive , slow and laborious and it is not easy to compare the raw data from different laboratories , the need of mass in-vitro culture makes it unsuitable for high throughput analyses (Andresen *et al*., 1996; Noyes *et al*., 1996; Jamjoom *et al.*, 2002) . A major disadvantage is that it determines phenotypes and not genotypes . In addition , any nucleotide substitution that does not change the amino acid composition remains undetected and the same is true for changes in the amino acid composition that do not influence the electrophoretic mobility (Jamjoom *et al.*, 2002) .

Another disadvantage is that the house-keeping genes analyzed in Multilocus enzyme electrophoresis are most probably under selective pressure so that mutations observed are not neutral , in addition , Multilocus enzyme electrophoresis relies on the assumption that the parasite's isoenzyme types (or zymodemes) represent stable multilocus genotypes , this is only true if genetic recombination is almost absent in natural populations of the parasite (Jiménez
etal., 1997) .

2.9. Microsatellites :

Microsatellites or simple sequence repeats (SSRs) , or Short Tandem Repeats (STRs) , discovered in 1981, are tandemly repeated motifs of 1-6 nucleotides found in all prokaryotic and eukaryotic genomes , they are present in both coding and non-coding regions (Ellergan , 2004) . In addition to being highly variable and polymorphic , microsatellites are also easy to genotype and densely distributed throughout eukaryotic genomes , making them the preferred genetic marker for high resolution genetic mapping (Dib *et al*., 1996; Dietrich *et al*., 1996; Schuler *et al*., 1996; Knapik *et al*., 1998; Cooper *et al*., 1999) .

Di nucleotide repeats dominate, followed by mono- and tetra nucleotide repeats, and tri nucleotide repeats are least dominant , repeats of five (penta-) or six (hexa-) nucutaneous leishmaniasis eotides can also be found , generally , among di nucleotides , (CA)n repeats are most frequent , followed by (AT)n , (GA)n and (GC)n , the last type of repeat being rare (Ellergan , 2004) . Microsatellite loci are characterized by high heterozygosity and the presence of multiple alleles , which is in sharp contrast to unique DNA sequences (Ellergan , 2004) .

Microsatellites account for 3% of the human genome (International Human

Genome Sequencing Consortium , 2001) . The genome of *Leishmania spp.* is relatively rich in micro satellites with about 600 (CA)n loci per haploid genome , currently , a *Leishmania spp.* genome project is being carried out which showed that *L. major* Friedlin genome , for instance , is 32.8Mb in size , with a karyo type of 36 chromosomes (Rossi *et al*., 1994) .

There are more applications of microsatellite analyses other than gene mapping which are ancient and forensic DNA studies : e.g. population genetics and conservation/management of biological resources (Jarne and Lagoda , 1996) , assessment of population subdivision and phylogenetic relatedness (Queller *et al*., 1993) , parentage analysis , phylogenetic

studies (Bowcock *et al*., 1994) , studies looking at population differentiation (Paetkau *et al*., 1995) and measuring inbreeding (Coltman *et al*.,1998; Coulson *et al*., 1998) .

2.10. Mutation Mechanism :

Microsatellite sequence variation results from the gain and loss of single repeat units or a single nucleotide . The most plausible explanation of loss of repeat units is slippage of polymerase during DNA replication (Schloetterer and Tautz 1992) . This is transient dissociation of the replicating DNA strands followed by misaligned re-association thought to be due to DNA polymerase pausing and then dissociating from the DNA (Levinson and Gutman , 1987; Ellergan , 2004) .

Replication slippage also occurs during PCR amplification of microsatellite sequences *in vitro* , characterized by the presence of 'stutter bands'that is minor products that differ in size from the main product by missing or additional repeat units (Shinde *et al*., 2003) . It is worth noting that most of these primary mutations *in vivo* are corrected by the mismatch repair (MMR) system , and only the small fraction that was not repaired ends up in the form of variable microsatellites (Strand *et al*., 1993; You *et al*., 2002) .

Replication slippage caused by dissociation of the two strands , re-aligning of the nascent strand out of the register (left) and then continued replication as part of the mismatch repair , this will cause misalignment producing a loop in the nascent strand and increase the repeat length , alternatively , the same will take place but on the template strand (right) causing decrease of repeat length , the emergence of microsatellite variation is explained by the 'length and point mutation model'(Ellergan , 2004) .

It is based on the existence of two opposing mutational forces operating on microsatellite sequences : length mutations , the rate of which increases with increasing repeat count , whereas point mutations

break long repeat arrays into smaller units . At equilibrium , there will be a steady-state distribution of repeat lengths governed by the rate of length mutation and the rate of point mutation (Dieringer and Schlotterer , 2003; Bell and Jurka , 1997; Ellergan , 2004) .

The rate of mutation at a given microsatellite locus is influenced by various factors : the repeated motif itself , allele size , chromosome position , GC content in flanking DNA , cell division (mitotic vs. meiotic) and the mismatch repair system (e.g. mutations at MMR genes) which is critical for the stability of the STR (You , 2002) .

In addition , recombination has been presented as a potential explanation for mutation , recombination changes the STR length/repeat number by unequal crossing over or by gene conversion (Brohele and Ellegren , 1999; Hancock , 1999; Jakupciak and Wells , 2000; Richard and P.ques , 2000 ; You , 2002) .

2.11. Vector :

It is commonly known that *L. Major* is transmitted by females of mainly *Phlebotomus papatasi* and *L. tropica* by mainly *P. sergenti* (Diptera , Nematocera , Psychodidae , Psychodinae) , sand flies are smaller than 3 mm and spend the day in burrows and cracks to prevent drying out , the female flies are active during the evening , hopping around silently for their blood meals for reproduction , sand flies have biting season during which they are active in transmission of infection , in the Middle East it extends from April/May to September/October (Killick-Kendrick , 1999; Jacobson , 2003; Wasserberg *et al*., 2003) .

There are other vectors for *L. tropica* such as *P. (Larroussius) guggisbergi* in Kenya with an infection rate of 4.3% and *P. (L.) arabicus* in Tiberias with an infection rate of 5% , vectors are either specific or permissive , permissive vectors like *P. argentipes* , *P. (L.) arabicus* , *P. perniciosis* and *P. halepensishave* O-glycosylated receptors on their mid

gut epithelium supporting the adherence of different *Leishmania spp*. (Lawyer *et al*., 1991 ; Jacobson *et al*., 2003) .

In contrast , specific vectors like *P. sergenti* and *P. papatasi* lack these receptors and harbour only single species of *Leishmania* , *L. tropica* and *L. major* , respectively (Peckova *et al*., 2005) . This is in agreement with the finding of Kamhawi *et al*. (2000) that *P. sergenti* does not support *L. major* and *L. donovani* .

2.12. Reservoir :

Cutaneous leishmaniasis has been given various names in different civilizations such as "Delhi boil" in India , "Baghdad boil" in Iraq and "saldana" in Afghanistan , the disease can present in various unusual clinical variants that can be difficult to diagnose , such as paronychial , chancre form , annular, palmo plantar , zosteri form and erysipeloid forms , the geographic distribution of cutaneous leishmaniasis is mainly determined by the sand fly vectors (*Phlebotomus spp*. and *Lutzomyia spp*.) (Killick-Kendrick , 1999) .

They live in dark , damp places ; these vectors do not fly high or far and they have a range of only 50 meters from their breeding site , Sand flies become infected through feeding on infected animals , once a sand fly is infected , it can transmit the parasite to both humans and animals for the rest of its life , unlike mosquitoes , they fly silently and their small size (2-3mm) allows them to penetrate through mosquito nets (Van Eys *et al.*, 1992 ; Wilson , 1995 ; Osman *et al.*, 1998) .

They are most active in the evening and at night , most infections exist as zoonoses among wild animals , such as rodents and dogs , and are most prevalent in rural or forest areas , although man is usually an incidental host , such infections are by no means uncommon , in endemic areas , up to 9 percent of the healthy population may have a positive Leishman in skin test , indicative of an earlier , often asymptomatic infection , in India and Pakistan simple cutaneous leishmaniasis is usually

caused by *Leishmania tropica* and man is the most common reservoir (Dujardin *et al*., 1995) .

A reservoir host is the ecological system in which an infectious agent survives persistently , cutaneous leishmaniasis is either anthroponotic where infection is transmitted by the vector from man to man , or zoonotic where an animal reservoir host is involved , *L. major* is completely zoonotic with various animals being confirmed as reservoirs (Ashford , 1996) . In North Africa and South-west Asia it is *Psammomys obesus* , a rodent living in underground burrows , in Iran and Central Asia , gerbils are the common reservoirs , either *Meriones libycus* or *Rhombomys opimus* (Klaus , 1999; Ashford , 1996) .

L. major has also been isolated from *Meriones shawi* and *Meriones lybicus* (Rioux *et al*., 1986 ; Ben-Ismail *et al*., 1987) . cutaneous leishmaniasis caused by *L. tropica* is anthroponotic in urban areas and zoonotic in rural villages (Klaus *et al*.,1999) . In several rural areas in Kenya and near Lake Tiberias , *L. tropica* has been isolated from hyraxes (*Procavia capensis*) (Ashford and Sang , 2001; Jacobson *et al*., 2003).

2.13. Treatment :

Most cutaneous leishmaniasis cases heal spontaneously in less than a year , however , living for one year with a lesion in the face which may be disfiguring and may complicate due to secondary bacterial infection is problematic , treatment is the sole choice for these patients , Dowlati (1996) reviewed types of therapeutic strategies for cutaneous leishmaniasis , among the methods tried with varying degrees of success are thermo therapy , cryo therapy with liquid nitrogen and surgery (Dowlati , 1996; Al-Majali *et al*.,1997; Reithinger *et al*., 2005) .

