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A Comparison between molecular and serological methods in the diagnosis of Kala-azar

A thesis

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Dedication

Iam pleased to dedicate this work

to my beloved,parents, wife,sons

and brother

(Abdul Raheem for his memory)

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List of Abbreviations

ACL	Anthroponotic cutaneous leishmaniasis
AIDS	Acquired Immunodeficiency Syndrom
AP-PCR	Arbitrarily Primed PCR
AVL	Anthroponotic visceral leishmaniasis
CL	Cutaneous leishmaniasis
DAT	Direct Agglutination Test
DCL	Diffuse cutaneous leishmaniasis
ELISA	Enzyme-linked immunosorbent assay
GIPL	Glycosylphospholipid
GPI	Glycosylphosphatidylinositol
HIV	Human Immunodeficiency Virus
IFAT	Indirect Immunofluorescent Antibody Test
IFN	Interferon
IL	Interleukin
IVDUs	Intravenous drug users
kDNA	kinetoplastic DNA
LPG	Lipophosphoglycan
MCL	Mucocutaneous leishmaniasis
MHC	Major histocompatibility complex
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
PKDL	Post – kala - azar dermal leishmaniasis
RAPDs	Random Amplified Polymorphic DNAs
RFLP	Restriction fragment length polymorphism
rK39	Recombinant kinesin-related protein
SSCP	Single strand conformation polymorphism analysis
WHO	World Health Organization
ZCL	Zoonotic cutaneous leishmaniasis
ZVL	Zoonotic visceral leishmaniasis
VL	Visceral leishmaniasis

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Summary

Eight hundred blood samples of patients with Kala-azar and one hundred blood samples of control group were studied. The number of the notified cases of Kala-azar in Baghdad and Wasit governorates during the period from January 2008 to August 2008 was 800 cases,fluctuated to reach a maximum of 165 cases (20.6%) in January 2008, and declined to reach 49 cases (6.1%) in July 2008.

It was also noted that all 300 / 300 (100 %) case-patients had fever, splenomegaly 290/300 (96.7%), hepatomegaly 250 (83.3%),weight loss 155 (51.7%), anemia 150 (50%) , paleness 106 (35.3%), diarrhoea 66 (22%), leukopenia 50 (16.7%) and abdominal pain 46 (15.3%).

The patients were divided according to the age criterion into seven groups from (G1 < 1year to G7 > 50 years), and the highest infection was appeared in children under one year old 560(70 %). There was male predominance which reached 436 (54.5%) while that female was 364 (45.5%). The proportion of Kala-azar infection in Baghdad (257- 85.7%) was lower than Wasit (460 - 92%). Most of the patients whose number reached 454 (56.8%) lived in different areas of rural Baghdad and Wasit governorates, and under poor conditions 509 (63.6%).

Anti-*Leishmania* antibody was detected in 717(89.62%) by using rK39; and from 100 blood samples ; 95, 70 and 88 were positive by using direct agglutination test (DAT), indirect immunofluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA), respectively. Based on these results, sensitivity and specificity of rK39 and DAT were found to be 89.6%, 95%, 100% and 100%, respectively. The sensitivity of IFAT and ELISA in the diagnosis of Kala-azar was 70% and 88% and their

specificity was 100% for both. ELISA showed a sensitivity, specificity, a positive predictive value (PPV) and negative predictive value(NPV) of 88%,100%,100% and 45.45% respectively. While for rK39 these values were 89.6%,100%,100% and 54.64%,respectively.The diagnostic accuracy for ELISA and rK39 was found to be 89.09% and 90.77 %, respectively.

Out of 100 patients, 95 appeared to be positive by PCR test and 5 were negative, giving a sensitivity for PCR of 95% and specificity of 100 %. Data analysis was done by Statistical Package for Social Sciences (SPSS) software and by using descriptive and analytical statistics.

Chapter One

Introduction and Literature Review

1- Introduction and Literature Review

1.1 Introduction

Leishmaniasis is a disease caused by protozoan parasites that belong to the genus *Leishmania* and is transmitted by the bite of certain species of sand fly,including flies in the genus *Lutzomyia* found in the New World and *Phlebotomus* in the Old World. The disease was named in 1901 by a Scottish pathologist William Boog Leishman, and this discovery was confirmed three years later by Charles Donovan. In year 1903, Sir Ronald Ross introduced the term *Leishmania* (Ross, 1903). These parasites are widespread in all continents.Leishmaniasis still constitutes major public health problem and the burden increases (Desjeux, 2001, 2004).

Leishmaniasis, a vector - borne disease caused by obligate intra-macrophage protozoa, is characterized by their diversity and complexity (Herwaldt, 1999; Desjeux, 2001).A total of about 21 *Leishmania* species have been identified to be pathogenic to humans. *Leishmania* is one of several genera within the family Trypanosomatidae, and is characterized by the possession of a kinetoplast, a unique form of mitochondrial DNA (Arias and Naiff, 1981).

Leishmaniasis is caused by different species of *Leishmania* . These flagellated protozoa, known as kinetoplastids, include a number of pathogens that are responsible for serious diseases in humans and other animals. They are characterized by the presence of kinetoplast, DNA-containing granule located within the single mitochondrion and associated with the flagellar bases(Webster and Russel, 1993). The members of family *Trypanosomatidae* have reduced or absent cytostomes that feed entirely through absorption (Sivakumar, 2004). They have a complex life-cycle, involving

more than one host, and go through various morphological stages. All members are exclusively parasitic.There are nine genera under this family: *Blastocrithidia , Crithidia , Endotrypanum , Herpetomonas , Leptomonas , Phytomonas,Wallaceina , Trypanosoma* and *Leishmania*.

There are mainly five types of leishmaniasis , caused by various species of *Leishmania*. The visceral leishmaniasis is caused by species of *L. donovani* complex that consist mainly of *L.(d) infantum*, *L .(d) donovani* and *L.(d) chagasi* (Ashford and Bates,1998). It is a severe form in which the parasites have migrated to the vital organs(Lainte *et al*.,2001). Mucocutaneous leishmaniasis(MCL),or espundia,produces lesions, which can lead to extensive and disfiguring destruction of mucousmembranes of the nose,mouth and throat cavities. The causative species of MCL are *L. (viannia) braziliensis* and *L. (viannia) guyanensis*. The cutaneous leishmaniasis (CL) can produce large numbers of skin ulcers, as many as 200 in some cases,on the exposed parts of the body(Sanyal,1985; Lainson and Shaw,1987). The causative species of CL are *L. major, L. tropica, L. mexicana* and *L. amazonensis*. The fourth form diffuses cutaneous leishmaniasis (DCL). It is an anergic variant of the localized cutaneous leishmaniasis in which lesions are disseminated, resembling lepromatous leprosy. The disease is caused by *L. (mexicana) amazonensis* and *L. aethiopica* (Lainson and Shaw, 1987; Pearson and Sousa,1996; Ashford and Bates, 1998).

Despite considerable progress in Cellular and Molecular Biology and in the evolutionary genetics, one is still far from understanding how these organisms act in natural populations. They have a complex life cycle, and present in very diverse ecological niches and can infect a wide range of hosts . Furthermore , *Leishmania* spp. can produce a great variety of clinical symptoms in humans.

Aims of the Study

1- Assessing the epidemiological and clinical aspects of the disease particularly in children with Kala-azar in Baghdad and Wasit governorates.
2- Estimating the sensitivity and specificity of PCR .
3- Diagnosing the parasite by different serological tests and evaluating these tests.
4- Comparing between molecular and serological tests in diagnosis of Kala-azar disease.

1.2- Literature Review

1.2.1 History Scope

The human leishmaniasis can present itself in man into five different forms:

a. Visceral leishmaniasis, also known as Kala-azar (in the Old world), is caused by the protozoan parasite *L. donovani* (Lainson and Shaw, 1987;Pearson and Sousa, 19٩٦). The *L. donovani* infects macrophages of liver, spleen and bone marrow. The infection is chronic and may be fatal in untreated cases. After successful treatment, 3 to 10 % of the patients develop post Kala-azar dermal leishmaniasis (PKDL), wart like nodules over the face and extensor surface of the limbs (El-Masum and Evans, 1995). Epidemics of Kala-azar are often associated with natural disasters or social upheavals. Visceral leishmaniasis is a severe form in which the parasites migrate to the vital organs (Liarte *et al*., 2001).

In central Iraq, as well as in the capital of the country, cases of visceral leishmaniasis caused by the *L. donovani* protozoon (with the bacterial strains) have been registered. This has been the result of the

increased number of the infection vectors, large concentration and the unsatisfactory health condition of the population. Since1991, incidences of leishmaniasis have started to occur in the southern provinces of the country(Misan,Thiqar,Basra),until that time its appearance was extremely rare. In 2001, 2893 cases of visceral leishmaniasis were diagnosed. in 2000, 2611 cases, and as many as 3866 cases were in the peak year-1992 (20 cases for every 100 thousand citizens) (Korzeniewski, 2004).

b.Cutaneous Leishmaniasis, is the most common that is infection characterized by ulcerative skin lesion in the face,arms or legs. The cutaneous leishmaniasis is principally, caused by *L. major*, *L. tropica* and *L. mexicana*. Although the lesions will persist and disseminate, cutaneous leishmaniasis is, generally, a self-healing disease (Traoré *et al*., 2001).

In all areas of Iraq,there had also been cases of cutaneous leishmaniasis. The course of disease is much more gentle than that of kala-azar. In 2001, there were 625 cases of cutaneous leishmaniasis, 955 cases were in 2000 and as many as 8779 cases were in peak year of 1992 (45 cases for every 100 thousand citizens) (WHO, 2003). Cases of cutaneous leishmaniasis, caused by *L. tropica* mostly occur in the suburbs of big cities such as Baghdad and Mosul and among large conglomerations of people where the sanitary conditions are unsatisfactory. The incidences caused by *L.major* are much more common ; they appear primarily in rural areas, especially in the northern and southern provinces of the country (WHO, 2003; Korzeniewski, 2005).

c. Mucocutaneous Leishmaniasis, is a variant form of cutaneous leishmaniasis, caused by *L . braziliensis*. This parasite has a tropism for macrophages of oro-naso-pharyngeal region, where it produces mucosal granuloma that eventually destroys the nose and mouth.

d. Diffuse cutaneous leishmaniasis(DCL) is a mutilating disease caused by *L. mexicana*. This infection disseminates with chronic skin lesions, resembling those of leprotamous leprosy, and is difficult to treat.

e - Post – kala - azar dermal leishmaniasis (PKDL) is caused by *L. donovani* , following the cure of the initial visceral leishmaniasis (Zijlstra *et al*., 2001).

Nevertheless, these are not absolute categories and there is a considerable overlap in the clinical manifestations, caused by the various species of *Leishmania*. For instance, cases have been described as visceral disease, caused by *L. amazonensis* (Amaral *et al*., 2000) and *L. tropica*(Magill, 1995).Other examples have been published about patients with mucosal leishmaniasis due to the infection with *L. donovani* or *L. major* in Sudan and Tunisia (Zijlstra *et al*., 1991 ,Aoun *et al*., 2000). Similarly, a form of disseminated leishmaniasis, following infection with *L.guyanensis* has been observed in French Guyana (Maggi *et al*., 2004).

The clinical forms of leishmaniasis resulted from the different affinities of the various species of *Leishmania* for macrophages, are located in different parts of the body. Temperature may be one of the major factors involved in this tropism. The *Leishmania* species that produce cutaneous and mucocutaneous diseases grow better at the slightly cooler temperatures(35ºC) of the skin of human. In contrast, *L.donovani*, the cause of visceral leishmaniasis, prefers the slightly higher temperature of 39°C for its growth (Berman and Neva, 1981).

1.2.2Transmission

The presence of leishmaniasis depends on a variety of ecological and biological factors. Since the various *Leishmania* species depend as much on the specific reservoir as on the specific vector species, a *Leishmania* focus can only exist if suitable ecological conditions are present for both the host animal species and the sandfly species. The topography and the climate are essential for the maintenance of the life cycle. Only if the reservoir and the vector live close enough together, the transmission of the parasite possible and the infectious cycle is maintained.

1.2.2.1 Zoonotic Cutaneous Leishmaniasis

Cutaneous leishmaniasis occurs either as a zoonotic or as an anthroponotic infection. Zoonotic cutaneous leishmaniasis is caused by *L.major*, with rodents serving as reservoir. These rodents live usually in colonies and are commonly found in vast uninhabited areas. The rodent burrows provide excellent breeding places for the sandflies. Through the coexistence of rodents and sandflies in the same habitat,the natural transmission cycle of *L. major* is maintained (Schallig *et al*., 2001; 2002). In zoonotic leishmaniasis humans are the only accidental hosts (Zilberstein and Shapira, 1994). Anthroponotic CL has been attributed to *L.tropica*. Major urban centers of Middle East (Aleppo, Damascus, Baghdad,Teheran and Kabul) were known to be highly endemic for *L tropica* (Aljeboori and Evans,1980). Humans were the only known reservoir.The infections were so numerous as to maintain the transmission cycle. it became,increasingly, obvious that the classical transmission pattern of *L. tropica* infections has changed (Sang *et al*.,1992; Ashford, 2000).The *L.tropica* is predominantly transmitted by sandflies of the species *Ph.sergenti* (Ashford *et al*. , 1993).

1.2.2.2 Zoonotic Visceral Leishmaniasis

Visceral leishmaniasis (VL) occurs either in a typical zoonotic or an anthroponotic pattern, depending on the species involved. *L. donovani* depends on the inter human transmission(Thakur, 2000). Subclinical infections are thought to exist at such a high number that would be sufficient to maintain the infectious cycle. Besides, PKDL patients are suspected to serve as a reservoir, especially, bridging long – term intervals between epidemics. The homophilic nature of the vector of Indian kala-azar, *Ph. argentipes* supports the hypothesis that humans are the only reservoir in India (Rittig and Bogdan, 2000).

In other regions of the world (Mediteranean countries, the Middle East, Central Asia and China) VL is a zoonosis . The causative agent is *L.d. infantum*, which is transmitted by phlebotomine sandflies, which are mainly of the subgenus *Larroussius* . Canids, predominantly dogs, serve as reservoir(Adler, 1962). Dogs suffer from canine visceral leishmaniasis (CVL), a disease closely related to human VL. In addition, dogs suffer, typically,from various dermal symptoms (Guevara *et al*., 1994). The distribution of the disease is typically rural. Wild canids(eg. foxes and jackals) seem to be an important factor for the distribution of the parasite over larger geographical areas (Belli *et al*., 1999). The epidemiology of *L.d. chagasi* in the New World is very similar,causing VL in humans and CVL in canids. The first reported case of VL in Sudan in 1938, the disease has become wide spread and is endemic in south and eastern parts of the White Nile and Upper Nile states(Hashim *et al*., 1995),besides other areas in the west and north of Khartoum (WHO, 1991). As in most countries, males are almost twice as likely to be affected by VL than females, with young children being at the highest risk. In the village of Um-Salala in eastern Sudan, the average age of VL patients was found to be 6.6 years with male to female ratio of 1.8:1. The annual incidence rate was 38.4 per 1000 population between 1991 and 1992, and 38.5 per 1000 during the period 1992 and 1993 (Zijlstra *et al*., 1998). In Somalia, sporadic cases of VL first appeared in 1984, mainly, in Middle Shabelle and Lower Juba areas. A recent retrospective study has shown that VL is endemic in these areas.Children below the age of 15years were at the highest risk and males were over three times more susceptible than females in Ethiopia (Ali and Ashford, 1994). Anthroponotic VL due to *L.donovani*, regularly causes severe outbreaks in Sudan with thousands of deaths and represents major problem in Syria, the desert zones in Egypt, Iraq, Jordan, Libya, Morocco, and Tunisia (WHO, 2006).

1.2.2.3 Zoonotic Mucocutaneous Leishmaniasis

Cutaneous leishmaniasis and mucocutaneous leishmaniasis of the New World are zoonotic, with small forest rodents and sloths serving as reservoir. Forest workers, hunters and settlers on cleared forest lands are, especially, at risk. Sandfly species of the genus *Lutzomyia* and *Psychodopogus* are the vectors (WHO, 1991; Ahluwalia *et al*., 2004).

1.2.3 Taxonomy

The classification of *Leishmania* was initially based on ecobiological criteria, such as vectors , geographical distribution , tropism , antigenic properties and clinical manifestation (Bray, 1974; Gardener *et al*., 1974). However, biochemical and molecular analysis showed that pathological and geographical criteria were often inadequate and thus other criteria, such as patterns of polymorphism,exhibited by kinetoplastic DNA(kDNA) markers, proteins or antigens, came to be used to classify *Leishmania* (Godfrey, 1979;Lanotte *et al*.,1981; Le Blancq *et al*., 1986). All members of the genus *Leishmania* are parasites of mammals (Ross, 1903). The two subgenera, *Leishmania* and *Viannia*, are separated on the basis of their location in vector's intestine (Lainson and Shaw, 1987). Initially, species classification was based on various extrinsic criteria, such as clinical, geographical and biological characteristics ; for example , *L. guyanensis* (isolated in Guyana), *L.peruviana*(isolated in Peru), *L.infantum* (isolated from a child in Tunisia) and *L. gerbilli* (isolated from gerbils). Since the 1970s,intrinsic criteria, such as immunological, biochemical and genetic data have been used to define species of *Leishmania* (Lumsden, 1974). The use of such molecular techniques has led to the publication of taxonomic scheme by the W.H.O (WHO, 1991)(figure 1.1).

Kingdom	**Protista**
Subkingdom	**Protozoa** (Anton van Leeuwenhoek, 1674)
Phylum	**Sarcomastigophora**
Subphylum	**Mastigophora**
Class	**Zoomastigophora**
Order	**Kinetplastida** (Honigberg, 1961)
Family	**Trypanosomatidae** (Mesnil and Brimont, 1908)
Section	**Salivaria**
Genus	***Leishmania*** (Leishman and Donovan,1901)
Species	***donovani, tropica, mexicana, braziliensis***

Figure (1.1) Taxonomy Scheme of *Leishmania spp.* (WHO, 1991)

New methods of detection, isolation and genetic identification were resulted from a massive increase in the number of the species described. Today, 30 species are known and approximately 20 are pathogenic for humans. These species generally represent different epidemiological and clinical characteristics,related to different genetic and phenotypic profiles (Cupolillo *et al*, 1995). Debate has centred on *L.panamensis*, *L.chagasi*, *L. peruviana, L.infantum,L.archibaldi, L.garnhami, L.pifanoi, L.forattinii* and *L. venezuelensis* (Ban˜uls *et al*., 2002 ; Mauricio *et al*., 2006).