The most common treatment in most parts of the world is the intra lesional application of pentavalent antimonials , e.g. sodium stibgluconate commercially known as Pentostam (Glaxowellcom) , the dose per lesion is 0.2-0.4 ml (100 mg/ml) or 15-20 mg /kg/day for 15-20 times every day ,

more or less depending on the lesion and its response to treatment (Croft and Yardley , 2002) . Pentostam can be given intramuscularly (IM) or intravenously (IV) depending on the progress and stage of the lesion (WHO , 1990) .

In the case of systemic treatment (IV) , a patient should be hospitalized and liver enzymes monitored for toxicity , Meglumine antimonate or Glucantime are other forms of antimony compound that was used once , topical ointments have been also used , Paromomycin , in combination with methyl benzethonium chloride gave different rates of success when applied in areas where either *L. major* or *L. tropica* is endemic , *L. tropica* tend to be less responsive to therapy , in a few cases it took more than 6 months for large lesions on the nose to heal up , to allow development of lasting immunity , patients with lesion(s) less than 3 weeks old are not treated and advised to come back after the 3-week period had elapsed (Klaus *et al*., 1999) .

2.14. Control :

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 (Klaus *et al*., 1999; Ashford , 1996) .

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 . Plants like Bougainvillea glabra were shown to decrease the risk for leishmaniasis by reducing the life span for sand

flies (Schlein *et al*., 2001) . Impregnated bed nets with various insecticides such as Delta methrin were applied as control measure with significant reduction in cutaneous leishmaniasis incidence rate (Alten *et al*., 2003) .

The human host was also a means for control either by 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).

2.15. Population Genetics Of *Leishmania* Parasites :

Population genetics dates back more than one hundred years , yet its modern form emerged only during the 1970s (Wakeley and Takahashi , 2003) . Hart l and clark (1997) defined population as a group of organisms of the same species living within a sufficiently restricted geographical area that any member can potentially mate with any other member , this definition bounds the population by mating of same species in a restricted geography , population genetics can be defined as 'the mathematical study of the dynamics of genetic variation within species .

Its main purpose is to understand the ways in which the forces of mutation , natural selection , random genetic drift , and population structure interact to produce and maintain the complex patterns of genetic variation that are observed among individuals within a species' (Wakely , 2005) . Clonality vs sexuality debate As for any parasite , the mode of reproduction of *Leishmania* can either be sexual or asexual (clonal) , sexual reproduction means passing half of the genes to the new progeny while asexual or clonal means passing all of the genes to the progeny (Ayala , 1998) .

Natural selection favors asexual reproduction , because given the same number of progeny , the asexual individual has double the fitness of the sexually reproducing one (Ayala , 1998; Victoir and Dujardin , 2002) .

Sexual reproduction has the advantage of creating variability for adaptation to changing environments , but has the disadvantage that advantageous gene combinations may be disrupted (Victoir and Dujardin , 2002) . Since sexual recombination in *Leishmania* seems to be either absent or very rare , the "clonal theory" was proposed to explain the population structure of different *Leishmania spp*. (Panton *et al*., 1991; Tibayrenc *et al*., 1990; Dujardin *et al*., 1995) .

According to this theory , genetic variability is due to gene mutations and their selection along clonal lineages . Clonality , but without solid prove was preferred by Lanotte *et al*.(1986) and Lainson and Shaw (1986) . Studies on *Trypanosoma cruzi* , another kineto plastid parasite , and other parasites concluded that natural populations reproduce predominantly clonally (Tibayrenc and Ayala , 1988; Tibayrenc *et al*., 1990) .

Ayala ,(1998) lists three reasons : First , in a sexually reproducing organism the individual genotype is ephemeral , the entity that persists and evolves is the gene pool and a few individuals encompass most of the genetic variability of the species , 0n the contrary , for a clonal organism , the entity that persists and evolves is the clonal lineage and the genetic diversity of the species can be captured only by extensive sampling of distinct lineages , second , extensive

genetic divergence among clonal lineages may reflect diverse biological characteristics , including pathogenicity , resistance to drugs and vaccines and other clinical parameters , third , in clonal organisms , epidemiological surveys , medical typing and drug development should be based on identification and characterization of clonal lineages , targeting those that are more pathogenic or ubiquitous (Dujardin *et al*., 1995) .

2.16. Genetic Diversity And Bottleneck Theory :

A population bottleneck (or genetic bottle neck) is usually defined as an evolutionary event in which a significant percentage of a population or species is killed or otherwise prevented from reproducing and the

population is reduced by 50% or more , bottlenecks reduce genetic variation and strongly disrupt the pattern of allele frequencies especially if the population has a low growth rate or high reproductively skew defined as a high variance in the reproductive success of either males or females (Hoelzel , 1999) .

It increases genetic drift which is inversely proportional to the population size, the overall and lasting result of a bottleneck is the reduction of genetic diversity , re distribution and reduction of allele frequencies and disappearance of rare alleles (Hoelzel , 1999 and 2002) . The immediate and transient observation following a bottleneck event is , however , the un expected increase in heterozygosity level (Cornuet and Luikart , 1996) due to sudden and rapid loss of rare alleles and resulting deficit of alleles (Hoezel , 2002) .

The bottle neck phenomenon has a detrimental effect on the population as the loss of genetic diversity and reduced polymorphism hinders the potential of a population to respond to a changing environment (Hoelzel , 1999) . This impact of bottle neck is dependent on two factors : the effective size of the population , and
the duration for which the population remains small , however , the duration of the bottle neck effect can be minimized if the growth rate of the population is high (Hoelzel , 1999) , the bottleneck hypothesis was often used to explain observations of low genetic variation (O'Brien *et al.*,1987; Gottelli *et al.*, 1994) .

An important aspect about bottle necks is that they may lead to the introduction of new species (Dodd and Powell , 1985; Ringo *et al.*, 1985; Meffert and Bryant , 1991; Galiana *et al.*, 1993) , though it is not the only reason for the rise of new species (Turelli *et al.*, 2001) .

Chapter Three
Materials and Methods

3.1 Materials

3.1.1. Equipments and Instruments

Table (3-1): The equipments and instruments that used in this study with their companies and countries of origin:

No.	Equipment & instrument	Company
1	Camera	Samsung /China
2	Disposable syringe 10 ml	Sterile EO. / China
3	Electrophoresis	Bioneer/ korea
4	Eppendorf tubes	Bioneer/ korea
5	Exispin vortex centrifuge	Bioneer/ korea
6	High Speed Cold centrifuge	Eppendorf /Germany
7	Micropipettes 5-50, 0.5-10, 100-1000µl	Eppendorf/ Germany
8	Nanodrop	THERMO/ USA
9	Oven	Memmert/Germany
10	Refrigerator	Concord /Lebanon
11	Sensitive Balance	Sartorius/Germany
12	Thermocycler PCR	BioRad /USA
13	Vortex	CYAN/ Belgium
14	Water Bath	Memmert/Germany

3.1.2. Kits

Table (3-2): The kits used in this study with their companies and countries of origin:

No.	Kit	Company	Country
1	**Genomic DNA Extraction Kit**	**Bioneer**	**Korea**
	Binding buffer		
	Proteinase K		
	Elution buffer		
	Washing buffer1		
	Washing buffer2		
	Binding column		
	Collection tube 2ml		
2	**AccuPower® PCR PreMix**	**Bioneer**	**Korea**
	Top DNA polymerase 1U		
	dNTP (dATP, dCTP, dGTP, dTTP) each: 250µM		
	Tris-HCl (pH 9.0) 10mM		
	KCl 30mM		
	MgCl2 1.5mM		
	Stabilizer and tracking dye		

3.1.3. Primers

The kinetoplast DNA (kDNA) Nested PCR primers for *L. major* and *L. tropica* were design and provided by (Bioneer company, Korea) as following table :

Table (3-3) : The primers with their sequences :

Primer	Sequence		Length	Temperature
Primary primer	**F**	**CGAGTAGCAGAAACTCCCGTTCA**	**23**	**59.3 ^{O}C**
	R	**ATTTTTCGCGATTTTCGCAGAACG**	**24**	**63.9 ^{O}C**
Secondary primer	**F**	**ACTGGGGGTTGGTGTAAAATAG**	**22**	**53.6 ^{O}C**
	R	**TCGCAGAACGCCCCT**	**15**	**52.5 ^{O}C**

3.1.4. Chemicals

Table (3-4): The chemicals with their companies and countries of origin used in this study:

No.	Chemical	Company and Origin
1	Agarose gel	Promega (USA)
2	Ethanol (96%)	Himedia (India)
3	Ethidium bromide	BIO BASIC INC/ USA
4	Nuclease free water	Bioneer/ Korea
5	TBE buffer	BIO BASIC INC/ USA
6	Loading dye	Bioneer/ Korea
7	(100bp) DNA ladder	Bioneer/ Korea

3.2. Methods

3-2-1. Data collection from patients :

A total of 62 cutaneous leishmaniasis were collected from patients who attended Al-Hussien hospital in Samawa from a period December 2014 to April 2015 .

Routinely diagnostic methods were done on these samples :

-Microscopical examination to detection a mastigote .

-Culturing these samples in NNN media to detection promastigote .

-Molecular method to detect the specimens of parasites (*L. tropica* , *L. major*) . Appendix-A .

3-2-2- Sample Collection :

The sample from the cutaneous lesion taken by aspiration with fine needle, Sterile syringe of (1) ml contain 0.9 ml of sterile normal saline was used to inject the fluid intra dermal through intact skin in to the active red border of the lesion , Aspirate the injected fluid as the needle draw back until the bloody stained fluid aspirate .

3-2-3-Direct Giemsa Stain:

1- A total of 20 micro liters were put on a glass slide and then brushed the glass droplet on a glass slide using another slide.
2-Proven swab using methanolic alcohol one minute duration.
3-examined prepared swabs and dye Giemsa stain compound microscope and on the strength of 100x magnification lens oily slide where examined to detect the presence of the parasite.

3-2-4-Culture on Novy-MacNeal-Nicolle(NNN) medium :

After sterilization of lesion area by ethyl alcohol 70% took a biopsy of the strong edge of ulcers and placed directly over the medium (NNN) record and pre configured and under sterile conditions Added 50 micrograms of antibiotic Gentamycin and sterility of the solution using the closed. Farm incubated at 23 ° C in an incubator for a period of one month

and examined every four days by compound microscope to follow the growth of the parasite , medium consists of the following **:**

1-NaCl	6.0gram
2-Agar	14.0gram
3-Distill water	900ml
4-Defibrenated rabbit blood	100ml
5-Gentamycin	50mg

3-2-5- Hematological parameters:

Collect blood samples from patients and controls and put in EDTA tubes for measured Hemoglobin level (Hb),white blood cells count (WBC's), and Eosinophil by sedimentation rate (ESR) in Ruby apparatuses in Al-Hussien hospital in Samawa

3-2-6- Genomic DNA Extraction:

Genomic DNA was extracted from wound lesion fluid by using AccuPrep®Genomic DNA extraction kit (Bioneer , Korea) and done according to company instruction as following steps:

1. Two hundred µl of wound lesion fluid was transferred to sterile 1.5ml micro centrifuge tube , and then added 20µl of proteinase K and mixed by vortex for one minutes .
2. After that , 200µl of Binding buffer were added to each tube and mixed by vortex for 25 seconds to achieve maximum lysis efficiency , and then all tubes were incubated at 60℃ for 10 minutes.
3. A 100µl of isopropanol were added to mixture and mixed well by pipetting , 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 tables were closed and centrifuged at 8000 rpm for 1 minute .

4. Throughout lysate was discarded in disposal bottle , and then 500µl Washing buffer 1 (W1) was added to each Binding filter column, and centrifuged at 8000 rpm for 1 minute .
5. Throughout washing buffer 1 was discarded in disposal bottle , and then 500µl washing buffer 2 (W2) was added to each Binding filter column , and centrifuged at 8000 rpm for 1 minute .
6. Throughout washing buffer 2 was discarded in disposal bottle , and then the tubes were centrifuged once more at 12000 rpm for 1 minute to completely remove ethanol .
7. After that , GD Binding filter column that containing genomic DNA was transferred to sterile 1.5ml micro centrifuge tube , and then added 50µl of Elution buffer and left stand the tubes for 5 minutes at room temperature until the buffer is completely absorbed into the glass filter of Binding column tube .
8. Finally , tables were centrifuged at 8000 rpm for 1 minute to elute DNA , and storage was at -20℃freezer .

3-2-7- Genomic DNA concentration examination

The extracted genomic DNA was checked by using Nanodrop spectrophotometer (THERMO . USA) , which measured DNA concentration (ng/µL) and checked the DNA purity by reading the absorbance at (260 /280 nm) as following steps:

1. After opening up the Nanodrop software , the appropriate application was chosen (Nucleic acid, DNA).
2. A dry chem-wipe was taken and cleaned the measurement pedestals several times. Then carefully pipette 2µl of free nuclease water onto the surface of the lower measurement pedestals for blank the system.

3. The sampling arm was lowered and clicking OK to initialize the Nanodrop, then cleaning off the pedestals and 1µl of blood genomic DNA was added to measurement.

3-2-8- Nested PCR master mix preparation

Nested PCR master mix were used by AccuPower® PCR PreMix (Bioneer , Korea) , and done according to company instructions as following table (3-5) :

Table (3-5): Nested PCR master mix preparation with their volumes

PCR master mix	Volume
Genomic DNA	5µL
Primary primers forward (10pmol)	1.5µL
Primary primers reverse (10pmol)	1.5µL
PCR water	12 µL
Total	**20µL**

After that, these PCR master mix reaction components that mentioned above, placed in standard PCR tubes containing the PCR PreMix as lyophilized materials containing all other components needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, MgCl2,stabilizer, and tracking dye). Then the tube placed in Ex is pin vortex centrifuge for 3 minutes. Then transferred in PCR thermocycler.

3-2-9- Primary thermocycler reaction conditions:

Polymerase Chain Reaction Thermocycler conditions was designed for primary primer was done according to as following table (3-6) :

Table (3-6): primary thermocycler reaction condition

PCR cycle	repeat	Temp.	Time
Initial denaturation	1	95C	5min
Denaturation	30	95C	30sec.
Annealing		55C	30sec
Extension		72C	1min
Final extension	1	72C	5min

Table (3-7): PCR master mix with their volume

PCR master mix	Volume
Primary round PCR product	3µL
Secondary primers forward (10pmol)	1.5µL
Secondary primers reverse (10pmol)	1.5µL
PCR water	14 µL
Total	20µL

After that, these PCR master mix reaction components that mentioned above, placed in standard PCR tubes containing the PCR PreMix as lyophilized materials containing all other components needed to

PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, MgCl2,stabilizer, and tracking dye). Then the tube placed in Exispin vortex centrifuge for 3 minutes. Then transferred in PCR thermocycler.

3-2-10- Secondary thermocycler reaction conditions :

Polymerase Chain Reaction Thermocycler conditions was designed for primary primer was done according to as following table (3-8):

Table (3-8): Secondary thermocycler reaction conditions .

PCR cycle	repeat	Temp.	Time
Initial denaturation	1	95C	5min
Denaturation	30	95C	30sec.
Annealing		55C	30sec
Extension		72C	1min
Final extension	1	72C	5min

3-2-11-Gel Electrophoresis:

Polymerase Chain Reaction products were analyzed by loading in 1% Agarose as following steps:

1- one hundred percent agarose gel was prepared in using 1X TBE and dissolving in water bath at 100 °C for 15 minutes, after that, left to cool 50°C.

2- Then 3µL of ethidium bromide stain were added into agarose gel solution.

3- Agarose gel solution was poured in tray after fixing the comb in the proper position after that , left to solidify for 15 minutes at room temperature , then the comb was removed gently from the tray and

10µl of PCR product were added in to each comb well and 5ul of (100bp Ladder) in one well .

4- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt and 80 AM for 1hour.

5-Polymerase Chain Reaction products were visualized by using ultraviolet trans illuminator.

3-2-12- Statistics analysis :

Data were analyzed , summarized and presented using SPSS (statistical package for social science) version 16 and Microsoft office excel 2010 .

Numeric variables were expressed as mean ± SD (standard deviation) , while nominal variable were expressed as number and percentage . Mann Whitney U- test was used to study difference in medium between two groups .

Chi – square test was used to study association between nominal variables . Kruskal Walls test was used to study difference in medium among more than two groups . Person correlation coefficient was used to study correlation . Sensitivity = true positivity *100/(true positive +false negative) , Specificity = true negative *100/(true negative + false positive), equations were used to calculate the value sensitivity and specificity . P. value was consider significant when it was $P \leq 0.05$.

Chapter Four
Results

4-Results

4-1- Demographic characteristics of the study groups

A comparison mean age between patients' group and control group, in which there was no significant difference . The current study showed the mean age of patients' group was 14.4±11.94 years with a range of (1-45 years) while the mean age of control group was 13.75±11.81 and a range of (2-46 years) , the P-value was (0.762) , the present study above mentioned results ensured a statistical match between patients' group and control group which is a basic requirement for such a cases control study (Table 4-1) .

Table 4-1: Mean age and age range in patients and control groups

Groups	N	Mean	SD	Minimum	Median	Maximum	P-value
Control	60	13.75	11.81	2	9.5	46	0.689*
Patients	62	14.40	11.94	1	10	45	

Mann Whitney U test

Standard deviation was relatively high due to a wide range of age in patients group and this reflected the trend of such disease to be seen in a wide range of age and being limited to a narrow age group . Median age was relatively less than the mean age in both groups giving an impression that the age variable is not normally distributed . This fact was proven by the application of Shapiro-Wilk test which showed a significant deviation in patients distribution by age from normal bell distribution (P<0.001) . For that reason Mann Whitney U test was used instead of t-test for purpose of comparison .

The present study showed the distribution of patients and control groups according to 10 years age intervals . Two important findings can be concluded from this , the first one is that the majority of patients were children (27% <10 years and 43.55 % 10-19 years) . The second point is

that the disease can affect any age and patients older than forty years were identified (Table 4-2) .

Table 4-2: Distribution of patients and control subjects according to 10 years age intervals

	Control		Patients	
Age intervals	**No.**	**%**	**No.**	**%**
<10 years	30	50	27	43.55
10-19 years	14	23.33	19	30.65
20-29 years	8	13.33	8	12.90
30-39 years	6	10.00	4	6.45
>40 year	2	3.33	4	6.45
Total	60	100.00	62	100.00

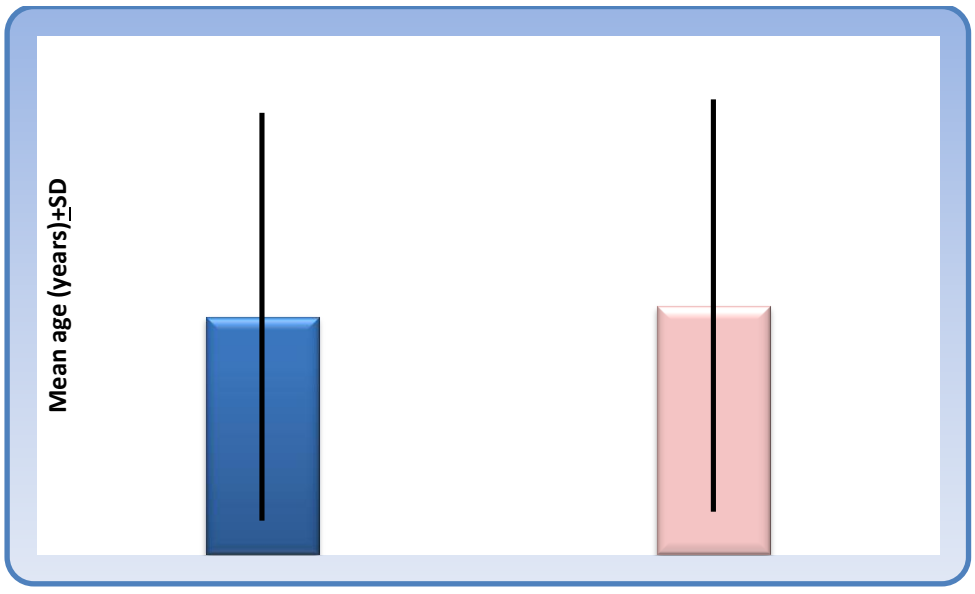

Figure 4-1: Bar chart showing mean age in patients and control groups

Regarding gender , the current study showed the patients group included 45 males (72.58%) and 17 females (27.42%) suggesting that the disease is more common in males with a male to female ratio of 2.65:1. The control group included 40 male subjects and 20 female subjects and the

Chi-square test revealed no statistical difference from gender distribution of patients group (P=0.477) .