1.2.4 Morphology

Leishmania exists in two forms:

a-Amastigote form

Amastigotes are ovoid and non flagellated forms of *Leishmania*, measuring 3-5 μm in length (Ross , 1903). On simple light microscopy, the nucleus is either central rounded or oval, while the kinetoplast is either small rounded or rod shaped.An infolding of the surface membrane creates an internal space, termed as 'flagellar pocket'. The flagellum is not functional in amastigotes and does not extend beyond the cell body (Allen and Aderem, 1995). In addition to anchoring the flagellum, the main function of the pocket is to function as a site of endocytosis and exocytosis (Webster and Russel, 1993).

Immediately below the origin of the flagellum lies a dense mass of mitochondrial DNA known as kinetoplast . The kinetoplast DNA is composed of several thousand circular DNA molecules, linked together in a catenated network (Shlomai , 1994). These DNA networks are of two types: each kinetoplast contains 25-250 maxicircles of approximately 30kb, and 5000 - 10,000 minicircles of about 2kb size each. Together, these constitute the mitochondrial genome . The cytoplasm contains both rough and smooth endoplasmic reticulum.The Golgi complex is typically found in the vicinity of the flagellar pocket, which probably reflects the role of this organelle in the endocytic and exocytic pathways. Lysosomes are also found in the cytoplasm together with an organelle that is unique to kinetoplastids,the glysosome (Opperdoes, 1991; Cupolillo *et al*., 2003).

The developmental cycle is initiated by the interaction of metacyclic promastigotes with skin macrophages.After the uptake and internalization

of metacyclic promastigotes in phagosome, fusion with lysosomes proceeds as normal and parasites inhabit a secondary lysosome or phagolysosome. During this process, the metacyclic promastigote transforms into an amastigote within 12-24 h and continues to grow and divide within the phagolysosomal compartment (Alberts *et al*., 2002). The amastigotes have to overcome two environmental challenges: the battery of lysosomal enzymes and low *p*H (4.5-5.5). Low *p*H is not a problem as amastigotes seem to be acidophiles: they are metabolically more active at low *p*H (Bates, 1993 ; Zilberstein and Shapira , 1994)(figure 1.2).

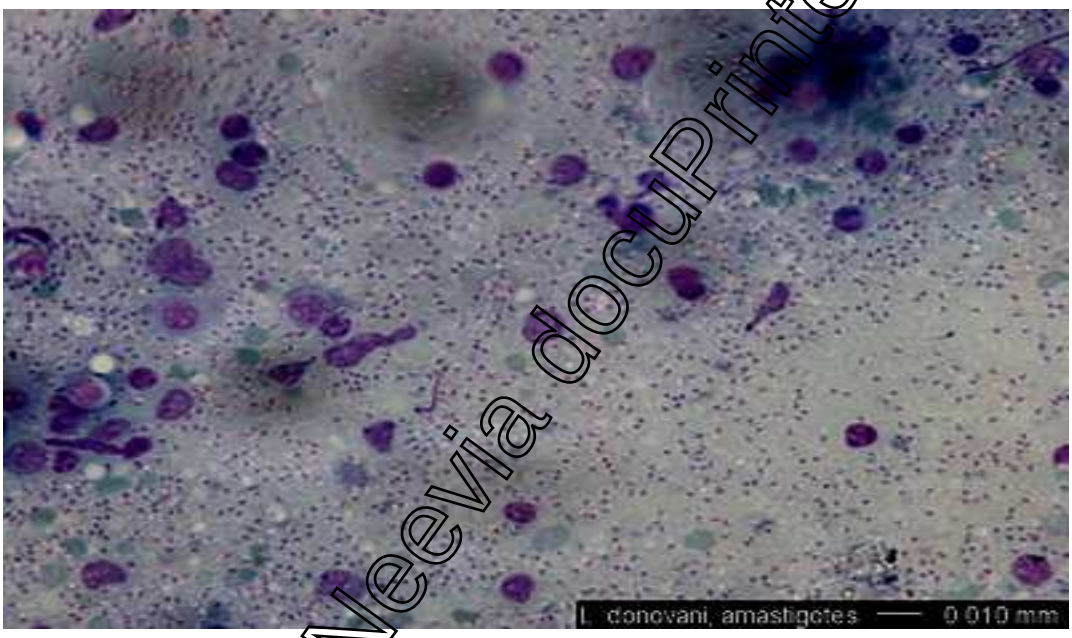


Figure (1.2) Amastigote of *L. donovani* in the Biopsy of Bone Marrow
(Centre for Disease Control and prevention ,USA)

b-Promastigote Form

In the sandfly host, the parasite is found in the promastigote form. The transformation of amastigotes to promastigotes starts within hours of ingestion of the amastigotes (either free or intracellular) and occurs,

exclusively, in the gut. The amastigotes are completely transformed into motile promastigotes within 24 - 48 h and they can keep on dividing by binary divisions. The mature metacyclic promastigotes are accumulated in the midgut and foregut (Ashford and Bates, 1998; Herwaldt, 1999).

The main difference from amastigotes is that the cell body is elongated and in the range of 8-15 μm, the flagellum emerges from the cell body,and functions to make these cells motile (Figure 1.3). The promastigote

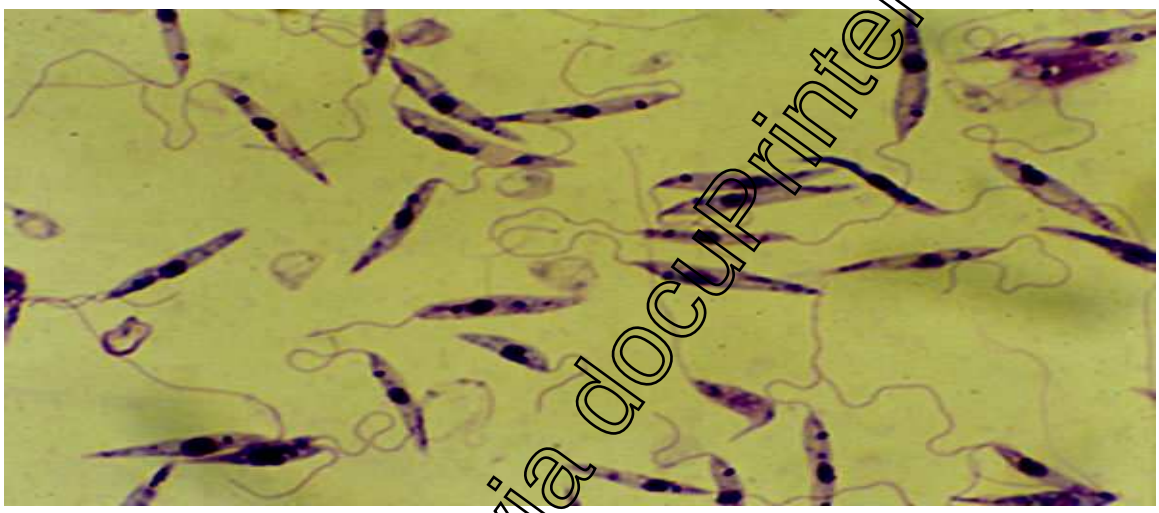


Figure (1.3) Promastigote of *L.donovani* in Blood Smear
(Centre for Disease Control and Prevention, USA)

flagellum has a paraxial rod, a paracrystalline structure running parallel to the microtubules of the axoneme. There is a variety of different promastigote forms that can be separated on morphological grounds, but functional distinction is less complete (*e.g.*, procyclic promastigotes, paramastigotes, nectomonad promastigotes, haptomonad promastigotes and metacyclic promastigotes) (Desjardins, 2003). The first developmental event in the sandfly is probably the transformation of amastigotes to procyclic promastigotes. These events occur in the posterior midgut of the sandfly (Bates,

1994; Ashford and Bates, 1998). When the multiplication of procyclic promastigotes occurs, they elongate and transform to nectomonad forms of 15-20 μm body length. Approximately, three days after blood feeding, the peritrophic membrane (a secretory sheath), which contains these parasites, usually begins to breakdown and so promastigotes start to set free forward to the anterior midgut (Lang *et al*., 1991; Walters, 1993).

Lipophosphoglycan (LPG) plays an important role in attaching and maturating the infection (Pimenta *et al*.,1992 ; Sacks *et al*., 1994). Within five days, the infection reaches the anterior midgut. Here, haptomonad promastigotes are attached to the stomodeal valve(Vickerman and Tetley, 1990). From the 5th day onwards, increasing numbers of small (5- 8 μm), narrow, highly motile, metacyclic promastigotes can be observed in the lumen of the anterior midgut or foregut,or both. The role of the fall in gut *p*H in inducing metacyclogenesis is more speculative, but promastigotes are known to acidify their culture media during growth *in vitro*(Bates and Tetley, 1993). In suitable culture medium and at appropriate temperature (26°C) within 24–28 hours at *p*H 7.0-7.5,these promastigotes are obtained.

1.2.5 Life Cycle

During their complex life cycle, *Leishmania* parasites are exposed to different extra-and intracellular environments. These organisms are digenetic parasites with two basic life cycle stages: an extracellular stage within an invertebrate host (phlebotomine sand fly) and an intracellular stage within a vertebrate host . Thus, the parasites exist in two main morphological forms, amastigotes and promastigotes, which are found in vertebrate hosts, invertebrate hosts, and in culture, respectively (Bogdan and Rollinghoff , 1999) (figure 1.4).

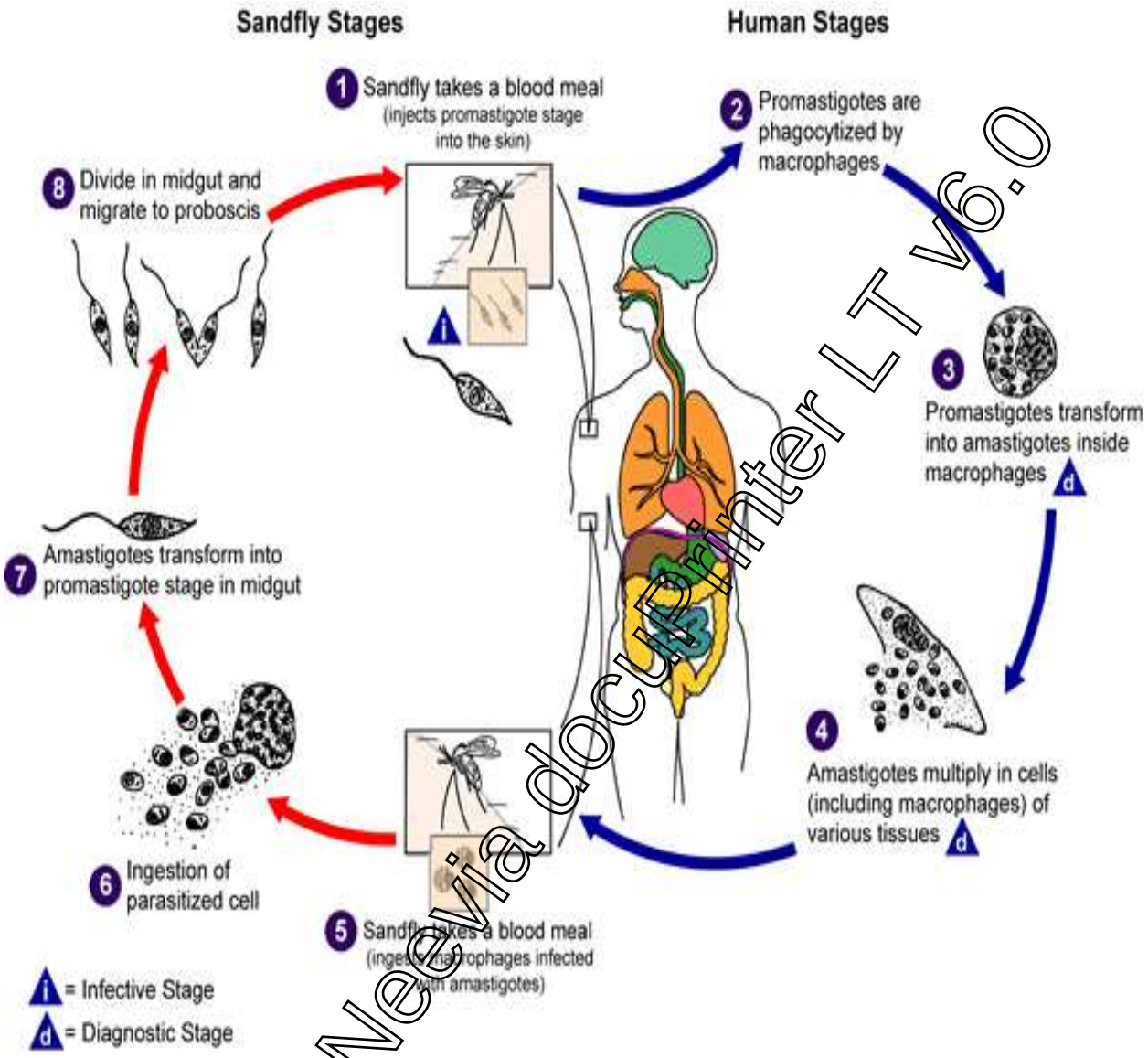


Figure (1.4) Life Cycle of *Leishmania* sp.

(Handman and Bullen, 2002)

1.2.5.1 Stages in the Invertebrate Host

The invertebrate hosts or vectors are small insects of the order Diptera,belonging to the subfamily Phlebotominae. They are commonly called phlebotomine sand flies. Out of the six genera described , only two are of medical importance : *Phlebotomus* of the 'Old World', divided into 12 subgenera, and *Lutzomyia* of the 'NewWorld', divided into 25 subgenera and species groups . All known vectors of the leishmaniasis are species of these two genera. Among the 500 known phlebotomine species,only 31 have been positively identified as vectors of pathogenic species of *Leishmania* and 43 as probable vectors(Killick-Kendrick, 1990, 1999).

The sand fly species, involved in the transmission of *Leishmania*, vary from one geographical region to another,but depend on the species of *Leishmania* (Killick - Kendrick, 1990, 1999). Like mosquitoes, the female needs a blood meal for egg development and only the female is haematophagous. Some phlebotomine species can support growth of only those species of *Leishmania* with which they are infected in nature, such as *Ph. papatasi* and *Ph.sergenti* ; these species are considered to be restricted vectors (Pimenta *et al*., 1994 ; Kamhawi *et al*., 2000). By contrast, other phlebotomine species, such as *Lu. longipalpis* and *Ph. argentipes* are permissive vectors since they are able to develop mature transmissible infections when infected with several *Leishmania* species (Rogers *et al*., 2004).

Within the intermediate host, *Leishmania* develops as promastigote forms,elongated motile extracellular stages, possessing a prominent free flagellum. Nevertheless, a variety of different promastigote forms have been distinguished on morphological grounds (Bates and Rogers, 2004).

1.2.5.2 Stages in the Vertebrate Host

The most remarkable accomplishments of *Leishmania* is that they successfully parasitize the mammalian cells that are responsible for killing invaders : macrophages. *Leishmania* are extremely successful parasites and natural infections are found in many different orders of mammals(Lainson and Shaw, 1987): rodents,canids,edentates,marsupials, procyonids, primitive ungulates and primates. All these mammals are considered potential reservoirs of the disease. Humans are possible hosts of these parasites, but in the majority of cases they are considered to be accidental hosts.

In the vertebrate host, the parasite evolves into an amastigote form. Amastigotes are ovoid (3-5 mm diameter), nonmotile intracellular stages. They do not have free flagellum and are located in the parasitophorous vacuoles of the host's macrophages (Handman and Bullen, 2002).

1.2.6 Modes of Transmission

Worldwide, vector-borne transmission is the most common mode of transmission (Lainson and Shaw, 1987; Ashford and Bates,1998; Kumar *et al*., 2001). Other modes of transmission such as transplacental, congenital, sexual, occupational (needle stick) exposures, and person-to-person transmission could also, theoretically,occur (Sivakumar, 2004).

1.2.6.1 Vector-Borne Transmission

When sandflies bite an infected host , they swallow *Leishmania spp.* amastigotes which circulate freely in the host's blood or inside peripheral blood mononuclear cells(PBMCs)(Lerner *et al*.,1991). These amastigotes migrate to the sandfly's proboscis, where they develop into stationary,

infective - stage organisms that could be qualified as "metacyclic" promastigotes (Lerner and Shoemaker, 1992).When this infected sandfly bites a second host, *e.g.*, a human being, these promastigotes are released and deposited on the site of bite or injected along with potent vasodilators (*i.e.*, maxadilan) that produce long-lasting erythema (Anez *et al.*, 2003). Macrophages phagocytize these promastigotes and enable them to survive inside the phagolysosome, and again transform into amastigotes. There, they proliferate by binary fission, ultimately causing lysis of the host cells and infecting the surrounding macrophages (Le Blancq *et al.*,1986; Sacks and Perkins, 1996). This cycle ends when another sandfly comes to feed on this host , carrying the infection to another host.

1.2.6.2 Transfusion-transmitted Leishmaniasis

Transfusion-transmitted leishmaniasis has been reported widely from many countries including India (Singh *et al.*, 1996;Mathur and Samantaray, 2004). It requires the parasites to be present in the peripheral blood of the donor, preferably asymptomatic, survive processing and storage in the blood bank,and infect the recipient(Popovsky,1991;Schreiber *et al.*, 1996). The *L.donovani* are expected to remain present in the blood for an undefined period between the bite of sandfly and their final localization to the target organs . By the time the clinical symptoms appear in the patient, the parasites may have already been circulating in the peripheral blood (Martin-Sanchez *et al.*, 2004). The first report of transfusion-transmitted kala-azar came from China in 1948(Chung *et al.*, 1948). Other reports of transfusion-transmitted kala-azar followed these two reports and have been published from France (Andre *et al.*, 1958), Sweden (Kostman *et al.*, 1963), Belgium (Cohen *et al.*, 1991), United Kingdom (Cummins *et al.*, 1995), India (Singh *et al.*, 1996) and Brazil (Luz *et al.*, 1997).

1.2.6.3 Needle-Sharing

At March(1993), 18, 347 cases of AIDS and about 200 cases of HIV-associated leishmaniasis were detected in Spain, of which more than 85% occurred among intravenous drug users (IVDUs) (Alvar *et al*.,1997). The infection is so common that 17% of 111 bone marrow aspirates(BMAs)in HIV-positive subjects with fever had amastigote. Other studies have been conducted with similar findings (Cruz *et al*., 2002).

1.2.6.4 Congenital Transmission

The first case of congenital leishmaniasis was reported in 1926 by Low and Cooke(Low and Cooke, 1926). Congenital VL manifests within three months of life and manifestations are by and largely similar to that of *Leishmania* acquired through sandfly bite, but the course is usually rapid (Sharma *et al*., 1996 ; Napier and Gupta, 1998). Pregnant women became more susceptible to leishmaniasis due to shift of cell mediated immunity to humoral immunity (Wegmann *et al*., 1993).

1.2.6.5 Sexual Transmission

Urine and prostatic fluid cultures from patients with VL have yielded promastigotes. Reports of sexual transmission (Mebrahtu *et al*., 1993; Paredes *et al*., 2003) include the transmission from man to woman, as well as the probable transmission in a homosexual man with AIDS, who had rectal lesion and an admitted frequent receptive anal intercourse while vacationing in endemic areas of Spain (Magill, 1995; Paredes *et al*., 2003).

1.2.6.6 Laboratory-Acquired Transmission

Laboratory acquired infections caused by *L. tropica* , *L. braziliensis* and *L. donovani* had been reported (Herwaldt and Juranek, 1993). Many of them were from needle-stick injuries, which led to ulcers at the inoculation site ; few were related to handling of contaminated specimens; and some to oral exposure, which led to visceral involvement.

1.2.7 Epidemiology of Leishmaniasis

1.2.7.1 Epidemiological and Ecological Diversity

Leishmaniasis is a typical example of an anthropozoonosis. The majority of infections are originally zoonotic (Grimm *et al*., 1996; Ahluwalia *et al*., 2003), although some cases are known to be a transmission of *L. donovani* from human to human. The different epidemiological cycles are :-

a- a primitive or sylvatic cycle (human infection is an accidental transmission, occurring in wild foci), e.g. *L. braziliensis*.

b- a secondary or peridomestic cycle (the reservoir is a peridomestic or domestic animal, parasite being transmitted to humans by anthropophilic sand flies), e.g. *L. infantum*.

c- a tertiary, strictly anthroponotic cycle, in which the animal reservoir disappears (or not yet been identified) and the sand fly vectors are totally anthroponotic e.g. *L.donovani*.Nevertheless,many unknown factors remain. For example, the main animal reservoir of *L. braziliensis* is still unknown (Cupolillo *et al*., 2003). *L. tropica* was considered to be a strict anthroponosis, but several cases of canine infection have been described (Dereure *et al*., 1991; Guessous-Idrissi *et al*., 1997).

One of the characteristics of the *Leishmania* cycle is that it is associated with various biotopes (Lainson and Shaw,1987). Thus, the cycle of a *Leishmania* species is not restricted to one specific environment, since a single species can be found in very different environments (Dedet, 1993; Lucas *et al*., 1998 ; Mauricio *et al*., 2001). Therefore , a single parasite species can develop in different sand fly species (Martin-Sanchez *et al*., 1994 ; Ferro *et al*., 1995). This environmental diversity also favours the ability of *Leishmania* to adapt to different vertebrate hosts (Rotureau, 2006; Tibayrenc, 2007). Another complicating factor is the possible presence of several *Leishmania* species in single leishmaniasis focus and thus sometimes in a single host (Antoine *et al*., 2004 ; Madeira *et al*., 2006). These various characteristics could have played important role in diversification of the *Leishmania* population and in speciation (Osorio *et al*., 1998).

1.2.7.2 Clinical Diversity and the Immune Response in Humans

(a)Clinical Expression in Humans

The hypothesis, based on epidemiological data, is that the majority of *Leishmania* species are adapted to a large range of hosts, and that the infections remain asymptomatic (Lainson and Shaw, 1987). On the other hand, in animals that are less well adapted, such as humans, infections can produce a wide range of diversified pathologies, from asymptomatic carriers and benign cutaneous lesions to more serious cases, such as the visceral form (Carroll, 1998). When humans are bitten by infective sand fly, parasite inoculation can lead to the development of leishmaniasis that has no effect on health. The rate of asymptomatic carriers (infected individuals without clinical manifestations) is not accurately known, but different studies have suggested that it may be higher than expected.

It appears that the different clinical forms are closely related to the adaptive immune response of the host , especially, the equilibrium between cellular and humoral immunity. The nature of the pathogen, notably,the species, seems to be strong factor as well (Salotra and Singh, 2006). Nevertheless, how *Leishmania* spp. cause human diseases and why the clinical symptoms are so variable remain enigmatic. These wide-ranging differences in clinical manifestations in human infection (virulence or degree of pathogenicity) have been discussed by Chang *et al*. (1999, 2003) and Chang and McGwire (2002).

(b) The Immune Response in Human Leishmaniasis

Since many individuals remain asymptomatic, it is obvious that the natural immune response of humans can eliminate or control the parasites. As described above,macrophages are the first host cells to contact and to be parasitized by *Leishmania*. They are key cells in the host immune defence (Basu and Ray, 2005). These cells, as well as the dendritic cells, present the parasite antigens to T cell receptors via the major histocompatibility complex (MHC) molecules (Klein and Sato, 2000; Zinkernagel and Hengartner, 2001). This is the acquired immune response. This step is fundamental since it may influence the type of immune response (Dennert, 1974 ; Kisielow *et al*., 1975 ; Mosmann *et al*., 1986), depending on the cytokine context and on the presented *Leishmania* peptides. Thus, *Leishmania* parasites have evolved mechanisms to evade or interfere with antigen presentation processes, making it possible to partially resist the T cell mediated immune responses (Antoine *et al*., 2004).

These escape strategies appear complex and various since,in humans, different patterns of immunological response are observed according to the clinical manifestation and exposure to different *Leishmania* species. Briefly, different Tcell responses are observed among the different cutaneous forms of leishmaniasis: an absence of a Th1 response (rather than presence of Th2) in diffuse cutaneous leishmaniasis; a Th1 response in patients with self-healing lesions (Kemp *et al*., 1994; Ajdary *et al*., 2000; Kemp, 2000) ; and a mixed Th1/Th2 response with high interferon-g (IFN-g) levels in patients with mucocutaneous leishmaniasis (Carvalho *et al*., 2003). In visceral leishmaniasis, a mixed Th1/Th2 response is observed with the production of IFN-g along with interleukin-10 (IL-10) (Ghalib *et al*., 1993 ; Kenney *et al*., 1998). However, individuals with asymptomatic or subclinical infections of visceralizing species of *Leishmania* show a peripheral blood mononuclear cell(PBMC) proliferation and a production of IL-2, IFN-g and IL-12; in cured patients, both Th1 and Th2 clones producing IFN-g and IL-4 have been isolated (Kemp *et al*., 1993).

The epidemiology of leishmaniasis in a given area is directly dependent on the behaviour of the human and/or animal population in relation to the cycle of transmission.There is a variety of factors that influence the transmission of the disease. Some are the following (Lang *et al*., 1991; Aguilar *et al*., 1998):

• Proximity of residence to sandfly breeding and resting sites.

• Type of housing.

• Occupation.

• Extent of exposure to sandfly bites.

• Natural resistance, whether genetic or acquired.

• Virulence of the parasite species.

• Zoonotic or anthroponotic reservoirs.

1.2.8 The Genetic Structures of *Leishmania*

Leishmania Genome

1.2.8.1 Nuclear Genome

The *Leishmania major* genome project, begun in 1994, has had a considerable success. The sequencing of *Leishmania major* is finished and the sequencing of *L. braziliensis* and *L. infantum* is in progress (Ivens *et al*., 2005). The determination of these *Leishmania* genome sequences will be a milestone for *Leishmania* research. The genome of *L. major* has been evaluated at 32.8 Mb, distributed on 36 chromosome pairs.'Old World' *Leishmania* species have 36 chromosome pairs (0.28 -2.8 Mb) (Wincker *et al*., 1996), whereas the 'New World' species have 34 or 35, with chromosomes 8 and 29 fused in the *L. mexicana* group and 20 and 34 in the *L. braziliensis* group (Britto *et al*., 1998). The chromosomes are linear,between 200 and 4000 kb in length,and possess telomeres, but centromeres have not been identified (Lighthall and Giannini,1992). Chromosome size variability is a characteristic of some *Leishmania* species, even between homologous chromosomes (Blaineau *et al*.,1991), which complicate the use of the karyotype for taxonomic studies. From the *L. major* genome, 911 RNA genes, 39 pseudogenes and 8272 protein-coding genes (36% of which have a putative function) were predicted. The genome has a G+C content of 59.7 % (Ivens *et al*., 2005). *Leishmania* genes are often organized in tandem arrays or at least have two or more copies spread through the genome (Bard, 1989).

1.2.8.2 Kinetoplast DNA

The kDNA is the mitochondrial DNA of the Kinetoplastida and it constitutes 10–20% of the total DNA (Simpson, 1987). It is a network of concatenated circular DNA, divided into two classes (Cortes *et al.*, 2004):

(a) The homogenous maxicircles (25–50 molecules of 20 kb) .

(b)The heterogeneous minicircles(0.8 kb),which have many(104)copies.

The maxicircle is the functional counterpart of the mitochondrial DNA even though its role in the editing of uracil residues into mRNA nucleotides has been demonstrated (Leo´ n *et al*., 1996). The minicircles encode guide RNAs (gRNA) for editing cytochrome oxidase subunit III mRNA (Figure 1.5).

Figure (1.5) Kinetoplast DNA(kDNA) of *Leishmania*. The kDNA is a network containing thousands of catenated DNA molecules (minicircles and maxicircles) (Leo´n *et al*., 1996)

1.2.9 Pathogenesis and Clinical Features of Leishmaniasis

Leishmaniasis is a variable disease with a variety of syndromes that are manifested alone or in combinations(Gangneux *et al*., 2000). The incubation period ranges from days to several months. The sandflies are biting humans and animals in the uncovered and hairless areas of the body. At the inoculation site, an erythematous nodule appears in case of CL. The nodule grows to ulcer with raised edge. This sore remains in that stage without further development and when it heals it leaves scar tissue.Scars can even disappear if they are on the face or over a joint (Lambrechts *et al*., 2004) (figure 1.6).

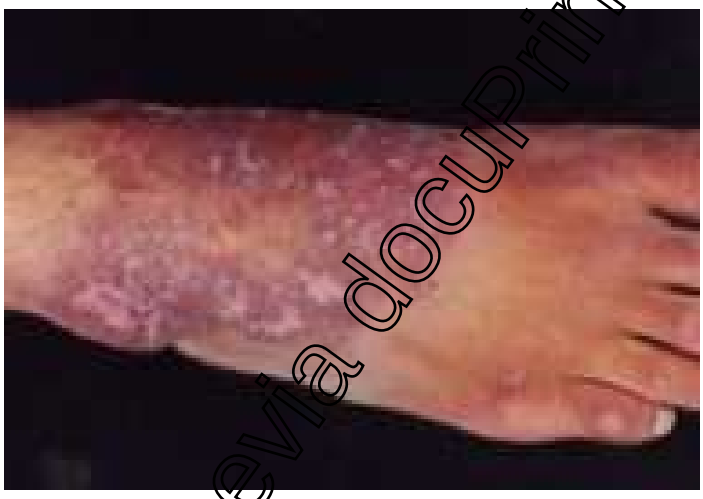


Figure (1.6)Scars of Cutaneous Leishmaniasis
(Lambrechts *et al*., 2004)

After inoculation of the parasite through the sandfly bite, *Leishmania* promastigotes are phagocytosed in the skin by activated macrophages. Patients with acute leishmaniasis fail to produce T helper cell 1 (Th1) cytokines and the parasite interferes with the killing mechanism of the macrophages (Rosenthal *et al*., 1991; Bogdan and Rollinghoff, 1999). The parasites are transformed into amastigotes to be divide. Amastigotes have affinity for macrophages and endothelial cells of arterioles and capillaries, leading to tissue lysis and necrolysis (Tanner, 1996). Then, one of the following events finds place:

• The immune system kills the parasites and the person becomes immune to reinfection by that species.

• A local infection develops until either the immune system of the host eradicates it or is defeated by it permitting dissemination.

• The infection disseminates to the viscera (*L.infantum* ,*L. chagasi*), oronasal mucosa (*L. brasiliensis*) or skin (*L. aethiopica*, *L. mexicana*).

Parasites multiply in the cells of the mononuclear phagocyte system like blood monocytes, macrophages, histiocytes, epithelioid cells, Kupffer cells of the liver , reticulo-endothelial cells in spleen and lymphoid tissue (West *et al*., 2000; Friedman *et al*., 2003) (figure 1.7).

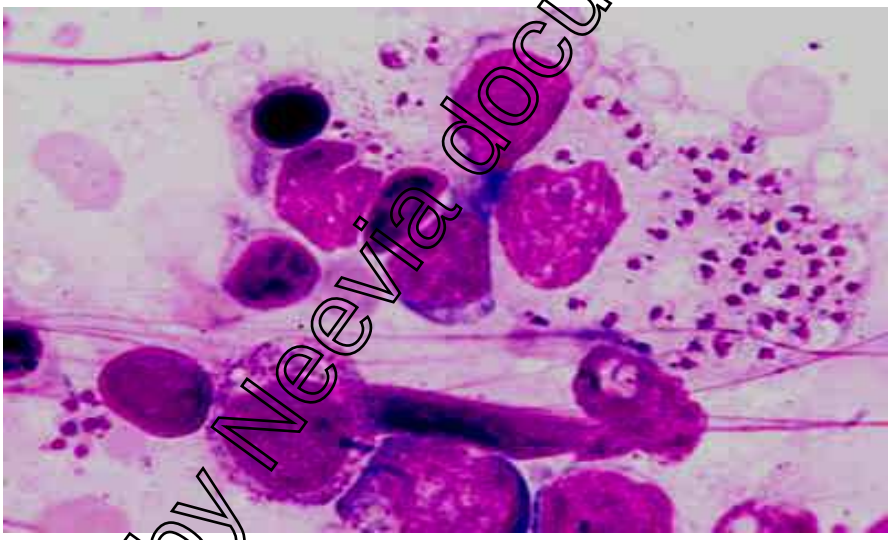


Figure (1.7) *Leishmania donovani* Diagnosed with a Wright's Stain on a Bone Marrow Aspirate
(West *et al*., 2000)

1.2.9.1 Variations in Pathogenic Potential among and within Species of *Leishmania*

a- Animal Models

Animal models are largely used for immunobiological studies to understand and characterize host–parasite interactions during infection. Both the host and the *Leishmania* species are determinants in the progression of the infection. Thus, animal models, such as mice,hamsters and non-human primates respond differently depending on *Leishmania* species involved (Wilson *et al*., 2005).

b- Experiments In Vitro

Studies in vitro of parasite dynamics have been conducted to compare virulent and avirulent strains at inter- and intra-specific levels. A study of growth behaviour in vitro of promastigotes of *L.braziliensis* (responsible for MCL), *L. peruviana* (responsible for benign CL) and putative hybrids between *L.braziliensis* and *L.peruviana* by Dujardin *et al*. (1995) showed that hybrids and *L. peruviana* presented similar growth characteristics and growth capacity (growth rate and cell density at the stationary phase) that significantly lower than that of *L. braziliensis* (Torrico *et al*., 1999).

1.2.9.2 Parasite Factors Involved in Pathogenicity and Virulence

The studies have concentrated on parasitic genetic factors and genetic markers that one directly related to virulence or to genetic markers that make it possible to apprehend them (Lambson *et al*., 2000). Direct genetic factors have been explored via genome manipulations, which can

be classified into two groups: forward and reverse methods (Panton,1991). The first is used to identify genes involved in given phenotype and the second provides a better understanding of gene function by manipulating it (Beverley *et al*., 2002). Based on these methods, many genetic and genomic tools have been used in the study of *Leishmania*, e.g. expression vectors, gene knockout and mutagenesis (Clayton, 1999 ; Motyka and Englund, 2004). With the development of these molecular tools, numerous potential *Leishmania* virulence and pathogenicity factors have been discovered (Robinson and Beverley, 2003) . To classify them, they have proposed a model for *Leishmania* virulence. Their model is based on the classification of parasite factors into invasive/evasive determinants and pathoantigenic determinants (Chang and Mc Gwire, 2002 ; Chang *et al*., 2003). Invasive / evasive determinants of *Leishmania* are crucial for infection, but they produce no pathology in the host, whereas *Leishmania* patho-antigenic determinants elicit antibodies at high titres and thus host immunopathology is a principal cause of the clinical symptoms (Chang and McGwire, 2002). This is not to say that patients with leishmaniasis do not produce any antibodies against invasive/ evasive determinants, but that titres against these determinants are insignificant in comparison to those produced by pathoantigenic ones (Turner *et al*., 2004).

(a) Invasive/Evasive Determinants

The invasive/evasive determinants(Chang and McGwire, 2002) relate to parasitic mechanisms that are necessary to establish infection, such as:

(i) *Leishmania*-macrophage attachment .
(ii) entry of *Leishmania* into macrophages.
(iii) intramacrophage survival.
(iv) differentiation and multiplication of *Leishmania* amastigotes.

(b)Pathoantigenic Determinants

The second group of factors comprises *Leishmania* pathoantigenic determinants (Steinkraus *et al*., 1993 ; Chang *et al*., 2003). This group includes all molecules that are capable of inducing host immunopathology as the principal cause of clinical symptoms. Thus, all *Leishmania* antigens eliciting antibodies at high titres and in comparison to those raised against invasive/evasive determinants can classified in this category. These patho-antigenic determinants are all conserved structural or soluble cytoplasmic proteins, which are often complexed with other molecules to form subcell-ular particles (Chang and McGwire, 2002; Chang *et al*., 2003).

These molecules clearly differ from those present in strains, isolated from cutaneous leishmaniasis. For example, the unique 117 bp repeat, encoding for a 39-amino acid peptide (recombinant products ¼ rK39) in the *Leishmania* kinesin - like gene, is expressed by the amastigotes of visceralizing *Leishmania* species(*L. donovani*, *L. chagasi*) not by dermo-tropic species (*L. major*, *L. amazonensis* and *L. braziliensis*) (Burns *et al*., 1993). Sera from VL patients contains high titres of antibodies specific to this 39-amino acid peptide and is called anti-rK39 (Singh *et al.*, 1995). This antigen has been, successfully, used for serodiagnosing active VL cases.

(c)Usefulness of Modern Tools for Gene Expression and Protein Analysis

Many new genetic tools are being developed for the post-genomic analysis of *Leishmania*. These include a large-scale sequence annotation and database building, microarray analysis and proteomics.These modern tools have been used mostly to explore differences between the develop-mental stages of *Leishmania*. But, the exploration of pathogenic diversity at the inter-and intra-species level has also begun (Li and Lazar, 2002).

1.2.10 Symptoms and Signs

The symptoms of VL vary between individuals and according to geographical foci. However, some of the common symptoms include high undulating fever often, with two or even three peaks in 24 hours and drenching sweats, which can easily be misdiagnosed as malaria. Chills, rigors, weight loss, fatigue, poor appetite, cough, burning feet, insomnia, abdominal pain, joint pain,anorexia,epistaxis and diarrhoea.Clinical sings include splenomegaly, hepatomegaly and lymphadenopathy are the main signs of leishmaniasis (Hashim *et al*., 1995; WHO, 2001).