This finding ensured a statistical match in gender between both groups which is a basic requirement to conduct such a case-control study (Table 4-3) .

Table 4-3: Distribution of patients and control subjects according gender

Gender	Control		Patients		P-value
	No.	%	No.	%	
Male	40	66.67 %	45	72.58	0.477
Female	20	33.33 %	17	27.42	
Total	60	100.00	62	100.00	

Mean age of male patiens was 16.1+12.92 years , while mean age of female patients was 9.91+7.42 years . Despite the apparent difference in mean age between male and female patients it was no signficant statistically (P=0.118) (Table 4-4) .

Mean age of male control subjects was not signficantly different from that of female control subjects, 12.86±11.6 versus 15.53±12.36 years; P= 0.451.

On the other hand there was no statistical difference in mean age between patients males and control males and also between patients females and control females (P>0.05) (Table 4-4) .

Table 4-4: Comparison of mean age by gender in patients and control groups .

Gender	Control			Patients			P-value
	N	Mean	SD	N	Mean	SD	
Male	40	12.86	11.60	45	16.10	12.92	0.191
Female	20	15.53	12.36	17	9.91	7.42	0.205
P-value	0.451			0.118			

The present study showed the distribution of patients according to residency was as (in table 4-5) : 23 patients (37.15 %) from Al-Warkaa , 10 patients (16.13 %) from Al-Hillal , 9 patients (14.52 %) from Al-Khther , 8 patients (12.9 %) from Al-Salman , 4 patients (6.45 %) from Al-Mamlaha , 3 patients (4.84 %) from Center , 2 patients (3.23 %) from Al-Swir, single patient from Al-Majd (1.61%) , single patient from Al-Najmi (1.61%), single patient from Al-Draji (1.61%) .

Table 4-5: Distribution of patients and control subjects according to residency

Residency	Control		Patients	
	No.	%	No.	%
Al- draji	1	1.67	1	1.61
Al- Salman	7	11.67	8	12.90
Al- Warkaa	21	35.00	23	37.10
Al-Hillal	10	16.67	10	16.13
Al-Khther	9	15.00	9	14.52
Al-Majd	1	1.67	1	1.61
Al-Mamlha	5	8.33	4	6.45
Al-najme	1	1.67	1	1.61
Al-swir	2	3.33	2	3.23
Centre	3	5.00	3	4.84
Total	60	100.00	62	100.00

Control subjects were selected in such a way to ensure matching in regional distribution (Table 4-5) .

4-2-Hematological parameters in patients and control groups :

Mean White Blood Cell (WBC) count was 15.52±3.64 ($X10^3$) in patient group while it was 7.44±1.43 ($X10^3$) in control group ; it was significantly higher in patient group than in control group (P<0.001) (Table 4-6) .

Mean Eosinophil count was (7.94±2) % in patient group while it was (3.73±1.13) % in control group; it was significantly higher in patient group than in control group (P<0.001) (Table 4-6) .

Mean Hb was 12.76±1.96 g/dl in patient group while it was 14.4±1.14 ($X10^3$) in control group ; it was significantly lower in patient group than in control group (P<0.001) (Table 4-6) .

Table 4-6: Mean hematological parameters in patients and control groups

Parameters	Control		Patients		P-value
	Mean	SD	Mean	SD	
WBC	7.44	1.43	15.72	3.64	<0.001
Eosinophil	3.73	1.13	7.94	2.00	<0.001
Hb	14.40	1.14	12.76	1.96	<0.001

In order to study the effect of age on hematological parameters, Pearson's correlation coefficient test was performed and it revealed the following :

1. There was no significant correlation between age of patients and WBC count (Table 4-7) .
2. Also there was no significant correlation between age of patients and eosinophil count percentage (Table 4-7) .

3. There was a significant positive correlation between age of patients and Hb, giving an impression that the higher the age of the patients the more is his hemoglobin concentration (Table 4-7) .

Table 4-7: Correlation between patients' age and hematological parameters

Correlation	WBC	Eosinophil	Hb
R	-0.186	-0.098	0.678
P	0.147	0.448	<0.001

There was no significant association between gender of patients and mean hematological parameters , as shown in table 4-8 . The table demonstrated that the mean WBC counts of male patients and female patients were (15.87± 3.99) $X10^3$ and (15.33±2.54) $X10^3$, respectively ; P=0.606 . Mean eosinophil counts of male and female patients were (8.00±1.92) % and (7.76±2.25) % , respectively ; P=0.683 . Mean Hb concentration of male and female patients were (12.94±2.08) g/dl and (12.29±1.56) g/dl , respectively ; P= 0.252 .

Table 4-8: Comparison of mean hematological parameters between male and female patients

	Male		Female		
Parameter	**Mean**	**SD**	**Mean**	**SD**	**P-value**
WBC	15.87	3.99	15.33	2.54	0.606
Eosinophil	8.00	1.92	7.76	2.25	0.683
Hb	12.94	2.08	12.29	1.56	0.252

4-3- Detection Of The Parasite:

The rate of light microscope detection was 62.9% , whereas the rate of detection by culture method was 83.87% and by PCR was 90.32% (Table 4-9) , figure (4-1),(4-2),(4-3).

Table 4-9: Rate of parasite detection by different methods

Result	Microscope		Culture		PCR	
	No.	%	No.	%	No.	%
Positive	39	62.90	52	83.87	57	90.32
Negative	23	37.10	10	16.13	5	8.04
Total	62	100.00	62	100.00	62	100.00

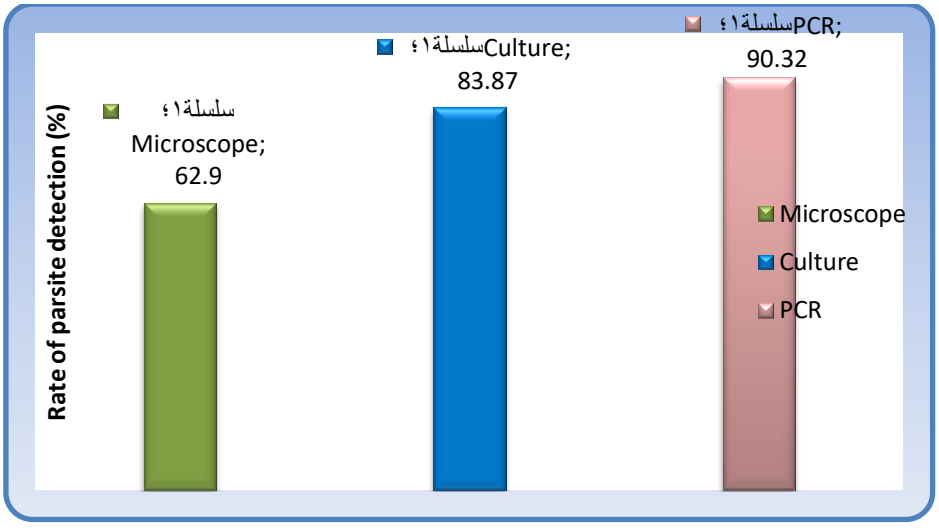

Figure 4-2: Rate of parasite detection by different methods

Figure 4-3: showing leishmanial amastigotes in a Giemsa-stained smear made from aspirates fluid of Cutaneous lesions (x1000) .

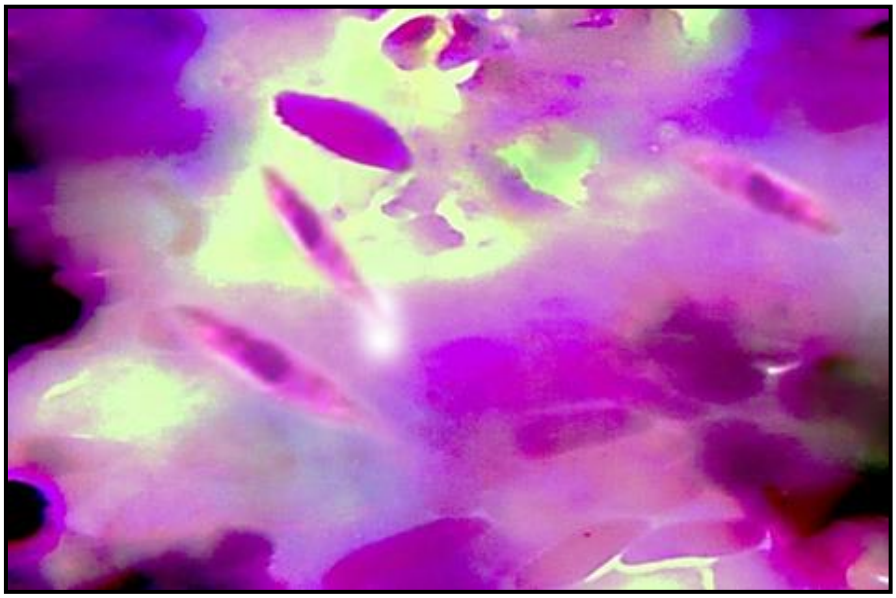

Figure 4-4: Promastigotes : flagellated motile forms found in culture (x1000) .

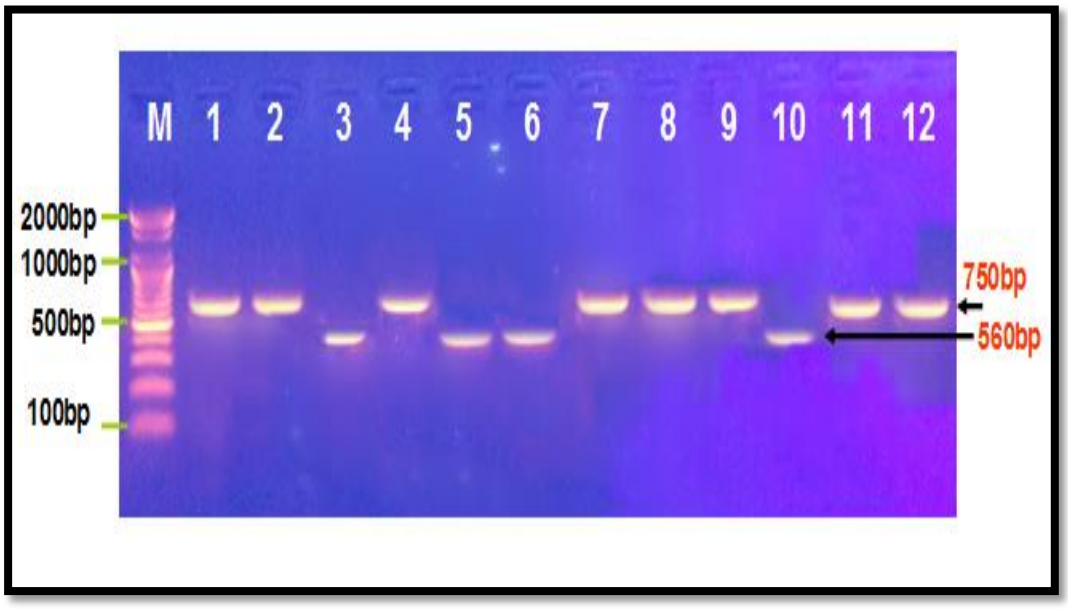

Figure 4-5 : Agarose gel electrophoresis image that show the Nested PCR product analysis of kinetoplast DNA (kDNA) in *Leishmania* samples . Where M: marker (2000-100bp) , lane (1, 2, 4, 7, 8, 9,11, and 12) some positive samples of *Leishmania tropica* isolates at (750bp) Nested PCR product , lane (3, 5, 6, and 10) some samples of positive *Leishmania major* isolates at (560bp) Nested PCR product .

On taking PCR as a reference gold standard method , the following data were obtained regarding both culture and microscope methods : Culture method was more specific and more sensitive than light microscope method (Table 4-10) .

Table 4-10: Comparison of sensitivity rate of the microscope and culture method in reference to PCR

Parameter	Microscope	Culture
Sensitivity	67.86	92.86
Specificity	83.33	100.00
PPV	97.44	100.00
NPV	21.74	60.00

4-4- Location And Number Of Lesions :

According to number of lesions , majority of patients (54.84%) had single lesion while the rest of patients had multiple lesions . Twenty three patients (37.10%) had 2 lesions , four patients (6.45%) had 3 lesions and only one patient (1.61%) had 5 lesions (Table 4-11) .

Table 4-11: Number of lesions

Number of Lesion	Number of patients	%
1	34	54.84
2	23	37.10
3	4	6.45
5	1	1.61
Total	62	100.00

According to lesion location , patients were classified as follows :

1. Thirty three patients (53.23%) had facial lesions .
2. Seventeen patients (27.42%) had hand lesions .
3. Three patients (4.84%) had neck lesions .
4. Nine patients (14.52%) had leg lesions (Table 4-12) .

Table 4-12: Location of lesions

Location	Number of patients	%
Face	33	53.23
Hand	17	27.42
Neck	3	4.84
Leg	9	14.52
Total	62	100.00

There was no significant difference between mean age of patients with multiple lesions and that of patients with single lesions (P=0.267) (Table 4-13) .

Table 4-13: Effect of age on number of lesions

Number of lesions	N	Mean	SD	P-value
Single lesion	34	15.94	13.68	0.267
Multiple lesions	28	12.54	9.33	
Total	62	14.40	11.94	

There was no association between gender of patients and number of lesions (P=0.499) (Table 4-14) .

Table 4-14: Association between patient's gender and number of lesions

Number of lesions	Male		Female		Total		P-value
	No.	%	No.	%	No.	%	
Single lesion	26	57.78	8	47.06	34	54.84	0.499
Multiple lesions	19	42.22	9	52.94	28	45.16	
Total	45	100.00	17	100.00	62	100.00	

There was no significant difference in mean hematological parameters (WBC , Eosinophil, Hb) between patients with single lesion and patients with multiple lesions (P>0.05) (Table 4-15) .

Table 4-15: The association between number of lesion and hematological parameters

Parameter	Multiple lesions		Single lesion		P-value
	Mean	SD	Mean	SD	
WBC	15.01	2.75	16.31	4.18	0.163
Eosinophil	8.21	2.04	7.71	1.96	0.323
Hb	12.38	1.93	13.08	1.96	0.165

There was no significant difference in mean age in relation to location of lesion(face , hand , neck and leg), (P>0.05) (Table 4-16).

Table 4-16: Mean age in relation to location of lesion

Location	Mean age	SD	P-value
Face	12.09	10.86	0.194
Hand	14.41	11.56	
Neck	22.67	19.76	
Leg	20.11	12.91	
Total	14.40	11.94	

There was no significant difference between location of lesion and gender(P>0.05) (Table 4-17).

Table 4-17: Association between location of lesion and gender

Location	Male	Female	Total	P-value
Face	21	12	33	0.334
Hand	14	3	17	
Neck	2	1	3	
Leg	8	1	9	
Total	45	17	62	

Also there was no significant difference between hematological parameters and location of lesion (P>0.05) (Table 4-18).

Table 4-18: Association between hematological parameters and location of lesion

Parameter	Face		Hand		Leg		Neck		P-value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
WBC	15.65	3.38	16.52	4.69	14.91	2.69	14.33	2.31	0.643
Eosinophil	8.18	2.17	7.71	2.05	7.56	1.42	7.67	1.53	0.785
Hb	12.60	2.03	12.74	2.02	13.56	1.75	12.30	1.76	0.610

4-5- *Leishmania* Subtypes :

The *Leishmania subtypes in* the present study were distributed as follows: *L. tropica* accounted for 69.35% while *L. major* accounted for 22.58% of cases (Table 4-19) .

Table 4-19: *Leishmania subtypes*

L. subtype	No.	%
L. tropica	43	69.35
L. major	14	22.58
Negative	5	8.06
Total	62	100.00

No significant association was found between *Leishmania subtypes* and mean age of patients (P>0.05) (Table 4-20) .

Table 4-20: Association between *L. subtypes* and mean age

Leishmania subtypes	Mean age	SD	P-value
Negative	15.60	12.56	0.928
L. tropica	14.01	12.16	
L. major	15.18	11.91	
Total	14.40	11.94	

Also there was no significant association between *Leishmania subtypes* and gender of patients (P>0.05) (Table 4-21) .

Table 4-21: Association between *L. subtypes* and gender

L. subtype	Male		Female		Total		P-value
	No.	%	No.	%	No.	%	
Negative	2	4.44	3	17.65	5	8.06	
L. tropica	33	73.33	10	58.82	43	69.35	0.218
L. major	10	22.22	4	23.53	14	22.58	
Total	45	100.00	17	100.00	62	100.00	

No significant association was found between *Leishmania subtypes* and any of hematologic parameters (P>0.05) (Table 4-22) .

Table 4-22: Association between *L. subtypes* and mean hematological parameters

Parameter	Leishmania subtypes	Mean	SD	P-value
WBC	Negative	16.60	3.91	0.857
	L. tropica	15.65	3.70	
	L. major	15.61	3.58	
Eosinophil	Negative	8.60	1.52	0.746
	L. tropica	7.88	2.20	
	L. major	7.86	1.51	
Hb	Negative	12.52	2.08	0.937
	L. tropica	12.82	2.09	
	L. major	12.68	1.61	

No significant association was found between *Leishmnia subtypes* and number of lesions (P>0.05) (Table 4-23) .

Table 4-23: Association between *L. subtypes* and number of lesions

L. subtype	Single lesion	Multiple lesions	Total	P-value
Negative	3	2	5	0.263
L. tropica	26	17	43	
L. major	5	9	14	
Total	34	28	62	

No significant association was found between *Leishmania subtype* and location of the lesion (P>0.05) (Table 4-24) .

Table 4-24: Association between *L. subtypes* and lesion location

L. subtype	Face	Hand	Neck	Leg	Total	P-value
Negative	3	1	1	0	5	0.379
L. tropica	23	13	2	5	43	
L. major	7	3	0	4	14	
Total	33	17	3	9	62	

Chapter Five
Discussion

5-Discussion

The present study was first molecular epidemiological study of leishmaniasis in the Al-Muthanna province and for the first time , molecular diagnostic tools have been applied to the identification of different species of *Leishmania* .