Visceral leishmaniasis can be complicated by serious secondary bacterial infections, such as: pneumonia, dysentery and pulmonary tuberculosis, which often contributes to the high fatality rate of VL patients. Other complications, though rare, include haemolytic anemia, acute renal damage and sever mucosal haemorrhage (WHO expert committee report, 1991). Systemic visceral infection in children usually begins suddenly with vomiting, diarrhea, fever, and cough. Adults usually have a fever from 2 weeks to 2 months, along with nonspecific symptoms, such as: fatigue,weakness, and loss of appetite. Weakness increases as the disease gets worse (Prasad *et al*., 2004; Markle and Makhoul, 2004).

1.2.11 Immunological Aspects

Leishmaniasis, like leprosy, are a spectrum of diseases ranging from self - healing cutaneous lesions to severe, non-healing disseminated cutaneous, mucocutaneous, or visceral infections. To a large extent, the clinical manifestations of the disease reflect the efficiency of the host's immune response to the parasite. Many of the clinical manifestations of human leishmaniasis can be mimicked experimentally and these experimental infections provide valuable models to study the immunological parameters associated with these diseases (Kropf *et al*.,1998).

During the past decade, impressive progress has been made in our understanding of immune mechanisms involved in leishmanial infections. T cells and the cytokines, release upon activation, play a critical role in determining the nature of the immune response to infect with *L. major* (Kropf *et al*., 1997) . Cytokines form a complex network of synergistic and antagonistic interactions, which not only induce, but also control immune responses. It is generally accepted that the nature of the T cell response is one of the crucial factors controlling experimental and human leishmaniasis (Kropf *et al*., 1999). Naive T helper cells can be differentiated into functionally distinct effector T cell subsets (Th1/Th2) and polarised T helper cell responses, which are crucial factors in mice and human leishmaniasis: Th1 responses lead to healing and immunity, whereasTh2 responses result in nonhealing disease.The mechanisms that lead to the differential expression of cytokines and ultimately to division of T helper cell responses are not entirely clear (Müller *et al*., 1997).

1.2.12 Diagnosis of Leishmaniasis

Visceral Leishmaniasis is usually diagnosed by demonstrating the Parasite in aspirates from the spleen, liver, bone marrow and lymph nodes, but this method is unsuitable in field settings. Quick differential diagnosis of leishmaniasis (Sundar *et al*., 2004) can be achieved with the direct agglutination test,which is quantitative, and which uses freeze-dried antigen, the urine antigen-detection test (dipstick rK39)that is particularly useful in immunocompromised patients and in evaluate treatment efficacy (Attar *et al*., 2001; Sundar *et al*., 2004). For CL, parasitological diagnosis is based on skin smears. For MCL cases,the diagnosis relies on serological tests. This is however, not helpful in most cases because the antibody levels are too low, whereas the manifestations of cell-mediated immunity e.g. Skin-test reactivity, usually develop during an active infection (Herwaldt, 1999).

1.2.12.1 Parasitological Diagnosis

In VL and CL, parasites may be isolated from 80% of the sores during approximately the first half of their natural course (Cuervo *et al*., 2004). Tissue juice,not blood is scraped with a scalpel blade from nodule. The nodule is grasped firmly between the pointed finger and thumb to exclude blood and an incision of a few mm long is made into the dermis. The material obtained can be used to prepare a smear that will be stained with Giemsa, Wright's or Leishman's stain,or inoculate culture media for the isolation and the culture of the parasite. Biopsy smears may be used for culture, inoculation into hamsters, impression smears or immunohistology in tissue sections (Romero *et al*., 1999). Lesion smears and culture are best in cases with cutaneous lesions and biopsy and hamster innoculation when mucosal lesions are predominant.

In VL splenic aspiration is the most sensitive method (Chulay *et al*., 1983). The obtained material is used to inoculate culture tubes and to make smears. The same procedure is followed with patients with AIDS (Delgado *et al*.,1998). Bone marrow and lymph node aspirations have proved to be very useful as well. Lymph node aspirations are, especially, useful in routine field diagnosis in both human and dogs(Kaul *et al*.,2000).

1.2.12.2 Serological Diagnosis of Leishmaniasis

a- Leishmanin (Montenegro) Test

The test measures delayed type hypersensitivity to *Leishmania* antigens. Leishmanin is a suspension of washed promastigotes in a solution of 0.5% phenol in saline.The antigen must be standardised against cases and controlled in the endemic area. An amount of 0.1ml solution is inoculated into the volar surface of the forearm. The area is measured from 48-72 h,

later. The test comes out positive in over 90% of the CL and ML cases, and less frequently in *L. aethiopica* infections and in ML with multiple sites of disease. In active VL, it is negative, but within several months to a year after recovery, individuals elicit a positive response (Weigle *et al*., 1991).

b- Indirect Immunofluorescent Antibody Test (IFAT)

The IFAT is one of the most sensitive tests available.The test is based on detecting antibodies which are demonstrated in the very early stages of infection and which are undetectable six to nine months after cure. If the antibodies persist in low litres, it is a good indication of probable relapse. Titres above 1/20 are significant and above1/128 are diagnostic (Williams, 1995). There is a possibility of across reaction with trypanosomal sera, however,this can be overcome by using *Leishmania* amastigotes as the antigen instead of the promastigotes (Gari-Toussaint *et al*., 1995).

The IFAT has proven to be very suitable for the detection of reservoir hosts of leishmaniasis like dogs, foxes and rodents in the Mediterranean area (Manciati *et al*., 1986; Sideris *et al*., 1999), in Brasil (Courtenay *et al*., 1996) and in Iran (Zovein *et al*., 1984). In western Turkey 490 dogs, were examined, using either IFAT or DAT. Anti-*Leishmania* antibodies were found by at least one test in 5.3 % (26/490) of the dogs. Infections were confirmed by a parasitological examination of polymerase chain reaction on lymph node aspirates in 65 % and 76.4 % of the seropositive dogs tested , respectively. The confirmation rate was 85 % by combining the results of PCR and microscopy (Ozbel *et al*., 2000).

c- Enzyme-Linked Immunosorbent Assay (ELISA)

In this method, the antigen is being absorbed on the surface of a well or on microtiter plate, then the patient's serum is added and the antibodies are bound to the antigen forming the antibody-antigen complex. Non reac-

ting molecules are washed away and an enzyme-linked anti-IgG is added, followed by the substrate.The enzyme is detected by the amount of colour produced and is relevant to the amount of antibodies present in the patient's serum (Mukhtar *et al*., 2000).

Engvall and Perlmann (1972) and Schnur and Zuckerman (1977) developed the first ELISAs for antibody detection and then many variations evolved from that. A modified DOT-ELISA was developed (Pappas *et al*., 1984; 1985) where formalin fixed *L.donovani* promastigotes were fixed on filter discs, placed in a microtiter plate. The sensitivity of this method was 98%, however, there was high cross reactivity with sera from patients,who are suffering from Chagas' disease, African trypanosomiasis and lupus erythematosus. Adhya *et al*.,(1995) found anti-*Leishmania* antibodies in the blood of 23 out of 39 early VL patients, using immobilized crude antigen of *L.donovani*,that captured antibody in serum 1:500 (Jaffe and McMahon-Pratt, 1987). The standard micro-ELISA, using intact promastogotes, obtained from *in vitro* culture as antigen, is in practice in cross-sectional and longitudinal studies of leishmaniasis, that give good results, but one still cross reacting in the co-endemic areas with sera from patients with African trypanosomiasis (El Amin and Omer, 1992).

To eliminate cross – reactions, different sets of chemically defined peptides are used, which are conjugated to a protein carrier, such as: bovine serum albumin (BSA) (Hommel *et al*., 1997). Application of different sets of synthetic peptides, such as a set of five peptides, derived from the amino acid sequence of gp63-like protein (Fargeas *et al*., 1996), lead to lower sensitivity (71%) but increase specificity (93%) in comparison to the crude antigen ELISA (80 % and 79%, respectively). The other sets of peptides that were used in other studies, were the fucose-mannose ligand (FML) (Palatnik-de-Sousa *et al*., 1995) and a glycoprotein which

was present on promastigotes and amastigotes of *L. donovani.* Furthermore, the C-terminal region of the 70 kilodalton (kD) heat shocks the protein of *L. brasiliensis* (Amorim *et al*.,1996), a 28 amino acid sequence derived from the repetitive element of gene B protein (GBP) of *L. major* (Jensen *et al*.,1999). The rK39, a recombinant product, consisting of the 39 amino acid repeat,which is part of 230-kD protein predominant in the *L. chagasi* amastigotes (Burns *et al*., 1993), was tryed as well. They all gave variable sensitivity and specificity numbers , dependent on the *Leishmania* species infecting the patients, but no cross reactivity gave a catholic sensitivity and specificity result for all different kinds of leishmaniasis.

A purified 200 - kDa antigenic fraction from *L. donovani* axenic amastigotes was, diagnostically, evaluated by ELISA for detecting antibody response in VL, PKDL and control patients. It seemed to have a potential prognostic significance and it may be able to differentiate between VL and PKDL(Kaul *et al*., 2000). The main problem with ELISA is that like other serological tests, it cannot distinguish between current, clinical and past infection (Hommel *et al*., 1997).

d- Western Blotting

For western blotting, proteins are originally separated by sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and are then transferred to a nitro-cellulose or nylon membrane. If there are antibodies present in the patient sample, they react with the components of the membrane and the antibody-antigen complex can be detected as in ELISA (Sallota *et al*., 1999).

e- Direct Agglutination Test (DAT)

In this test, the antigen preparation consists of all the organisms and a serological response (mainly IgG) recognises surface-born antigens of the parasite. DAT is a relatively fast and techniqually simple, with high sensitivity and specificity (Meredith *et al*., 1995 ; Hommel *et al*., 1997). Thus, it is one of the most widely that was used immunological tests that has been applied in diagnostic and epidemiological studies.

The main disadvantages of it are cross - reactivity (Hommel *et al*., 1997), the persistence of the antibodies after apparent cure (Zijlstra *et al*., 1991), and the thermal instability of the aqueous antigen (Zijlstra *et al*., 1998). An improved DAT, based on stable, freeze-dried antigen, has been developed to be use on canine serum samples (Dog-DAT) which showed sensitivity of 100% and specificity of 98.8% (Oskam *et al*.,1996).

f- Widal Reaction in Kala-azar Diagnosis

Kala-azar usually has an indolent course, but it can be presented as an acute disease with fever, anorexia and vomiting (Manson - Bahr, 1987; Koehler *et al*., 2002). Widal test inspite of its limitations and fallacies, is still widely used as a proof of enteric fever. It was found that some of the widal positive patients did not respond to the conventional treatment for enteric fever (Sharma *et al*., 1996). Later on, they were proved to be cases of Kala - azar (by demonstration of *L. donovani* bodies, in bone marrow or splenic aspiration) and to respond to anti kala-azar treatment (Singh *et al*., 2002).

1.2.12.3 Molecular Diagnosis of Leishmaniasis

a- Polymerase Chain Reaction (PCR)

The PCR is the amplification of a known specific nucleic acid sequence, using oligonucleotide primers (around 20-mers),which are, specifically,bound to the DNA, flanking the region of interest. The amplification is achieved by using heat-stable DNA polymerase, isolated from *Thermus aquaticus* (Saiki *et al*., 1988). The target DNA is denatured at 94°C and the double strands (ds) become single strands (ss). Then the primers are allowed to anneal at temperature specific for each set(somewhere between 50-65°C). So, after each amplification cycle, each double stranded DNA molecule gives rise to two double stranded copies of target DNA. The PCR products are visualised after gel electrophoresis and Southern blotting (Osman *et al*., 1997; Salotra *et al*., 1999).

A variety of clinical materials have been used for detection of *Leishmania*,like bone marrow,lymph node aspirates and peripheral blood, skin scrapings or sand flies. A variety of target sequences has been used as well, but maximum sensitivity has been achieved by using multicopy sequences like ribosomal RNA genes , kinetoplast DNA , mini-exon-derived RNA genes and genomic repeats (Mathis and Deplazes, 1995; Pizzuto *et al*., 2001). The specificity of the PCR can be adapted to the specific needs by the conserved targeting or variable regions (Piarroux *et al*., 1994; Weiss, 1995).

In peripheral blood, preserved in filter papers, from parasitologically confirmed VL patients, sensitivity was 63 % (Meredith *et al*., 1993), and 70% (Osman *et al*.,1997,1998). Andresen *et al*.,(1996) used venous blood and the sensitivity was 92.5 %. Adhya *et al*., (1995) found that PCR was

able to detect *Leishmania* DNA in 77% of Indian VL patients at an early stage of disease. Mathis and Deplazes, (1995) demonstrated *Leishmania* DNA in leukocytes from the Ficoll-Plaque, in 64 % of blood samples from HIV/*Leishmania* co-infected patients, both in PCR and in culture.

In the case of epidemiological studies, PCR has been used in limited extent, but with very impressive results though. El-Hassan *et al*.,(1993), reported the presence of *Leishmania* in *Arvicanthis niloticus* rodents, a vector of *L. donovani* in eastern Sudan called unstiped grass mice,while Mathis and Deplazes,(1995), using canine samples, had 100% sensitivity in lymph node aspirates, but 38.5% in blood samples. Carreira *et al*., (1995) in Central America showed that PCR provided a rapid diagnosis with a sensitivity of 60% and with a degree of concordance of 87 % to the different molecular techniques used.

b-Polymerase Chain Reaction Solution Hybridisation Enzyme-Linked Assay (PCR-SHELA)

In the case of *L. donovani* complex, a 60 bp repetitive degenerate sequence (Lmet2) was found (Howard *et al*., 1991). Qiao *et al*.,(1995) developed an assay, which targets this sequence that is combined with post-PCR hybridisation and colorimetric detection in microtiter plates, coated with avidin. One of the primers was labelled with digoxigenin,and biotinylated probe was used for hybridisation that was binding to avidin and to the labeled primer. Substrate addition produced a colorimetric reaction and absorbency was measured at 405 nm after 1h using an ELISA reader.

c- Use of Species Specific Primers in PCR

The PCR can be adapted to the specific needs by targeting conserved or variable regions and thus it is possible to characterise the parasite

present in the specimen to the genus complex or species level (Schönian *et al*., 2000 ; Lachaud *et al*., 2001). Primers were designed to amplify a *L. donovani* minicircle sequence. Cross-reactions were present with other *Leishmania* sp.(Smyth *et al*.,1992). Because of the great variability within the kDNA, the use of probes is limited to the geographical region from which the isolate comes from (Meredith *et al*., 1993). Bhattacharya *et al*., (1993), were able to detect CL species, using primers derived from the conserved and variable regions of a kDNA minicircles.

d- Restriction Fragment Length Polymorphism (RFLP) of an Amplified Region

Restriction enzyme digestion of PCR products allowed further differentiation. Van Eys *et al*., (1992) could distinguish *L. donovani* and *L. brasiliensis* from the other species by their characteristic restriction patterns after digesting the PCR product (small subunit ribosomal RNA genes) with restriction enzymes, such as *Rsal* and *Hhal*. Cupolillo *et al*., (1995) , digested the internal transcribed spacer (ITS) region with 10 different restriction enzymes, demonstrating a variety of intra-and inter-specific variations , in a number of New World *Leishmania* isolates. Espinoza *et al.*, (1995), amplified the Gp63 region in 58 *L. peruviana* isolates and exposed the amplicons *Eco*RI and *Sal*I, demonstrating 19 and 16 distinct RFLP patterns, respectively.

e- Arbitrarily Primed PCR (AP-PCR) and Random Amplified Polymorphic DNAs (RAPDs) as Fingerprinting Methods

Three PCR- based methods for DNAfingerprinting were developed in early 1990s. These include : Arbitrarily primed PCR (AP-PCR) by Welsh and McClelland (1990), random amplified polymorphic DNA (RAPD) assay by Williams *et al*., (1990) and DNA amplification fingerprinting

(DAF) by Caetano-Anollés *et al.*,(1991). The common strategy that underlies them all is that they are all based on the use of arbitrary primers that perform PCR amplification of random genomic DNA fragments. Each primer or combination of them generates a characteristic pattern of amplification products, which is visualised by either radionuclide incorporation, ethidium bromide or silver staining. Polymorphisms between individuals or strains are detected as differences between the patterns of DNA fragments from different DNAs using a given primer or a set of primers (Williams *et al*., 1990,1995). This strategy provides a number of advantages over the classic DNAfingerprinting through RFLP because it permits easy and rapid generation of polymorphic markers since it uses very small amounts of DNA and does not require any knowledge of the target DNA sequence (Fisa *et al*., 2002).

The RAPD is often used in molecular studies (Cuervo *et al*., 2004; Indiani de Oliveira *et al*., 2004 ; Martin-Sanchez *et al*., 2004 ; De Castro *et al*., 2005). The main drawbacks of this technique are that :-

(i) The bands of equal electrophoretic mobility may not be homologous.
(ii) The recognition of allelic variants of randomly amplified polymorphic DNA markers is difficult in the absence of crossing data, and so it is normally impossible to distinguish homozygous from heterozygous genotypes at specific loci with this technique.
(iii) the technique is not very reproducible.

1.2.12.4 Species and Strain Identification

a- Enzymatic Identification (isoenzyme analysis)

Isoenzymes are variant enzymes with identical functions. Each of Them in sample may appear as one of the several alternative bands after electrophoretical separation. A pattern is defined as a unique arrangement

of one or more bands displayed by one or more enzymes. The series of bands (one per enzyme), produced by a given isolate is its enzyme profile (Pratlong *et al*.,1994;Taylor *et al*.,1999;Veeken, 1999). Groups of isolates having same isoenzyme profile are called zymodemes (Rioux *et al.*,1990).

b- Southern Blotting Using DNA Probes

Van Eys *et al*., (1989) developed the method first, using two recombinant probes pDK10 and pDK20 which was derived from nuclear DNA. The pDK 10 probe could differentiate the Old World CL-causing species from the *L. donovani* complex and the pDK20 probe was able to distinguish between all Old World *Leishmania* species (Van Eys *et al*., 1992). Most researchers that moved to that direction targeted mainly kine-toplast DNA (kDNA), because its molecules exist in 10 000 copies and have variable regions that differ amongst minicircle classes in the same network (Simpson,1987; Barker,1989).

c- The Sequence-Confirmed Amplified Region Analysis (SCAR)

Genetic markers, identified by sequence-confirmed amplified region analysis (SCAR), have been successfully, applied to population genetic studies in fungi (Taylor *et al*.,1999; Brisse *et al*.,2000; Lewin *et al*.,2002). These markers have two advantages:

(i) they seem to represent a random sample of the genetic variation in natural populations.

(ii) they can easily be amplified by conventional PCR techniques, using only minute quantities of even partially degraded DNA.

d- Kinetoplast DNA

Leishmania kDNA has mainly been used as a diagnostic tool to detect small amounts of parasite DNA in biological materials because of its highly repetitive character (Barker, 1989; Wilson, 1995). kDNA is useful target for PCR due to the abundance of minicircles. The high degree of sequence heterogeneity makes kDNA useful for taxonomic and genetic purposes. Studies have shown that kDNA, and, especially, the minicircle sequences,have a significant degree of polymorphism together with some highly conserved regions (McCann *et al*., 1999; Franco *et al*.,2000; Brewster and Barker, 2002). Based on these properties, kDNA is also used as a target to study genetic polymorphism at specific and intraspecific levels.

Two types of approach have been developed:

(i)PCR amplification of kDNA minicircles by specific primers followed by a cleavage of PCR fragments by different restriction endonucleases (RFLP) (Kapoor *et al*., 1998 ; Berzunza - Cruz *et al*., 2000 ; Rodriguez-Gonzalez *et al*., 2006).

(ii) PCR amplification of kDNA minicircles (Brenie` re *et al*., 1999).

e- Single Strand Conformation Polymorphism Analysis (SSCP)

The SSCP analysis represents comparatively new technique (Orita *et al*., 1989), which is easily implemented and which generates useful markers. The SSCP analysis is based upon principle that electrophoretic mobility of single-strand DNA molecule in a non-denaturing gel is dependent upon both its size and its shape (Axton and Hanson, 1998).

1.2.12.4 Advanced Genetic Researchs of Leishmaniasis

Comparative genomics and single nucleotide polymorphism genotyping are two existing tools that could be applied to *Leishmania* research.

a- Comparative Genomics

The comparative genomics approach has already been tested for other parasites, such as *Plasmodium* by Carlton *et al*. (2002) and has recently begun to be used on the Kinetoplastida by El-Sayed *et al*. (2005). Many species-specific genes,especially, large surface antigen families, occur at non - syntenic chromosome - internal and subtelomeric regions. Retroelements,structural RNAs and gene family expansion are often associated with syntenic discontinuities that-together with gene divergence, acquisition and loss, and rearrangement within the syntenic regions- have shaped the genomes of each parasite (El-Sayed *et al*., 2005) (figure 1.8).

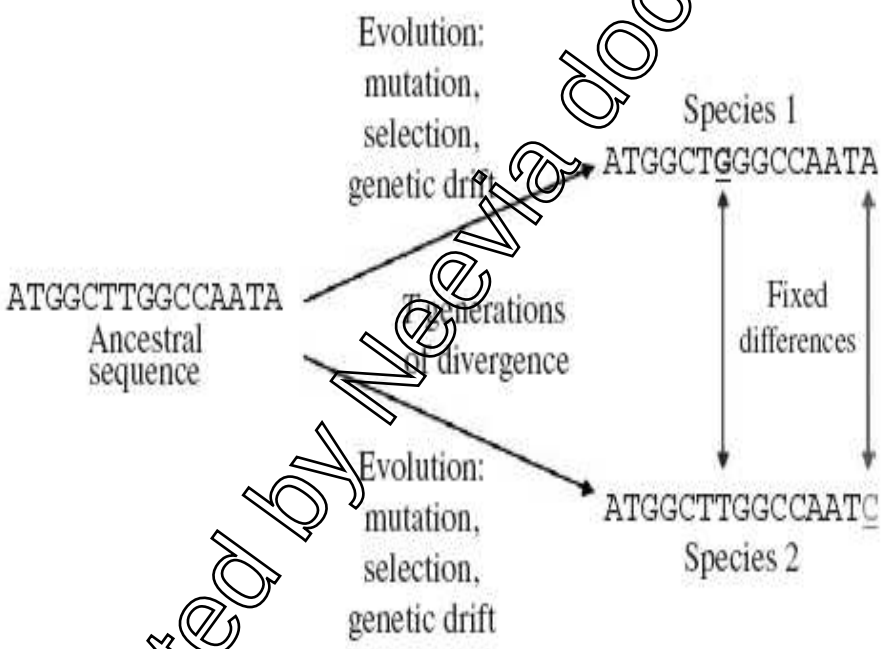


Figure 1.8) Schematic Representation of the Divergence of Two Base Sequences after Speciation Process which showed *T* generation ago. (El-Sayed *et al*., 2005)

The major principle of comparative genomics is very simple: the two (or more) genomes under examination once had a common ancestor (Gutierrez, 2000; Carlton, 2003; McCarter, 2004). Therefore,every nucleotide base, present in the genome of each species, is a result of both nucleotide composition of the original ancestral genome and of the action of evolution on the genome of each species since their divergence (Itagaki *et al*.,1995; Fraser *et al*., 2000; Gutierrez,2000; Ozbel *et al*., 2000). In this figure, evolution can be regarded as a combination of three major forces that independently affect and modify the composition of the genome of each species:mutation, selection and genetic drift (International Mouse Genome Sequencing Consortium, 2002).

b- Other Tools

The example of Single Nucleotide Polymorphisms (SNPs) are single nucleotides that are polymorphic in the population. SNPs represent the most widespread type of sequence variation in genomes and are quite easily characterized and genotyped.As a consequence,they constitute interesting genetic markers that can be used for various purposes : revealing and analyzing the evolutionary history of populations (Brumfield *et al*., 2003), analysing the recombination rate in the genome, mapping genomes or conducting association studies,i.e. linking particular genotypes to particular phenotypes.The SNP discovery is process by which single nucleotide position in the genome is determined to be polymorphic in the population ofinterest (Marth *et al*., 1999; Lindblad-Toh *et al*., 2000 ; Kwok, 2001).

(1) Recombination Studies

The nature and scale of recombination rate variation are largely unknown for most species. Recombination allows sites to evolve independently, and thus may act as a diversifying force, generating new variants

of the parasite. SNPs can help to analyse and / or reveal patterns of recombination in the genome of pathogens (Mu *et al*., 2005). Different methods, parametric and nonparametric (Awadalla, 2003), can be used to estimate the effective recombination rate within populations of pathogens. Such SNP - based approaches have been used in several species, including *Plasmodium falciparum* .

(2) Association Studies and their Limitations

The SNPs can also be used to genetically map DNA sequences that contribute to heritable phenotypes (Pardi *et al*., 2005). While most SNPs are found outside genes and probably do not have an effect.Those located in and near genes may contribute to the genetic basis of certain phenotypes, for example pathogenicity, virulence or resistance to certain drugs. The approach used to identify SNP alleles associated with particular phenotypes is conceptually quite straight forward (Wickstead *et al*.,2003).

c- Towards an Integrated Approach

As mentioned above,the life cycle of *Leishmania* basically includes the vector, the parasite and the host, but the outcome of transmission of infection and disease is dependent on the intrinsic characteristics of these three factors. Indeed,the epidemiology of leishmaniasis reflects the particular combination of interactions among these factors, but in many endemic areas, the exact role of each factor and its relation to human infection are unknown (Volf *et al*., 1994, 2002 ; Ramalho-Ortigao *et al*., 2001; Boulanger *et al*.,2004). It is known, however, that all of them have an impact on the manifestation of the disease:

(i) there is a strong vector–parasite specificity.

(ii) *Leishmania* species are, statistically, associated with certain clinical forms and various factors have been described as associated with clinical diversity.

(iii) the risk of leishmaniasis is markedly increased by allelic variants at specific host genetic loci. This strongly suggests that it is necessary to study all these factors in a single-focus population in order to understand fully the epidemiology of leishmaniasis (Rogers *et al*., 2004).

d- Recombinant Kinesin-Related Protein(rK39)

A kinesin - related protein - encoding gene has been discovered in *Leishmania chagasi* that contains repetitive 117- bp sequence encoding 39 amino acid residues (K39), conserved at the C- terminal end in all of the VL-causing isolates examined so far (Burns *et al*., 1993). The recombinant product of K39(rK39)has proven to be very sensitive and specific antigen in an ELISA for the serodiagnosis of VL from the endemic foci in Brazil, China, Pakistan, and Sudan (Burns *et al*., 1993).

Serological tests have been developed, using the cloned antigen of 39 amino acid repeats of a kinesin like gene, found in *L. chagasi*, instead of the whole *Leishmania* parasites. Studies, using the rK39 antigen, either in ELISA(Badaro *et al*.,1996) or dipstick form(Sundar *et al*.,1998), performed well in Brazil,India and Europe (Badaro *et al*.,1996;Houghton *et al*., 1998). In Sudanese kala-azar patients, a rK39-based ELISA was 93% sensitive, but only 80% specific (Zijlstra *et al*., 1998).

1.3.13 Biochemistry of *Leishmania* Species

Since 1980, there has been a burst of published reports describing the biochemistry of various human parasites. Interest in the biochemistry

and molecular biology of the human parasites has increased greatly, in part because of the growing awareness of the morbidity and mortality, especially, in the developing countries, and because of the increased support for parasite research in developing countries through programs sponsored by governments and various foundations and philanthropic groups concerned about global health problems.

New knowledge about enzymes, proteins, and other macromolecules (e.g., leishmanial excreted factor), synthesized by parasites in general, is important for at least three reasons. First, such information may provide an insight about the function of these macromolecules or may inform us about which ones play some key role in the life cycle or contribute to the infectivity of the parasite. Second, these proteins, especially, if they are essential to the organism living extracellularly or if they play some important pathophysiologic role in the host-parasite relationship,could provide targets for innovative therapeutic strategies.Third,assays for these macromolecules,whether enzymologic or immunologic, might be useful in the diagnosis of leishmaniasis and in the identification of various strains within a particular clinical form of the disease, thereby helping to guide the selection of most appropriate therapy (Mundodi *et al*., 2002; Salotra *et al*., 2006).

Chapter Two
Materials and Methods

2- Materiales and Methods

2.1 Instruments

Instruments	Origin
ELISA reader	Italy
Gamma counter	England
Refrigerator	Iraq
Deep freezer	India
Votex mixer	India
Rotator shaker	India
Centrifuge	Germany
Fluorescent microscope	France
Timer	Swiss
Light microscope	Japan
Water bath	Germany
Incubator	Germany
Polymerase Chain Reaction	India
BioDoc-imaging system	India
Microcentrifuge	India
Laminar flow	India
Gel electrophoresis	India
Thermocycler	India
UV transilluminator	India

2.2 Equipments

Equipment	Origin
Microtiter plate	Germany
Plan tube	China
Centrifuge tube	China
Micropipette	India
Tips	India
Slides	China
Coverslips	China
Rack	England
Syringes	Turkey
Aluminum foil	China

2.3 Laboratory Kits

Laboratory Kits	Origin
ELISA kit	Spain
IFAT kit	Italy
Serum rK39 dipstick kit	USA
DAT kit	Spain
Primer	India
Taq DNA polymerase	India

2.4 Reagents

Reagents	Concentration
Phosphate buffer saline	0.05%
Bovine serum albumine	0.05%
O-phenelenediamine dihydrochloride	5%
H_2SO_4	5%
Tween-20	2%
Glycerol	50%
NaCl	0.9%
Sodium dodecyl sulphate	1%
Sterile deionized water	Variable
EDTA	1mM
Tris-HCl	10mM
$(NH_4)_2SO_4$	40mM
$MgCl_2$	3mM
dNTP	0.4mM
Agarose Gel	1%
Boric acid	9%
Ethanol	70%

2.5 The Area of the Study

This study was carried out in clusters of villages and cities that represent an endemic focus of visceral leishmaniasis . These villages and cities were located in Baghdad and Wasit governorates, mid-south of Iraq. The disease is well known among people in these areas as Black fever.The climate of the study area could be divided through the year into a hot dry summer(May-July),a warm wet autumn(August-November), a cold winter (December-February) and moderately warm spring(March-April). The average minimum / maximum temperature of the area is 28 / 55ºC in

summer and 0 / 34ºC in autumn , winter and spring . This study area is generally characterized by flat and reduced vegetation and by grass surro-unded by large scale of lakes that are supplied by water sewage.

2.6 Clinical Cases

The total human patients involved in this study were divided into seven age groups and males were represented 54.5% (436 / 800) and females 45.5% (364/ 800) in patient groups.

2.7 Sample Collection

Ten ml of blood was collected from a total of 900 individuals. They were categorised as 800 Kala-azar cases and 100 controls by physians according to clinical signs and symptoms. The confirmed cases of Kala-azar had fever, hepatosplenomegaly, anaemia and weight loss from hospitals and central laboratories in different parts of Baghdad and Wasit governorates (Zahra'a hospital, laboratory of public health and health centers). The controls consisted of 100 clinically healthy people.

2.8 Laboratory Diagnosis

Conventional and molecular methods for diagnosing Kala-azar, such as parasitological, serological, and PCR were used in this study. The identification of the causal agent of human leishmaniasis by these tests is difficult even in a well-equipped hospital and because improved tools for this purpose are needed (Sundar *et al*., 2004; da Silva *et al*., 2005).

2.8.1 The Detection of Anti-*Leishmania* Antibodies

The presence of anti-*Leishmania* antibodies in the sera and blood samples was determined by four different tests : serum rK39 dipstick,

ELISA, IFAT, and DAT. All samples were tested by serum rK39 dipstick, While only 100 of them were also carried out by ELISA, IFAT, and DAT.

2.8.1.1 Serum rK39 Dipstick

The rapid immunochromatography test kit "Dipstick rK39" (In Bios International Inc., USA) was used. This rapid assay is for the qualitative determination of antibodies to the recombinant antigen specific for visceral leishmaniasis (Burns *et al*., 1993) caused by parasite members of the *L. donovani* complex.

Principle

The Serum rK39 dipstick test for VL is a qualitative membrane-based immunoassay for the detection of antibodies to Visceral Leishmaniasis in human serum. The membrane is pre-coated with a novel recombinant VL antigen on the test line region, and chicken anti-protein A is on the control line region. During testing,the serum sample reacts with the dye conjugate (protein A-colloidal gold conjugate) which has been pre-coated in the test device. The mixture, then, migrates upward on the membrane chromatographically by capillary action to react with recombinant VL antigen on the membrane and it generates a red line. Presence of this red line indicates a positive result,while its absence indicates a negative result. Regardless of the presence of antibody to VL antigen, as the mixture continues to migrate across the membrane to the immobilized chicken anti-protein A region, a red line at the control line region will always appear. The presence of this red line serves as verificating sufficient sample volume and proper flow and as a control for the reagents (Sundar *et al*., 2004).

Storage

The sealed pouch or vial, containing the test strip, is designed to be stored at room temperature (20°C-28°C). The bottle, containing the Chase Buffer,is designed to be stored at 2°C- 8°C. Exposure to temperatures over 30°C can impact the performance of the test and should be minimized. The strips should not be frozen.The test should be used within 1hour after its removal from the pouch or vial to prevent exposure to humidity.

Sera Collection

1- Human serum was tested with this test strip. Whole blood should not be used with this test as it may affect ones ability to read the test line correctly due to excessive background. Dilutions of serum in buffer cannot be tested directly.

2- The serum was removed from the clot of red cells as soon as possible to avoid hemolysis.

3- The test was performed after sera collection. Do not leave sera at room temperature for prolonged periods. Sera can be refrigerated at 2-8°C up to 3 days. Otherwise, sera should be stored at -20 °C.

4- The sera was bring to room temperature prior to testing.

Kit Contents

A rK39 test strip's membrane is pre-coated with a recombinant VL Antibody (rK39) on the test line region, and chicken anti-protein A is on the control line region.The Kit contains the following:

1.Twenty-five individually pouched test strips .

2. One vial of Chase Buffer solution.

Test procedure

The procdures followed in this test involve the following :-

1. allowing the sera and Chase Buffer to reach room temperature prior to testing.

2. removing the rK39 dipstick test for VL from the foil pouch or vial.

3. adding 20 µl of sera to the test strip in the area beneath the arrow.

4. placing the test strip into a test tube,or into a wells of a 96 well tissue culture plate so that the end of the strip face downward, as indicated by the arrows on the strip.

5.adding 2-3 drops (150 µl) of the Chase Buffer solution, provided with this test kit.

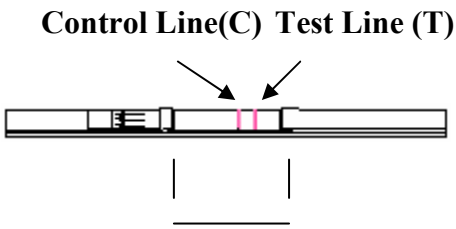
6. reading the results in 10 minutes. It is significant that the background Is clear before reading the test,especially, when samples have low titer of anti-Leishmanial antibody,and only a weak band that appears in the test region(T).The results interpreted after10 minutes can be misleading.

Types of Results

1-Positive Resut

The test is positive when the control line and test line appear in the test area as shown in Figure 2.1. A positive result indicates that the rK39 dipstick detected antibodies to members of *L. donovani* complex. A faint line is considered a positive result. As a guide for interpretation, the red color in the test region will vary depending on the concentration of anti-Leishmanial antibodies present. The test line for "weakly positive" sera

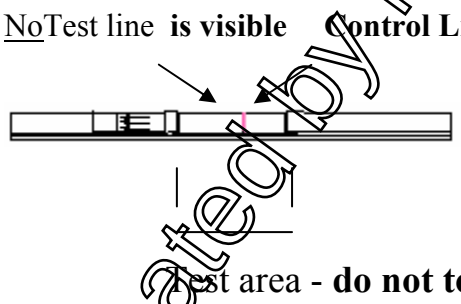
samples may show results between a weak positive red line to a faintly red, almost white background. ("Weakly positive" samples are those with low affinity or low titer antibodies against the recombinant test antigen.)



(Figure 2.1 positive result of rK39)

2- Negative Result

The test is negative when only the control line appears. A negative result indicates that the rK39 dipstick did not detect antibodies to members of *L.donovani* complex. No test line is present as in Figure 2.2.



(Figure 2.2 Negative result of rK39)

3- Invalid Result

No lines appear at either the control or test line areas. The test is also invalid if no control line appears,but a test line is seen.It is recommended to retest using a new *rK39* test for VL and fresh serum.

٢.8.1.2 Enzyme Linked Immuno Sorbent Assay (ELISA)

The ELISA test was performed using the standard procedure described earlier with modifications(Hommel *et al*.,1988; Srivastava and Singh,1988). The antigen was prepared from the promastigotes of *L. donovani* (MHOM/ IN/80/Dd8) grown in monophasic medium(Lowry *et al*.,1951). The protein content of the antigen was estimated by Lowry's method(Harith *et al*.,1988). The standardization of optimum concentration of antigen , was done by chequer board titration method. For coating the wells,0.3 µg of *L.donovani* antigen, diluted in antigen coating buffer (carbonate-bicarbonate buffer, *p*H 9.6) was used and the plates were incubated at 4°C overnight. On the following day the wells were washed thrice with PBS (*p*H 7.2) containing 0.05% (V/V)Tween 20 (PBS-Tween 20) and blocked with 100 µL of 0.5% (w/v) BSA in PBS and incubated for 2 hours at room temperature. Sera from different categories of cases and control were initially diluted to 1:100 with 0.5% (W/V) BSA in PBS. The sera were serially diluted in two fold and 100 µL diluted sera were added to each well.