It is in this study , *L. tropica* was first recognized as causative agent in Al-Muthanna province , while a classical *L.major* focus , as early as in 1998 (Coltman *et al*., 1998) . The other striking finding came from the microsatellite analysis which showed that two genetically distinct populations of *L. major* are present in the Middle East (Cooke *et al*., 2003) . The Jordan Valley and the Arava/Negev desert populations genotypes are found in geographically close areas , less than 100 km apart from each other (El Tai *et al*., 2000) .

Molecular epidemiological studies will impact the look on leishmaniasis in Al-Muthanna province , as it showed differences in epidemiology patterns for both *L. major* and *L. tropica* species most likely reflecting different life cycles . This finding will , if taken into consideration by the authorities in Al-Muthanna province , lead to a revision of control measures and even treatment regimens .

5.1. Method Comparison

5.1.1.Microscopy , Culture Method And Molecular Methods:

All conventional methods employed for the diagnosis of Cutaneous leishmaniasis have modest to low rates of positivity , such as microscopy 42% by Aviles *et al.* (1999); 46.7% by Weigle *et al.* (2002); 48% by Andresen *et al*. (1996); 66.7% by Rodrigues *et al.* (2002); and 72% by Rodriguez *et al*. (1994), histopathology 33% by Aviles *et al*. (1999); 76% by Andresen *et al*. (1996) and 66.2% by Rodrigues *et al*. (2002) and culture 46.5% by Rodrigues *et al*. (2002) .

Molecular method have been proven as sensitive and specific method for direct detection and identification of *Leishmania* parasites in different clinical specimens (Schönian *et al*. 2001) . So the present study showed that there were no positive PCR reactions in the negative group and that all negative controls were negative , confirmed that we obtained real positive results . PCR proved to be more sensitive (87 %; 52/60) in comparison to graded microscopy regardless whether all samples (37%; 22/60) , or only those that were stained correctly (49%; 22/45) were used.

In general , the sensitivity of PCR was consistent with results of other studies that evaluated microscopy and PCR-based methods and revealed sensitivity ranging from 75.7% to 100% depending on the targeted DNA sequence amplified , i.e kinetoplast DNA , ribosomal RNA genes , mini circle DNA *etc*. the type of primers , i.e. genus-specific , sub-genus specific or species specific and the nature of clinical sample (Piarroux *et al.*, 1994; Andersen *et al.*, 1996; Belli *et al.*, 1998; Aviles *et al*., 1999; Rodrigues *et al*., 2002; Motazedian *et al*.,2002) .

5.2. Demographic Characteristics Of The Study Groups :

The current study demonstrated a comparison in mean age (in years) between patients' group and control group , in which there was no significant difference . Age of patients' group with a range of (1-45 years) while a control group range of (2-46 years) . The above mentioned results ensured a statistical match between patients' group and control group which is a basic requirement for such a cases control study .

The present study showed the distribution of patients and control groups according to 10 years age intervals . Two important findings can be concluded ; the first one is that the majority of patients were children (27% <10 years and 43.55 % 10-19 years) . The second point is that the disease can affect any age and patients older than forty years were

identified . These results differ from study by Al-Jawabreh *et al*., (2004) , them noticed that the gender-age difference was clearer in adults between 20-29 years .

These results favour the explanation that differences in exposure to the disease is based mainly on host behaviour for adults in their 20s . For children , it may be due to naivety of the immune system and to genetic factors which have been suggested to explain the sex-age variance in other parasitic infections such as malaria and schistomiasis (Cooke *et al*., 2003; Henri *et al*., 2002) . Immune regulatory mediators have been shown to develop with age (Sack *et al*., 1998; Tsaknaridis *et al*., 2003) .

Using hamsters infected in the laboratory it was found that the burden of *Leishmania* infection was more on males than females and this was attributed to different levels of sex hormones and of cytokines known to promote experimental leishmaniasis : interleukin 4 and transforming growth factor (TGF) (Travi *et al*., 2002) .

Regarding gender , patients group included 45 males (72.58%) and 17 females (27.42%) suggesting that the disease is more common in males with a male to female ratio of 2.65:1, this result agreement with Amin Masoumeh *et al*., (2014) .

The distribution of patients according to residency was as follows : 23 patients (37.15 %) from Al-Warkaa, 10 patients (16.13 %) from Al-Hillal , 9 patients (14.52 %) from Al-Khther , 8 patients (12.9 %) from Al-Salman , 4 patients (6.45 %) from Al-Mamlaha , 3 patients (4.84 %) from Center , 2 patients (3.23 %) from Al-Swir , single patient from Al-Majd (1.61%) , single patient from Al-Najmi (1.61%) , single patient from Al-Draji (1.61%) .

There are many factors that play important roles in the presence and distribution of CL in different parts of Iraq particularly middle regions , including the presence of animal reservoirs such as rodents , dogs , and

the use of clay to build some of the houses in villages in these areas . Furthermore, presence of agricultural areas that attracts and harbors many kinds of insects , and there population work long hours in the farms where they are more exposed to insects bites .

Also , CL in our country may primarily affect families of farmers and nomads who are chiefly exposed to night biting sand flies . The presence of high gerbil population densities in the areas may be blamed as reservoir of infection that is supported by the crops for which the irrigation canals had been constructed . Furthermore , the canal embankments serve as densely populated and favored rodent and sand fly infestation areas (Al-Samarai and Al-Obaidi , 2009) .

The emergence of leishmaniasis in some foci may be the result of interruption of previously applied methods of control , like insecticide spraying on early diagnosis and treatment of positive cases . It is believed that reduction in insecticide spraying for malaria control contributes to the increase in the population of synanthropic sand flies and results of incidence of the disease in some endemic foci of VL and CL (Choi and Lerner , 2001 ; Neoumine , 1996) . Control subjects were selected in such a way to ensure matching in regional distribution .

5.3. Hematological Parameters In Patients And Control Groups :

In order to study the effect of age on hematological parameters , Pearson's correlation coefficient test was performed and it revealed the following :

4. There was no significant correlation between age of patients and WBC count (Table 4-15) .
5. Also there was no significant correlation between age of patients and eosinophil count percentage (Table 4-15) .

6. There was a significant positive correlation between age of patients and Hb , giving an impression that the higher the age of the patients the more is his hemoglobin concentration (Table 4-15) .

In the patients , the most frequent sign and symptom was fever which predominant in all patients 100% , followed by hepatosplenomegaly , while the least frequent feature was abdominal pain which present in only 30.8% , which is in agreement with previous reports (Al-Ani *et al*., 2012 ; Al-Hamash , 2012) .

5.4. Methods Of Parasite Detection :

The present study , three methods were used to identify the presence of the parasite for purpose of comparison . These were light microscopic examination , culture and PCR . The rate of light microscope detection was 62.9% , part of this broad range may be explained by the differing parasite densities in various types of materials , where as the rate of detection by culture method was 83.87% and by PCR was 90.32% .

Skin-scrapings in comparison to other materials showed heterogeneity with regard to the number of parasites in a smear , as the parasites are not equally distributed in the tissue and the number of cells fixed on the slide may differ too . In addition , in all cases of leishmaniasis , the host defense influences the density of parasites . In leishmaniasis diagnostics , the real problem is the cases with only few parasites in the material .

To diagnose these cases correctly , one needs a very sensitive method. The method we have used was PCR which has been proven as sensitive and specific method for direct detection and identification of *Leishmania* parasites in different clinical specimens (Schönian *et al*. 2001) . The fact , that there were no positive PCR reactions in the

negative group and that all negative controls were negative , confirmed that we obtained real positive results .

Taking PCR as a reference gold standard method , this result agreement with (Abdullah Qader *et al*., 2009) , the following data were obtained regarding both culture and microscope methods : Culture method was more specific and more sensitive than light microscope method , these results were in agreement with many studies in Iraq (AlSamarai and AlObaidi , 2009) Afghnistan (Faulde *et al*., 2008) , Iran (Talari *et al*., 2006) , Colombia
(Ramirez *et al*., 2000) , but disagreement with other study done in India (Singh and Sivakumar , 2003) .

The high frequency of wet lesions may be due to the presence of reservoir animals in large number in some areas in Iraq especially rodents and dogs (AlSamarai and AlObaidi , 2009) or because of this may be due to the difference in number of patients in these studies .

5.5. Location And Number Of Lesions :

According to number of lesions, majority of patients (54.84%) had single lesion while the rest of patients had multiple lesions. Twenty three patients (37.10%) had 2 lesions , four patients (6.45%) had 3 lesions and only one patient (1.61%) had 5 lesions , These results were in agreement with many studies in Iraq (AlSamarai and AlObaidi , 2009) Afghnistan (Faulde *et al*., 2008) , Iran (Talari *et al*., 2006) , Colombia (Ramirez *et al*., 2000) , but disagreement with other study done in India (Singh S. and Siva kumar , 2003) . The high frequency of wet lesions may be due to the presence of reservoir animals in large number in some areas in Iraq especially rodents and dogs (AlSamarai and AlObaidi , 2009) .

According to lesion location , patients were classified as follows:

5. Thirty three patients (53.23%) had facial lesions.

6. Seventeen patients (27.42%) had hand lesions.
7. Three patients (4.84%) had neck lesions.
8. Nine patients (14.52%) had leg lesions .

Similar to the findings of some other studies (Yaghoobi-Ershadi *et al*., 2001; Fazaelia *et al*.,2009) , the most affected part of the body was upper limb followed by the face . This is one of the usual characteristics of the zoonotic Cutaneous leishmaniasis ; whereas , for the anthroponotic Cutaneous leishmaniasis , the most commonly involved sites are face and upper limbs (Sharma *et al*., 2005) . However , this is not always consistent , and there are some reports indicating predominant involvement of the face in zoonotic Cutaneous leishmaniasis (Talari *et al*., 2006; Momeni and Aminjavaheri , 1995) .