Plates were incubated at 37°C for one hour. After washing three times with washing solution, 100 µL of antihuman gammaglobulin conjugated with horseradish peroxidase reagent (HRP)(1:1000) was added to each well and incubated at 37°C for one hour. The plates were thoroughly washed and 100 µL of O-phenylenediamine dihydrochloride (OPD) solution(as substrate) was added to each well. The reaction was stopped after 15 minutes with 100 µL of 5N- H_2SO_4. The results were read on an automatic ELISA

reader using a filter of 492 nm. An optical density above 0.270 was taken as positive for presence of antileishmanial Antibodies (Sarkari *et al*., 2002).

ELISA has been used as a potential serodiagnostic tool for almost all infectious diseases,including leishmaniasis.The technique is highly sensitive, but its specificity depends upon the antigen used.Several antigens have been tried. The commonly used antigen is a crude soluble antigen (CSA). It is prepared by repeated freezing & thawing (four to six cycles) of a suspension of promastigotes in phosphate – buffered saline , followed by cold centrifugation at 10,000 to 20,000 _ *g*. The supernatant is used as soluble antigen and is used to coat ELISA plates after estimation of protein content (100 to 5,000 ng/ml).

2.8.1.3 Indirect Immunofluorescent Antibody Test (IFAT)

The IFAT using commercial kit (Germany) for the diagnosis of human leishmaniasis (FIOCRUZ/BIO-MANGUINHOS) was performed according to the instructions of the manufacturer for detection of antibodies in serum diluted from 1:40 up to 1:640.

The procedure of the IFAT test uses as antigen whole *Leishmania* promastigotes. Dried antigen slides are stored at -70°C until use. A two-fold serial dilution of the test serum in phosphate buffered saline (PBS) is placed on 12 spots slides. The dilution may vary from 1:1 up to 1:1040 or further.Fluorescein-conjugated anti-human IgG at optimal dilution is added to each spot. The slides are considered to be positive when more than 50% of the parasites show complete peripheral flourescence (pappas *et al*., 1985).

Test procedure (WHO, 2001)

1. The frozen antigen-coated slides were washed in PBS and allowed to dry at room temperature.

2. The sera were inactivated for 30 minutes in water bath at 56 C.

3.The amount of 30 µl of diluted serum were distributed on each slide circle and incubated for 30 minutes at 37 C.

4. The serum were removed by vigorous washing in PBS.

5. An amount of 30 µl of diluted fluorescein-conjugated anti-human IgG were distributed on each slide circle and incubated for 30 minutes at 37 C.

6.A cover slip was mounted in a few drops of PBS/glycerol (50% v/v each).

7. The slide was read under fluorescent microscope the highest dilution show fluorescent promastigote was taken as antibody titer.

2.8.1.4 Direct Agglutination Test (DAT)

The DAT is a highly specific and sensitive test. It is cheap and simple to perform making it ideal for both field and laboratory use. The antigen is prepared from promastigotes of *L. donovani* and test can be carried out on plasma, serum, blood spots and whole blood. Serum titres of 1:3200 are considered positive (El-Masum and Evans, 1995).

The direct agglutination test was performed basically as described by Harith *et al*.(1988) and Meredith *et al*. (1995). In brief, the serum samples were diluted in physiological saline (0.9% NaCl) containing 0.78%-mercaptoethanol and by using microtiter plates. Two - fold dilution series of the sera were made, starting at a dilution of 1:100 (step1) and going up to a maximum serum dilution of 1:6400 (step 11). Well 12 was used as a negative control. Fifty µL of DAT antigen (concentration of 5 x 10^2 parasites / ml) was added to each microtitre plates with V-shaped wells containing 50 µL of diluted serum. After completion of test, microtitre plates were shaken on a plane surface and were incubated at 22°C overnight after wrapping it with aluminum foil. The results were recorded in titres.

2.8.2 Molecular Tests

Polymerase Chain Reaction

In Baghdad and Wasit governorates, Centeral health laboratories of Iraq, are a reference center for diagnosis of VL, receiving patients from various cities of Iraq for diagnostic confirmation or exclusion.

Hundred blood samples from the governorate of Baghdad and Wasit, admitted for diagnosis of VL from January 2008 to August 2008 were studied by using PCR. Diagnosis of VL was based on suggestive clinical presentation, associated with a positive DAT test.

Procedure of PCR

Materials

1- BioDoc-ItTM imaging system (Bangalore Genie, India)
2- Microcentrifuge (India)
3- Laminar flow (India)
4- Gel Electrophoresis (San Leonardo, CA)
5- Thermocycler (India)
6- pipette
7- Lysis Buffer /50 cc
8- Londing buffer
9- Tris base, Boric acid, EDTA [TBE(50x)]
10- NaCl
11- NaOH

Protocol for PCR

1-DNA Extraction (Maniatis et al., 1982)

1. The blood sample was homogenized by using vortex.
2. An amount of 100µl of the sample was added to 200 µl of lyses buffer.
3. An amount of 20 µl of Protease K was added to the above prepared sample and shake well for 5 second.

4. The sample was centrifuged at 8,000 rpm for a short period of time then transferred to 56 ºC water bath and keep it for 1 hour.
5. 300 µl of Phenol + Chloroform was added (Phenol: Chloroform; 1:1).
6. The prepared sample was centrifuged at 5,000 rpm for 5 minutes, and 3 phases were formed.
7. The top phase was separated in a new tube which contains DNA.
8. The same volume of Chloroform was added to the separated DNA and shake well.
9. The tube was centrifuged at 5,000 rpm for 5 minutes.
10. The top phase was separated in a new tube which contains DNA.
11. The same volume of Isopropanol was added to the separated DNA.
12. An amount of 0.1 sodium acetate was added (3M: pH=5.2),and mix.
13. The tube was transferred to -20 °C freezer for 10 minutes.
14. The tube was centrifuged at 12,000 rpm for 15 minutes.
15. The supernatant was discarded and dry the pellet (the tube was put upside down on a piece of paper).
16. The same volume of 70% Ethanol was added to the tube.
17. The tube was centrifuged at 5,000 rpm for 5 minutes.
18. The supernatant was discarded and dry the pellet again (the tube was put upside down on a piece of paper).
19. An amount of 20-50 µl of distilled water was added and mix it.
20- The tube was stored at -20 °C until being used.

Lysis Buffer

Tris-HCL	10 mM ; pH= 8
EDTA	1 mM ; pH=8
Tween 20	2 %
SDS	1 %
NaCl	100 mM

2-PCR Primers

- Forward Primer: 5' TCGCAGAACGCCCCTACC 3' (Indiagen, India)
-Reverse Primer: 5' AGGGGTTGGTGTAAAATAGG 3' (Indiagen, India)
-DNA
- Mastermix (Indiagen, India)
- Diethyl pyrocarbonate treated water (DEPC).

PCR Reagent for One Sample

REAGENT	**VOLUME ADDED**	**FINAL CONCENTRATION**
DEPC water	**9.5 µl**	
-Mastermix: **150mM Tris-HCl PH 8/5,** **40mM $(NH_4)_2SO_4$,** **3mM $MgCl_2$*** **2% Tween 20** **- dNTP's** **- Taq DNA Polymerase** **- Loading buffer** **- DEPC water**	**12.5 µl**	 **0/4mM** **0/05 u/µl**
Forward Primer	**1 µl**	
Reverse Primer	**1 µl**	
DNA	**1 µl**	
Final volume	**25 µl**	

1. All the reagents were thawed from ice.

2. The DNA templates were thawed at room temperature,and vortex lightly before use.

3. The PCR mix was prepared according to the above table and distributed into 0.2 ml for each tube.

4. The PCR reaction was performed according to the following cycling program, and disabled the heated lid:

No.	Temperature	Time	Cycles	
1	**95°C**	**5 sec**		**Initial Denaturation**
2	**94°C**	**30 sec**		**Cycle Denaturation**
3	**60°C**	**45 sec**	**31**	**Primer Annealing**
4	**72°C**	**1 min**		**Primer Extension**
5	**72°C**	**5 min**		**Substrate Clearance**
6	**4°C**	∞		**Storage**

3-Electrophoresis

Purpose: To determine the presence or absence of PCR products and quantify the size (length of the DNA molecule) of the product.

Materials Needed: Agarose
TBE Buffer
DNA ladder standard
Electrophoresis chamber
Gel casting tray and combs
DNA stain
Staining tray
Pipette and tips

Recipes:TBE Buffer

- Tris base 107.81 g/l (0.89 M)
- EDTA 7.44 g/l (0.02 M)
- Boric acid 55.0 g/L (0.89 M); added Tris base to ~900 ml H_2O, then added boric acid and EDTA to solution and mixed. The mixture was poured into 1L graduated cylinder and added H_2O to total volume of 1L.

Agarose Gel Electrophoresis Protocol (Ogden and Adams, 1987)
Preparing the Agarose Gel (1%)

• 0.35 g of agarose powder was added into 500 ml flask.
• An amount of 35 ml TBE Buffer was added to the flask (the total gel volume well vary depending on the size of the casting tray)
• The agarose was melted in a microwave or hot water bath until the solution becomes clear.
• The solution was let at cool temp. (50-55°C), then the flask was swirl occasionally to cool evenly.
• The ends of the casting tray was sealed with two layers of tape.
• The combs was placed in the gel casting tray.
• The melted agarose solution was poured into the casting tray and added 1µl Ethidium Bromide and let cool until it is solid (20-40 min) (it appeared milky white).
• The combs was pulled out and removed the tape.
• The gel was placed in the electrophoresis chamber.
• The enough TBE Buffer was added so that there is about 2-3 mm of buffer over the gel.

Loading the Gel

• An amount of 1 µl of each sample/Sample Loading Buffer mixture was added into separate wells in the gel.
• An amount of 4 µl of the DNA ladder standard was added into at least one well of each row on the gel.

Running the Gel

• The power supply was turned on to about 100 volts(80 volts,40 Mili amper for 80 min).Maximum allowed voltage will vary depending on the size of the electrophoresis chamber –**it should not exceed 5 volts / cm**
• The current is running was check in the correct direction by observed the movement of the blue loading dye (run in same direction of DNA).
• The gel was placed on a UV transilluminator for nucleic acid visualization and analysis.

2.9 Statistical Analysis

The suitable statistical methods were used in order to analyze and assess the results (Sorlie, 1995), they include the followings:

1- Descriptive Statistics

A) Statistical tables including observed frequencies with their percentages.

B) Summary statistic of the readings distribution (mean, SD, SEM, minimum and maximum).

2 – Inferential Statistics

These were used to accept or reject the statistical hypotheses, they include the followings:

A) Chi-square (χ^2), B) Binomial test(Z), C) Evaluating the validity of the tests.

Note: The comparison of significant (P-value) in any test were:

S= Significant difference ($P<0.05$).

HS= Highly Significant difference ($P<0.01$).

NS= Non Significant difference ($P>0.05$).

3-Computer and Programs

All the statistical analysis were done by using Pentium-4 computer through the SPSS program (version-10) and Excel application.

Chapter Three

Results and Discussion

3- Results and Discussion

3.1 Results

3.1.1 Distribution of Kala-azar Patients According to the District

A comparison between Baghdad and Wasit governorates showed higher infection in Wasit than Baghdad governorate. Therefore, the proportion of Kala-azar infection in Baghdad andWasit governorates revealed 257(32.1%) and 460 (57.5%) were positive results respectively (table and figure 3.1).

Table (3.1) Distribution of Kala-azar according to the District

		Kala azar patients		Total
		Baghdad province	Wasit province	
Serum rK-39 dipstick	Positive	257	460	٧١٧
	Negative	٤٣	٤٠	٨٣
Total		٥٠٠	٥٠٠	٨٠٠

	P-value	C.S
District Wasit and Baghdad	.000	Highly Sig. (P<0.01)

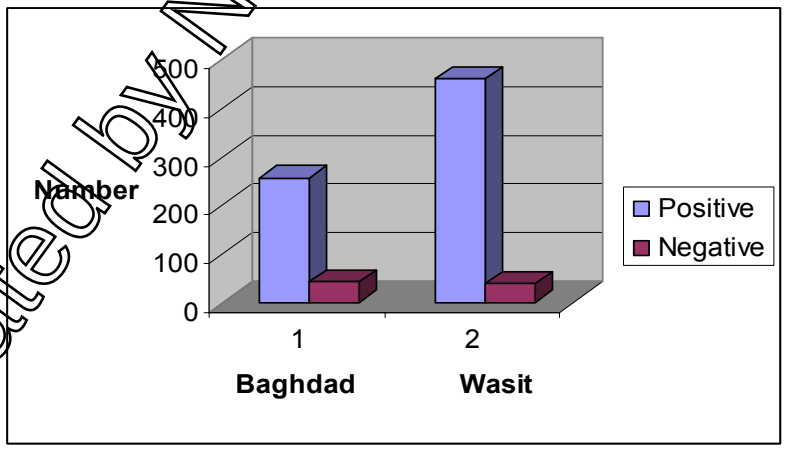


Figure (3.1) Distribution of Kala-azar according to the District

3.1.2 Distribution of Kala-azar Patients According to the Age

The total human patients involved in this study was divided into seven age groups (Table and figure 3.2). Kala-azar infection found among children under one year old of the patient groups 560(70 %) This higher rate than the other age groups which was began to decrease reaching to(1%) in age more than 50 years of age groups,while in control groups there were no infection was recorded (0.00) in all age groups (Table and figure 3.2).

Table(3.٢)Distribution and percentage of Kala-azar according to the age

Age groups / Year		Studied groups		Total
		Kala-azar patients	Apparently healthy control	
G1(<1)	N	560	34	594
	%	70	34	66.0%
G2=1-10	N	115	26	141
	%	14.4	26	15.7%
G3=11-20	N	53	14	67
	%	6.6	14	7.4%
G4=21-30	N	38	8	46
	%	4.8	8	5.1%
G5=31-40	N	15	8	23
	%	1.9	8	2.6%
G6=41-50	N	11	5	16
	%	1.4	5	1.8%
G7(>50)	N	8	5	13
	%	1	5	1.4%
Total	N	800	100	900
	%	100.0%	100.0%	100.0%

P-value	C.S
	Highly
.000	Sig. (P<0.01)

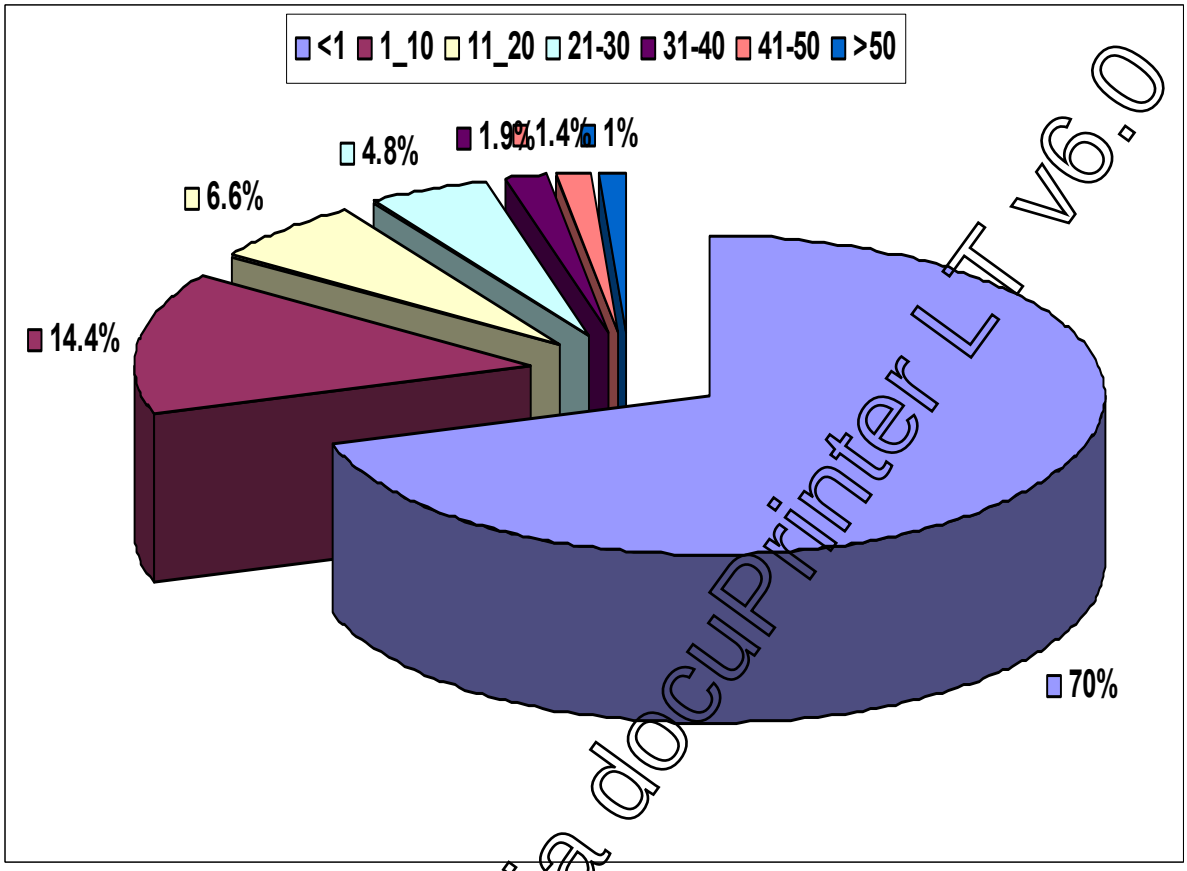


Figure (3.٢) Distribution and percentage of Kala-azar according to age

3.1.3 Prevalence of Kala-azar in Relation to Gender

Males were higher infected 54.5% (436/800) than females 45.5% (364/ 800) in patient groups, while no infection for both sexes in control groups (Table and figure 3.3). Therefore, the ratio of male/ female was 1.2 / 1.

Table (3.3) Gender distribution of Kala-azar

Age groups / Year		Gender		Total
		Male	Female	
<1	N	294	266	560
	%	67.4%	73.1%	70.0%
1-10	N	68	47	115
	%	15.6%	12.9%	14.4%
11-20	N	32	21	53
	%	7.3%	5.8%	6.6%
21-30	N	20	18	38
	%	4.6%	4.9%	4.8%
31-40	N	9	6	15
	%	2.1%	1.6%	1.9%
41-50	N	7	4	11
	%	1.6%	1.1%	1.4%
>50	N	6	2	8
	%	1.4%	.5%	1.0%
Total	N	436	364	800
	%	100.0%	100.0%	100.0%

P-value	C.S
	Non
0.5%	Sig.