There was no significant difference between mean age of patients with multiple lesions and that of patients with single lesions (P=0.267) , as shown in table 4-13. There was no association between gender of patients and number of lesions (P=0.499) . There was no significant difference in mean hematological parameters between patients with single lesion and patients with multiple lesions (P>0.05) .

5.6. *Leishmania Subtypes* :

The *Leishmania subtypes* in the present study were distributed as follows : *L. tropica* accounted for 69.35% while *L. major* accounted for 22.58% of cases , as shown in table 4-18 , this result agreed with (Shahid Niaz Khan *et al*., 2013) . No significant association was found between *Leishmania subtypes* and mean age of patients (P>0.05) .

Also there was no significant association between *Leishmania subtypes* and gender of patients (P>0.05) . No significant association was found between *Leishmania subtypes* and any of hematologic parameters (P>0.05) . No significant association was found between *Leishmnia*

subtypes and number of lesions (P>0.05) . No significant association was found between *Leishmania subtype* and location of the lesion (P>0.05) . This variation in seasonal peak could be due to the existence of various dominant reservoir species in these areas as well as to the activity of the sand flies . The differences in monthly distribution of CL patients might also be related to the sand fly activities and requirement of blood meal .

Chapter Six
Conclusions
&
Recommendations

6.1- Conclusion

1. The current study showed the mean age of patients' group was 14.4±11.94 years with a range of (1-45 years) . The patients group included 45 males (72.58%) and 17 females (27.42%) .

2. The present study showed the distribution of patients according to residency was as : 23 patients (37.15 %) from Al-Warkaa , 10 patients (16.13 %) from Al-Hillal , 9 patients (14.52 %) from Al-Khther , 8 patients (12.9 %) from Al-Salman , 4 patients (6.45 %) from Al-Mamlaha , 3 patients (4.84 %) from Center , 2 patients (3.23 %) from Al-Swir, single patient from Al-Majd (1.61%) , single patient from Al-Najmi (1.61%) , single patient from Al-Draji (1.61%) .

3. White Blood Cell (WBC) count was 15.52±3.64 ($X10^3$) in patient group . Mean Eosinophil count was (7.94±2) % in patient group . Mean Hb was 12.76±1.96 g/dl in patient group .

4. Polymerase Chain Reaction is gold standard method . Culture method was more specific and more sensitive than light microscope method .

5. According to lesion location , patients were classified as follows :

a. Thirty three patients (53.23%) had facial lesions .

b. Seventeen patients (27.42%) had hand lesions .

c. Three patients (4.84%) had neck lesions .

d. Nine patients (14.52%) had leg lesions .

6. The *Leishmania subtypesin* the present study were distributed as follows: *L. tropica* accounted for 69.35% while *L. major* accounted for 22.58% of cases .

6.2- Recommendation

1- Educational programs that promote personal, household hygiene and provide new techniques to control on vectors such as sand fly insect should be implemented by the health and environmental authorities in our country during peak times will help minimize exposure to infection by this parasite.

2- Genotyping study is needed using RFLP-PCR to record the genotypes of Leishmania Spp.to provide data base on circulating species, genospecies and their modes of transmission in our community especially in outbreak cases.

3- The use of plant extracts instead of chemotherapy for being highly expensive and have side effects.

4-Further studies are necessary to understand the survival times and effects of environmental factors on parasites, such as temperature, humidity, and sunlight to identify the risk as a possible source of infection for human.

5- The preparation of a vaccine for the treatment of cutaneous leishmaniasis lesions with fewer side effects.

Chapter Seven
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Appendixes

Appendix-A : Questionnaires Paper Used In The Study .

Ser.	Gender	Micro.	Culture	PCR	Age	Area	No. Lesion	Location	Esionophile	Hb	W.B.C	L.Sp.
1												
2												
3												
4												
5												

Appendix-B : Images illustrate the location of lesion with leishmaniasis .

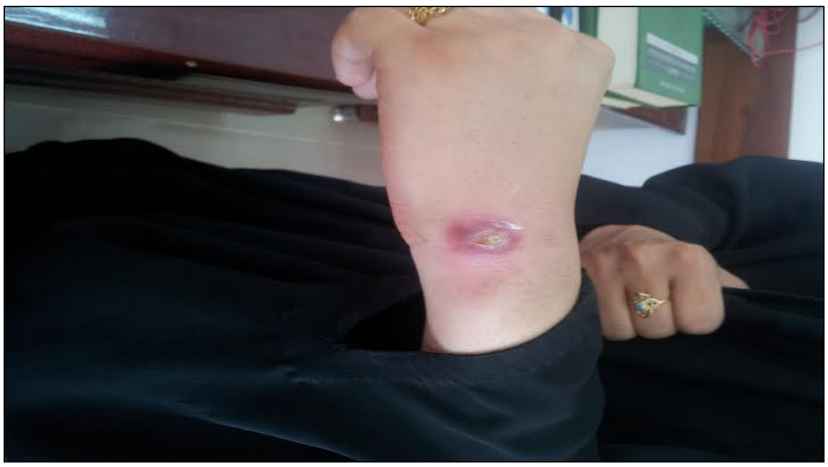

Image-1-Women infect by Cutaneos leishmaniasis in the hand

Image-2-Child infect by Cutaneos leishmaniasis in the foot

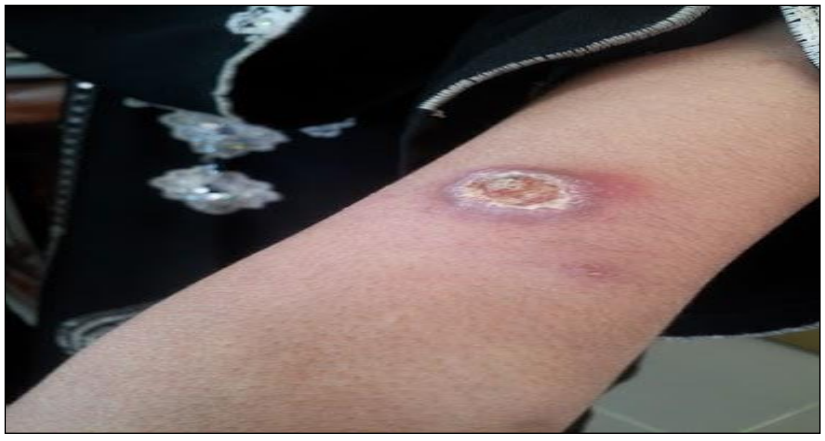

Image-3-Child infect by Cutaneos leishmaniasis in the arm

Image-4-Girl infect by Cutaneos leishmaniasis in the hand

Image-5-Child infect by Cutaneos leishmaniasis in the face

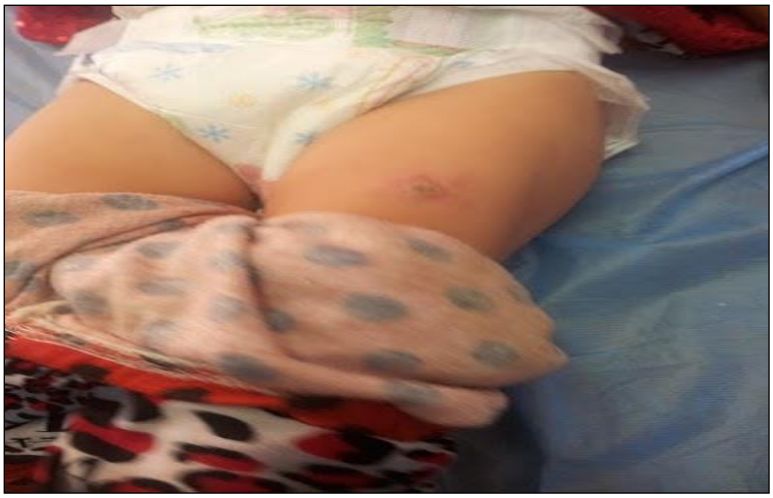

Image-6-Child infect by Cutaneos leishmaniasis in the thigh

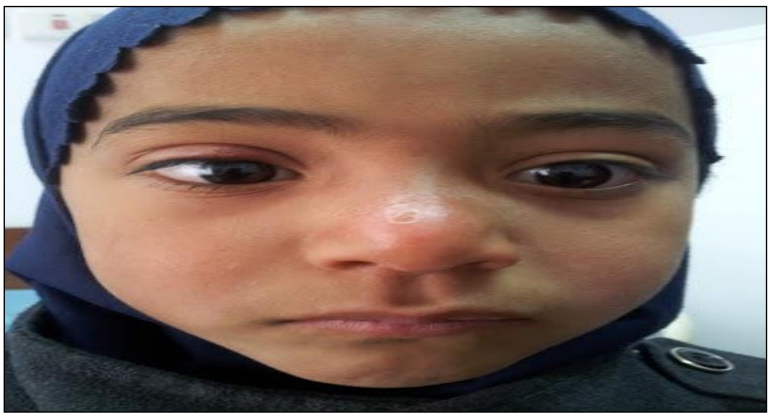

Image-7-Child infect by Cutaneos leishmaniasis in the nose

Image-8-Child infect by Cutaneos leishmaniasis in the face

Image-9-Child infect by Cutaneos leishmaniasis in the face

Image-10-Child infect by Cutaneos leishmaniasis in the face

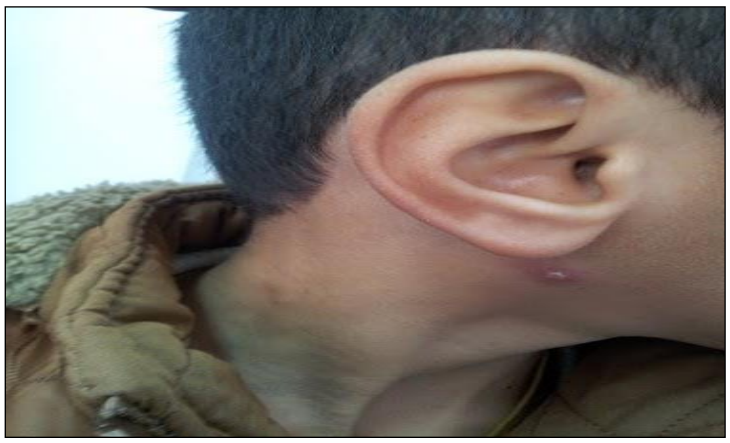

Image-11-Child infect by Cutaneos leishmaniasis in the neck

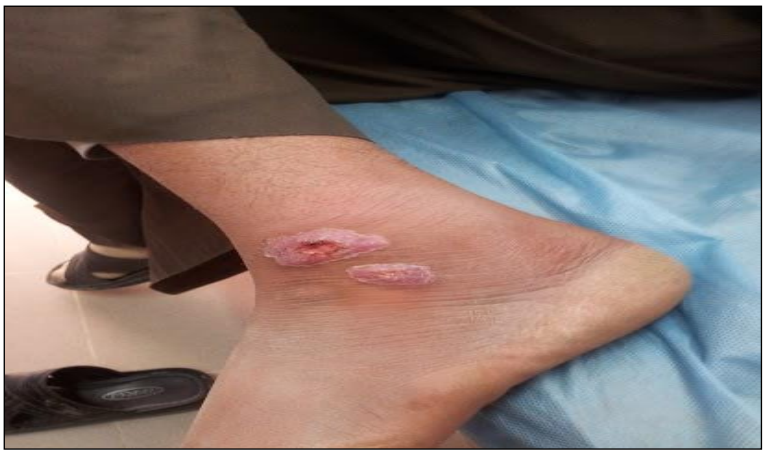

Image-12-Adult infect by Cutaneos leishmaniasis in the foot

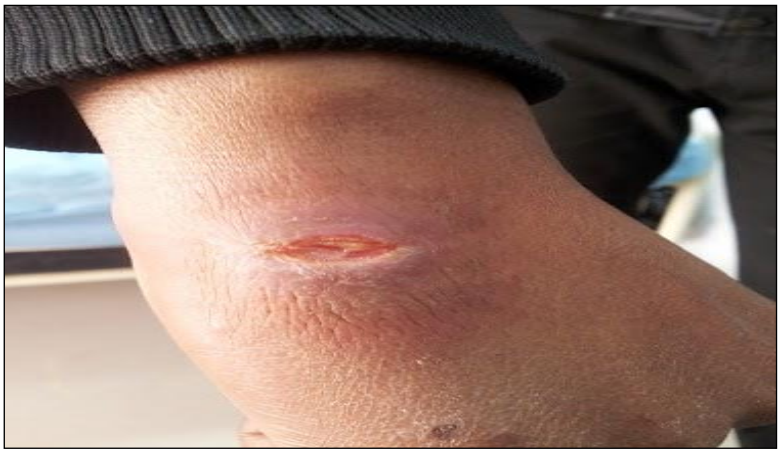

Image-13-Adult infect by Cutaneos leishmaniasis in the hand

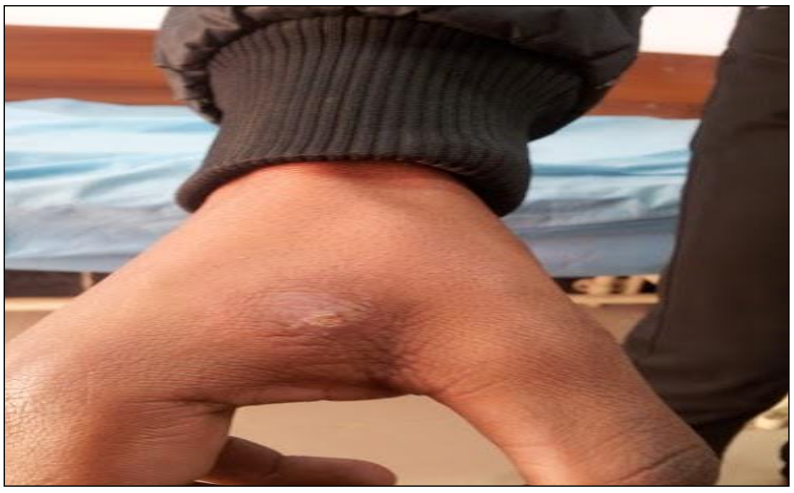

Image-14-Child infect by Cutaneos leishmaniasis in the hand

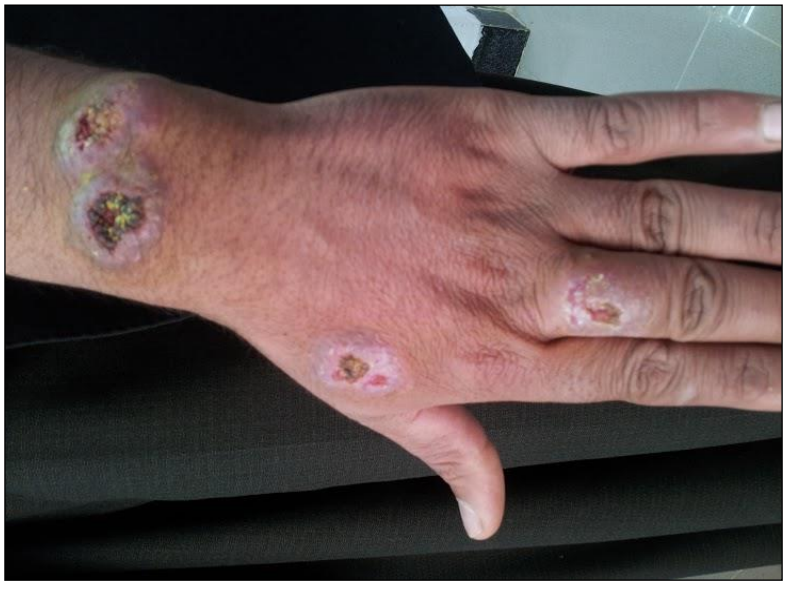

Image-15-Adult infect by Cutaneos leishmaniasis in the hand

Appendix-D : Images illustrate the instruments that used in this study .

Appendix-E : Images illustrate the drug that used in this study .

الخلاصة

تهدف هذه الدراسة لمقارنة حساسية وخصوصية طرق PCR المستخدمة لتشخيص الطفيلي بطريقة الزرع المختبري والفحص المجهري لأثبات دقة تقنيات PCR لتشخيص اللشمانيا الجلدية .

متوسط عمر المرضى كان (14.4±11.94) بمعدل (1-45 سنة) بينما متوسط عمر مجموعة السيطرة كان (11.81 ±13.75) وبمعدل (2-46 سنة) . فيما يتعلق بالجنس ، مجموعة المرضى تتضمن 45 ذكر (72.58 %) و17 أناث (27.42 %) نستنتج من ذلك أن المرض أكثر أنتشارا في الذكور . متوسط عمر المرضى الذكور كان (12.92 ±16.1) سنة ، بينما متوسط عمر المرضى الأناث كان (7.42±9.91) سنة .

نسبة الأصابة بالنسبة للمكان كان كالتالي : 23 مريض (37.15 %) من الوركاء ، 10 مرضى (16.13 %) من الهلال ، 9 مرضى (14.52 %) من الخضر ، 8 مرضى (12.9 %) من السلمان ، 4 مرضى (6.45 %) من المملحة ، 3 مرضى (4.48 %) من المركز ، 2 مرضى (3.23 %) من السوير ، مريض واحد من المجد (1.61 %) ، مريض واحد من النجمي (1.61 %) ، ومريض واحد من الدراجي (1.61 %) .

ثلاث طرق أستعملت لتحديد مكان الطفيلي لأغراض المقارنة . منها الفحص المجهري والزرع المختبري والفحص الجزيئي بطريقة PCR .

أنواع اللشمانيا في الدراسة المقدمة كانت كالتالي : *L. tropica* تقدر ب(69.35%) بينما *L. major* (22.58 %) من الحالات .

اقرار المناقشين

نشهد أننا أعضاء لجنة التقويم والمناقشة قد اطلعنا على هذه الرسالة الموسومة ب "دراسة مقارنة بين الطرق التقليدية والجزيئية لتشخيص اللشمانيا الجلدية في محافظة المثنى) " وناقشنا الطالبة "جميلة عبيد مزهر " في محتوياتها وفيما له علاقة بها بتاريخ 15 /3/ 2017 ووجد انها جديرة لنيل درجة ماجستير علوم في علم الاحياء المجهرية

ا. د.غازي يعقوب عزال

كلية الطب البيطري / جامعة البصرة

رئيسا

ا.م.د. حازم عزيز ناجي

كلية العلوم / جامعة الكوفة

عضوا

ا. م. د. وئام سعد عبد الحمزة

كلية العلوم/ جامعة المثنى

عضوا

ا.م.د غادة باسل علي

كلية الطب / جامعة القادسية

عضوا ومشرفا

م.د.ياسر دخيل كريمش

كلية التربية للعلوم الصرفة/جامعة المثنى

عضوا و مشرفا

ا. م. د. حسين جابر عبد الحسين

عميد كلية العلوم

التاريخ: / /2017

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة المثنى
كلية العلوم
قسم علوم الحياة

دراسة مقارنة بين الطرق التقليدية والجزيئية لتشخيص اللشمانيا الجلدية في محافظة المثنى

رسالة

مقدمة إلى مجلس كلية العلوم - جامعة المثنى كجزء من متطلبات نيل

درجة

ماجستير في علم الأحياء المجهرية

من قبل

جميلة عبيد مزهر

بكالوريوس علوم حياة

كلية التربية/ جامعة البصرة

1980

بإشراف

الأستاذ المساعد الدكتورة
غادة باسل علي

الدكتور
ياسر دخيل كريمش الاسدي

2017 م

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