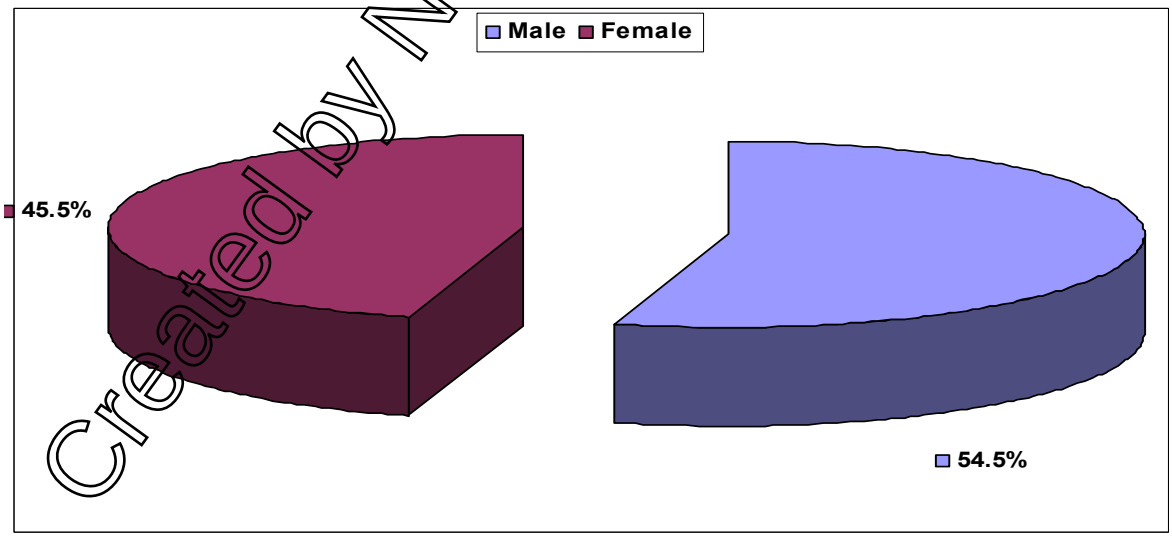


Figure (3.3) Gender distribution of Kala-azar

3.1.4 Signs and Symptoms of Kala-azar cases

All case-patients were investigated by physian had fever 300/300 (100 %) , splenomegaly 290/300 (96.7 %) , hepatomegaly 250 (83.3 %), weight loss 155 (51.7),anemia 150 (50 %), paleness 106 (35.3), diarrhoea 66(22%), leukopenia 50(16.7) and abdominal pain 46(15.3%) (Table 3.4).

Table (3.4) Signs and Symptoms of kala-azar cases

Signs and Symptoms	No.(%) of patients / 300
Fever	300 (100)
Splenomegaly	290 (96.7)
Hepatomegaly	250 (83.3)
Anemia	150 (50)
Leukopenia	50 (16.7)
Weight loss	155 (51.7)
Diarrhoea	66 (22)
Paleness	106 (35.3)
Abdominal pain	46 (15.3)

	P-value	C.S
Signs and Symptoms	.000	Highly Sig. (P<0.01)

3.1.٥ Prevalence of Kala-azar According to the School Age

The infection was higher among below school age patients 67.3 % (538/800)than unknown and school age patients 16.8% (134/800), 16.0% (128/800) respectively (Table 3.5).

Table (3.5) Distribution of Kala-azar according to the school age

Age	N	% of Total N
Below school age	538	67.2%
School age	128	16.0%
Unknown	134	16.8%
Total	800	100.0%

	P-value	C.S
School age		Highly
	.000	Sig.
		(P<0.01)

3.1.6 Prevalence of Kala-azar in Relation to the Kind of Living

The higher infection was appeared in population who lived in poor conditions 63.6 % (509/800) than in good condition 36.4 % (291 / 800) (Figure 3.6).

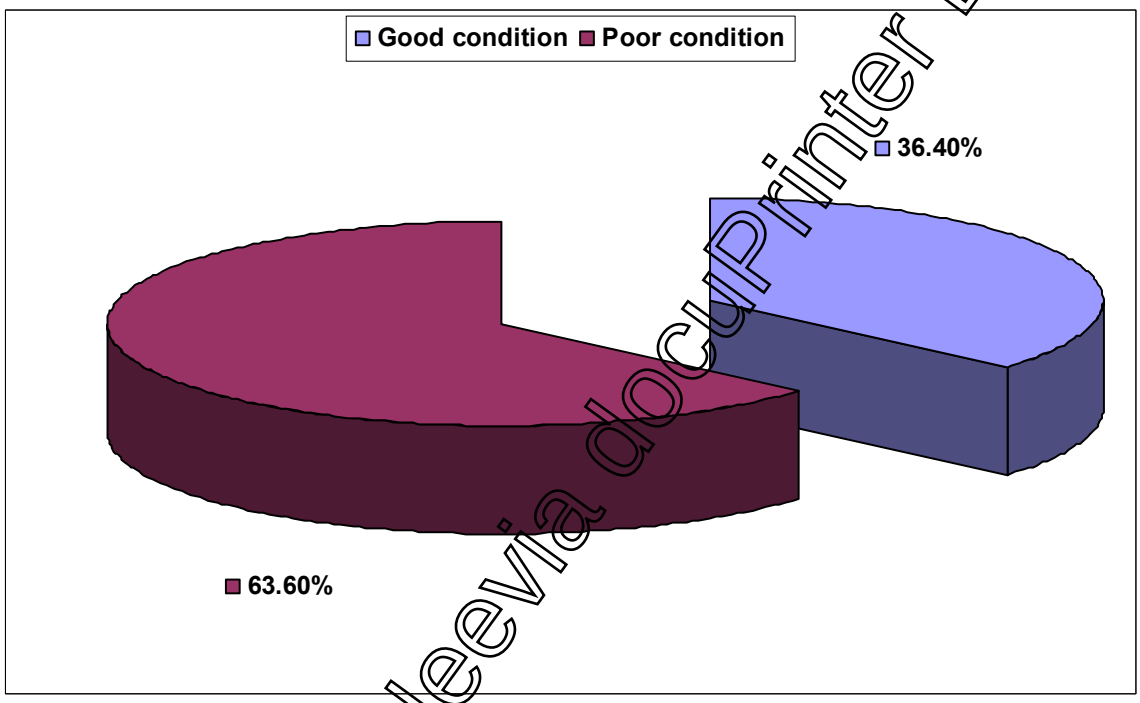


Figure (3.6) prevalence of Kala-azar according to the kind of living

3.1.7 Prevalence of Kala-azar According to the Presence or Absence of Domestic Animals

The Kala - azar infection was higher among patients with direct contact of domestic animals 61.8%(494/800) than others 38.3% (306/800) (Figure 3.7).

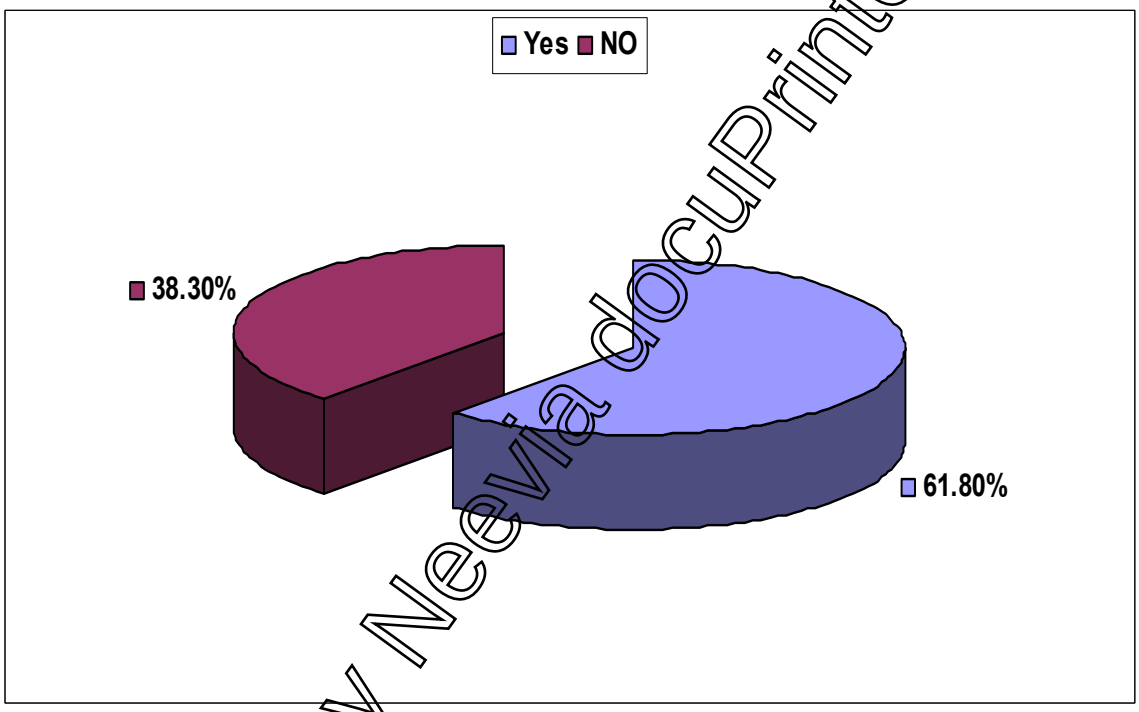


Figure (3.7) Comparison between the presence or the absence of domestic animals in Kala-azar cases

3.1.8 Prevalence of Kala-azar in Relation to the Residence

The higher infection was seen among rural population 56.8 % (454 / 800) than in urban 43.3 % (346 / 800) (Figure 3.8).

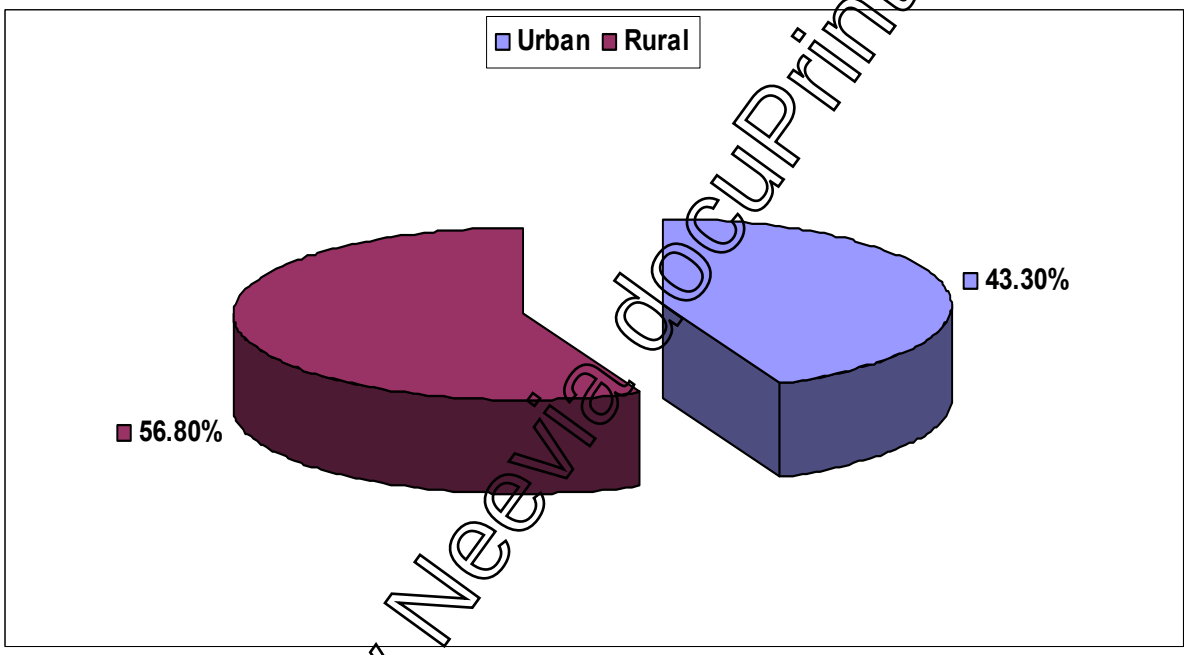


Figure (3.8) prevalence of Kala-azar according to the residency

3.1.9 Monthly Diagnosis Rates of Kala-azar Infection

The highest infection rate of Kala-azar were appeared during January 2008 (20.6 %), while the lowest were during July 2008 (6.1%) (Figure 3.9).

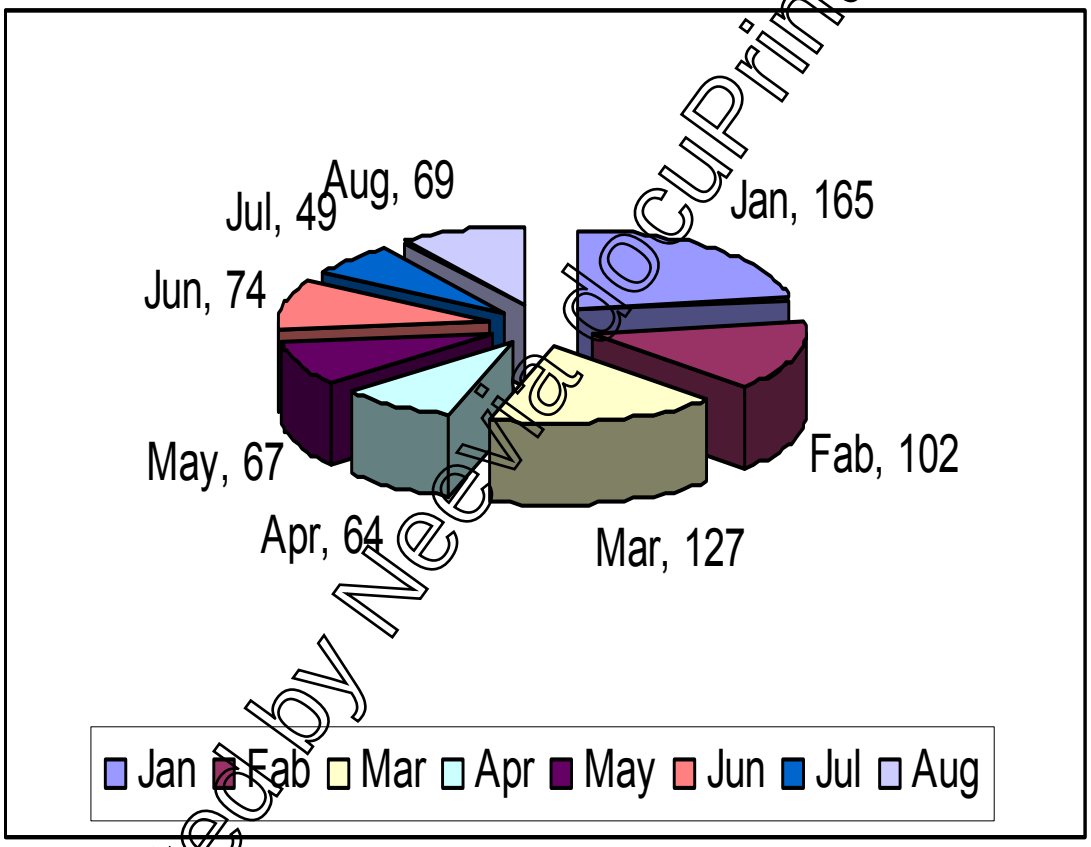


Figure (3.9) Monthly distribution of Kala-azar

3.1.10 Dignosis of Kala-azar by Serum rK39 Dipstick

Out of 800 patients, 717(89.6%) were found to be positive by serum rK39 dipstick test and 83 (10.4 %) were negative (Table 3.10), giving a sensitivity for rK39 dipstick of 89.6% and specificity of 100 %.

Table (3.10)Distribution of Kala-azar cases and controls according to Serum rK39 dipstick

		Cases		Total
		Kala azar patients (Positive)	Apparently healthy control (Negative)	
Serum rK-39 dipstick	Positive	٧١٧		٧١٧
	Negative	٨٣	١٠٠	١٨٣
Total		٨٠٠	١٠٠	٩٠٠

	P-value	C.S
Serum rK-39 dipstick		Highly
	.000	Sig.
		(P<0.01)

Validity tests	**%**
Sensitivity	**89.625**
Specificity	**100**
PPV	**100**
NPV	**54.64**
Accuracy	**90.77**

3.1.11 Distribution of Kala-azar cases according to IFAT

Out of the 100 patients, 69(69 %)were positive for IFAT and 31(31%) were negative (Table 3.12), giving a sensitivity for the IFAT of 70.5 % and specificity of 100 % (Table 3.13).

Table (3.11)Distribution of Kala-azar cases and controls according to IFAT

		Cases		Total
		Kala azar patients (Positive)	Apparently healthy control ((Negative	
IFAT	Positive	٧٠		٧٠
	Negative	٣٠	١٠	٤٠
Total		١٠٠	١٠	١١٠

	P-value	C.S
IFAT		Highly
	.000	Sig.
		(P<0.01)

Validity tests	**%**
Sensitivity	**70**
Specificity	**100**
PPV	**100**
NPV	**25**
Accuracy	**72.72**

3.1.12 Dignosis of Kala-azar by ELISA

Out of 100 confirmed Kala-azar cases, 88(88 %) were appeared to be positive by ELISA and 12 (12 %) were negative (Table 3.12), giving a sensitivity 88 % and specificity of 100 % .

Table (3.12)Distribution of Kala-azar cases and controls according to ELISA

		Cases		Total
		Kala azar patients (Positive)	Apparently healthy control ((Negative	
ELISA	Positive	٨٨		٨٨
	Negative	١٢	١٠	٢٢
Total		١٠٠	١٠	١١٠

	P-value	C.S
ELISA		Highly
	.000	Sig.
		(P<0.01)

Validity tests	**%**
Sensitivity	**88**
Specificity	**100**
PPV	**100**
NPV	**45.45**
Accuracy	**89.09**

3.1.13 Dignosis of Kala-azar by DAT

Out of 100 patients, 95 (95 %) were appeared to be positive by DAT test and 5 (5%) were negative(Table 3.13), giving a sensitivity for the DAT of 95 % and specificity of 100 %.

Table (3.13)Distribution of Kala-azar cases and controls according to DAT

		Cases		Total
		Kala azar patients (Positive)	Apparently healthy control ((Negative	
DAT	Positive	٩٤		٩٤
	Negative	٦	١٠	١٦
Total		١٠٠	١٠	١١٠

	P-value	C.S
DAT		Highly
	.000	Sig.
		(P<0.01)

Validity tests	**%**
Sensitivity	**95**
Specificity	**100**
PPV	**100**
NPV	**66.66**
Accuracy	**95.45**

3.1.14 Dignosis of Kala-azar by PCR

Hundred of the selected serologically positive clinical samples,which were collected from the field work were also positive in 95 (95%) for the PCR and 5(5%) were negative (Table 3.14), giving a sensitivity 95 % and specificity of 100% .

Table (3.14) Dignosis of Kala-azar by PCR

PCR	N	% of Total N
Positive	95	95%
Negative	5	5%
Total	100	100.0%

	P-value	C.S
PCR		Highly
	.000	Sig.
		(P<0.01)

Validity tests	**%**
Sensitivity	**٩٥**
Specificity	**100**
PPV	**100**
NPV	**90**
Accuracy	**95**

3.1.15 PCR amplification

The following figure (3.10) was appeared that the size of the PCR product was consistent within each species. Whole minicircles of kDNA were amplified, the PCR products therefore reflected the size of the mini-circles in *L.donovani* (600 bp) from lane 1 to lane 10, and negative control was used, containing DNA from a healthy donor for each experiment (lane 11).

Description of 1 Kb Ladder from Gibco BRL:

The 1 Kb DNA Ladder (USA) is suitable for sizing linear double-stranded DNA fragments from 500 bp to 12 kb. The bands of the ladder each contain from 1 to 12 repeats of a 1018-bp DNA fragment. In addition to these 12 bands, the ladder contains vector DNA fragments that range from 75 to 1636 bp. The 1636-bp band contains 10% of the mass applied to the gel (figure 3.10).

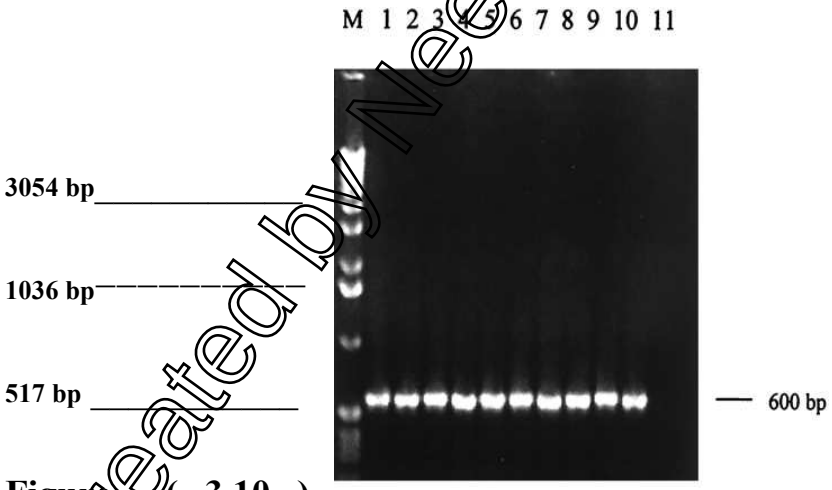


Figure (3.10) Results of PCR amplification of *L. donovani* DNA analyzed on agarose gel. Lane M, 1 kb Ladder(Gibco BRL); lane 1-10 represent the positive results of PCR and lane 11 represents the negative result as control.

3.1.16 PCR in relationship to age and gender groups

The following table shown the positive clinical samples which were dignosed by PCR distributed according to age and gender groups.

Table (3.16) PCR in relationship to age and gender groups

Age groups / Year	PCR	Gender		Total
Positive		Male	Female	
<1	N	22	11	33
	%	22 %	11%	33%
1-10	N		18	18
	%		18%	18%
11-20	N	11		11
	%	11%		11%
21-30	N	11		11
	%	11%		11%
41-50	N	11	11	22
	%	11%	11%	22%
Total	N	55	40	95
	%	55%	40%	95%

P-value	C.S
	Non
0.370	Sig.

3.1.17 Serum rK39 Dipstick in relationship to age and gender groups

The summarized results for rK39 in relation to age and gender groups, presented in (Table 3.17) showed that 717 (89.6%) of confirmed Kala-azar were found positive.

Table (3.17) Serum rK39 dipstick in relationship to age and gender groups

Age groups / Year	Serum rK-39 dipstick	Gender		Total
Positive		Male	Female	
<1	N	268	244	512
	%	37.4%	34.0%	71.4%
1-10	N	60	42	102
	%	8.4%	5.9%	14.2%
11-20	N	27	18	45
	%	3.8%	2.5%	6.3%
21-30	N	16	15	31
	%	2.2%	2.1%	4.3%
31-40	N	6	5	11
	%	.8%	.7%	1.5%
41-50	N	5	4	9
	%	.7%	.6%	1.3%
>50	N	5	2	7
	%	.7%	.3%	1.0%
Total	N	387	330	717
	%	54.0%	46.0%	100.0%

P-value	C.S
	Non
0.795	Sig.

3.1.18 ELISA in relationship to age and gender groups

The following table shown the positive clinical samples which were dignosed by PCR distributed according to age and gender groups.

Table (3.18) ELISA in relationship to age and gender groups

Age groups / Year	ELISA	Gender		Total
Positive		Male	Female	
<1	N	24	18	42
	%	27.3%	20.5%	47.7%
1-10	N	10	8	18
	%	11.4%	9.1%	20.5%
11-20	N	6	4	10
	%	6.8%	4.5%	11.4%
21-30	N	6	3	9
	%	6.8%	3.4%	10.2%
31-40	N	3	2	5
	%	3.4%	2.3%	5.7%
41-50	N	2	1	3
	%	2.3%	1.1%	3.4%
>50	N	1		1
	%	1.1%		1.1%
Total	N	52	36	88
	%	59.1%	40.9%	100.0%

P-value	C.S
	Non
0.980	Sig.

3.1.19 IFAT in relationship to age and gender groups

Table (3.19) showed non significant differences (p > 0.05) in Kala-azar patients were dignosed by IFAT in relation to age and gender groups.

Table (3.19) IFAT in relationship to age and gender groups

Age groups / Year	IFAT	Gender		Total
Positive		Male	Female	
<1	N	13	11	24
	%	18.8%	15.9%	34.8%
1-10	N	8	5	13
	%	11.6%	7.2%	18.8%
11-20	N	6	5	11
	%	8.7%	7.2%	15.9%
21-30	N	5	3	8
	%	7.2%	4.3%	11.6%
31-40	N	3	2	5
	%	4.3%	2.9%	7.2%
41-50	N	4	1	5
	%	5.8%	1.4%	7.2%
>50	N	2	1	3
	%	2.9%	1.4%	4.3%
Total	N	41	28	69
	%	59.4%	40.6%	100.0%

P-value	C.S
	Non
0.967	Sig.

3.1.20 DAT in relationship to age and gender groups

The following table shown the positive clinical samples which were dignosed by DAT distributed according to age and gender groups.

Table (3.20) DAT in relationship to age and gender groups

Age groups / Year	DAT	Gender		Total
Positive		Male	Female	
<1	N	25	20	45
	%	26.3%	21.1%	47.4%
1-10	N	12	6	18
	%	12.6%	6.3%	18.9%
11-20	N	8	5	13
	%	8.4%	5.3%	13.7%
21-30	N	6	2	8
	%	6.3%	2.1%	8.4%
31-40	N	4	1	5
	%	4.2%	1.1%	5.3%
41-50	N	3	2	5
	%	3.2%	2.1%	5.3%
>50	N	1		1
	%	1.1%		1.1%
Total	N	59	36	95
	%	62.1%	37.9%	100.0%

P-value	C.S
	Non
0.828	Sig.

Table (3.21) Comparison between PCR and Serum rK39 dipstick

		PCR		Total
		Positive	Negative	
Serum rK-39 dipstick	Positive	89	0	89
	Negative	11	0	11
Total		100	0	100

Validity tests	**%**
Sensitivity	**88.88**
Specificity	**100**
PPV	**100**
NPV	**50**
Accuracy	**90**

Table (3.22) Comparison between PCR and ELISA

		PCR		Total
		Positive	Negative	
ELISA	Positive	٨٨	0	٨٨
	Negative	١٢	0	١٢
Total		١٠٠	٠	١٠٠

Validity tests	**%**
Sensitivity	**88.88**
Specificity	**100**
PPV	**100**
NPV	**50**
Accuracy	**90**

Table (3.23) Comparison between PCR and DAT

		PCR		Total
		Positive	Negative	
DAT	Positive	٩٥	٠	٩٥
	Negative	٠	٥	٥
Total		٩٥	٥	١٠٠

Validity tests	**%**
Sensitivity	**٩٥**
Specificity	**100**
PPV	**100**
NPV	**90**
Accuracy	**95**

Table (3.24) Comparison between PCR and IFAT

		PCR		Total
		Positive	Negative	
IFAT	Positive	69	0	٦٩
	Negative	26	5	٣١
Total		٩٥	5	١٠٠

Validity tests	**%**
Sensitivity	**100**
Specificity	**100**
PPV	**100**
NPV	**100**
Accuracy	**100**

Table (3.25) Comparison between DAT and Serum rK39 dipstick

		DAT		Total
		Positive	Negative	
Serum rK-39 dipstick	Positive	٨٠		٨٠
	Negative	١٤	٦	٢٠
Total		٩٤	٦	١٠٠

Validity tests	**%**
Sensitivity	**84.21**
Specificity	**100**
PPV	**100**
NPV	**25**
Accuracy	**85**

Table (3.26) Comparison between DAT and ELISA

		DAT		Total
		Positive	Negative	
ELISA	Positive	٨٨		٨٨
	Negative	٦	٦	١٢
Total		٩٤	٦	١٠٠

Validity tests	**%**
Sensitivity	**92.63**
Specificity	**100**
PPV	**100**
NPV	**41.66**

Accuracy	**93**

Table (3.27) Comparison between DAT and IFAT

		DAT		Total
		Positive	Negative	
IFAT	Positive	٦٦	٤	
	Negative	٢٨	٢	٣٠
Total		٩٤	٦	١٠٠

Validity tests	**%**
Sensitivity	**70.52**
Specificity	**40**
PPV	**95.71**
NPV	**6.66**
Accuracy	**69**

3.2 Discussion

Kala-azar or visceral leishmaniasis, is a serious health hazard of tropical and subtropical countries, has plagued mankind since antiquity. The main problem to control the VL is to establish a definite diagnosis. Till date the demonstration of the parasite in splenic / bone marrow aspirates is considered to be gold standard (Bain, 2001; Wernecki *et al*., 2002), but these invasive procedures have their own drawbacks. These tests may sometimes be found to be false negative, if the parasite density is low. Hence, there is a need for an alternative approach for diagnosis of VL. In the present study, ELISA showed a sensitivity of 88% and specificity of 100% by using the sonicated soluble antigen at a cut off OD value of 0.270.

Edrissian and Darabian, (1999) and Grech *et al*., (2000) showed the sensitivity of ELISA 91% with a specificity of 91.6% . Hommel *et al*., (1988), Srivastava and Singh, (1998) showed the sensitivity of ELISA in the range of 97-100%. Our results showed a sensitivity and specificity of 88%, 89.6%, 100%, and 100% for ELISA and rK39 respectively. These results well corroborate with the results of others (Singla *et al*., 1993; Garcez *et al*., 1996 ; Boelaert *et al*., 1999 ; Aoun *et al*., 2000 ; Singh and Sivakumar, 2005) who showed a sensitivity and specificity in the range of 95-100 %.

The sensitivity of ELISA using these concentrations of CSA is reported to range from 80 to 100% , but cross-reactions with sera from patients with trypanosomiasis, tuberculosis, and toxoplasmosis have been recorded (Bray *et al*.,1973; Smrkovski and Larson, 1977 ; Choudhry *et al*.,

1990 ; Singh *et al*., 1995 ; Kumar *et al*., 2001). On the other hand, when various selective antigenic masses (116 kDa, 72 kDa , and 66 kDa) were used, a specificity of 100 % could be achieved, but only at the cost of

sensitivity, which went down to as low as 37.5% (Vinayak *et al*., 1994; De Colmenares *et al*.,1995). Its use in ELISA has been found to result in 100% sensitivity and 96 % specificity (Palatnik- de - Souza *et al*., 1995). In a recent study, it was found that the sensitivity and specificity of ELISA in diagnosing VL could also be increased by the use of soluble antigens derived from promastigotes cultivated in a protein-free medium.

In the present study, A comparison between Baghdad and Wasit governorates showed a highly significant difference ($P < 0.01$). Therefore, the proportion of Kala-azar infection in Baghdad was 257 (32.1 %) is lower than Wasit governorate 460 (57.5 %), this may be due to many reasons, the living standard, way of living, the presence of reservoir or domestic animals, and above all the viability of infected sandfly due to suitable environmental conditions (Yassin, 2004).

An rK39 showed a higher sensitivity, specificity, PPV and NPV in comparison to ELISA. The diagnostic accuracy (DA) of rK39 was also higher. In addition, rK39 did not show any cross reactivity with other diseases like malaria where as many cases of malaria were found positive by ELISA. Srivastava and Singh, (1998), Edrissian and Darabian, (1999) observed similar cross reactivity between VL and malarial fever patients. A rK39 failed to detect antileishmanial antibodies in four parasitologically confirmed cases of VL while ELISA failed to detect in seven parasitologically confirmed cases, the negative reaction in these cases may be due to prior treatment of the patients. The other reasons could be advanced stage of the disease, which may lead to a state of anergy (Kala-azar is known to produce immune suppression). Hence these patients may be considered

as low responders so far as antibody production is concerned,These results well corroborate with the results of others (Sinha and Sehgal, 1994; Basu and Ray, 2005).

The clinically suspected persons who showed positive reactions by rK39 and ELISA may indicate low parasitaemia in these patients which could not be detected by parasitological examination of their splenic/bone marrow aspirates. One of the endemic control / contact by rK39 and two by ELISA showed positivity for Kala-azar. This positivity in endemic healthy individuals is explained by constant exposure of individuals to sand fly bite resulting in latent or subclinical infection. Alternatively, the patients might be in the incubation period yet to show sign and symptoms of disease. From our study,it appears that rK39 is a sensitive and specific test with high diagnostic accuracy. It is a cost effective test, as it does not require any sophisticated equipments. It is also a simple test and can be carried out as well as interpreted even by unskilled paramedical staff. Considering the merits of rK39, in our opinion, it is an ideal test for diagnosis and seroepidemiological studies of Kala-azar. The high sensitivity and ease of performance make the dipstick test (rK39) very suitable for surveillance surveys, This fact has already been documented by (Singh *et al*., 2005 ; Sivakumar *et al*., 2006). However, the lower specificity of the test compared to DAT may result in some cases being misdiagnosed as false positives. Therefore, the diagnosis of Kala-azar must be made on the basis of the outcome of the diagnostic test in combination with clinical and epidemiological information.

All the selected DAT positive clinical samples, which were collected from the field work were also positive in PCR. All negative controls were negative ; i.e. no contamination or inhibition was detected. The PCR provides a useful tool for the typing of parasites while diagnosis is being performed on clinical samples. Such a tool is necessary to complement

diagnostic assays since most of them do not furnish the taxonomic information about the parasite required to determine the appropriate therapeutic and control measures.Early detection and typing would enable implementa-

tion of specific treatment. The test has other potential values in detecting and typing parasites in vectors for epidemiological surveys and in retrospective studies (Saltora *et al*., 2001).

The PCR assay that is specific for *L. donovani* kDNA was used in our study, and it could detect the parasite in highly sensitive manner in clinical samples from Iraqi patients with Kla-azar. The assay could detect as little as fragment of parasite DNA from *L. donovani*, an amount that represents the equivalent of approximately 0.1 parasite. DNAs from several parasite obtained from patients with Kala-azar were found to amplified with high sensitivity (Grimaldi and Tesh, 1993). Therefore, the assay is theoretically capable of detecting a single parasite in biological sample. The high sensitivity of our detection was evident by its ability to amplify parasite DNA from peripheral blood of patients with Kala-azar patients in large majority of cases. A total of 100 clinical samples from patients with Kala-azar were examined,and 95% tested positive by PCR.Our PCR described in this work yielded a unique product of approximately 600 bp,and specific side product appeared on the gel. It had the advantage that results were easily interpreted upon analysis of agarose gels. The high level of sensitivity was reflected by the ability of assay to detect parasite DNA in peripheral blood of patients with Kla-azar with 95% sensitivity in the cases examined. Use of peripheral blood is advantageous because the collection procedure is less invasive and safer than the splenic or bone marrow biopsy specimen collection(Adhya *et al*., 1995). Bone marrow is known to have a high load of parasites, while in peripheral blood the parasites are relatively scarce. Studies reporting PCRs with detection sensitivities comparable to ours did obtained sensitivities as

same as that of our assay when using blood samples of patients with Kala-azar (Lachaud *et al*., 2001).

The sensitivity of PCR assay may be affected by several factors such as accessibility of the DNA in parasite-containing biopsy samples and the conditions used in PCR amplification. In our study, The phenol-chloroform

extraction was used. It was very efficient and thorough purification method, which is comparatively hazardous and time consuming. Many steps of lysis, digestion, extraction and washing require much opening and closing of the 1.5 ml tubes,implying an increased risk of sample cross-contamination. The phenol-chloroform extraction is highly efficient in removing inhibiting factors, such as hemoglobin. It therefore proved to be reliable also for samples with a higher blood content. Proteinase-K digestion with subsequent phenol-chloroform extraction is the best known classical extraction method, which was successfully employed also in many previous studies on clinical samples (Smyth *et al*., 1992 ; Ashford *et al*., 1995 ; Andresen *et al*., 1996 ; Osman *et al*.,1997; Mimori *et al*., 1998; Campino *et al*., 2000; Singh, 2003). The advantage of the Proteinase K method is that it did not lost any DNA,as the whole procedure is accomplished in the same tube. One disadvantage in using the Proteinase K is that it may have DNA inhibitors. Inhibition of the PCR reaction in blood samples however is a rare phenomenon(1%) (Cascio *et al*., 2002).

In this study, serological surveys using DAT analysis showed high sensitivity and specificity of the population in the study areas, our results were in accordance to the observation of researcher (Schallig *et al*., 2002). A number of seropositive cases were found among children with no previous history of Kala-azar. Examination of the history of Kala-azar patients showed that Kala-azar cases are much more frequent in childrens, and most seropositive cases were found among children under one years, The results are similar to other studies carried out (Cascio *et al*., 2002; Nuwayri-Salti *et al*.,2000). The peak number of cases was in children under

one years old and the seropositive rate decreased with increasing of age of the individuals. Prior studies in the world have shown a seropositive rate more than 50% in age group under one year and decreased with increasing of age (Belhadj *et al*., 2000; Sunder *et al*., 2004). One important characteristic of visceral leishmaniasis is that the greater the incidence of the disease,

the greater the risk to the youngest children. This fact has already been documented in Iraq (Korzeniewski, 2004), Sudan (Hashim *et al*., 1995), and Brazil, where the disease's preference for the infant population has remained constant over the years (Carreira *et al*., 1995; Campos-Ponce *et al*., 2005).

It was found that the majority of cases were below the age of 3 years; this suggests that small children are at higher risk probably because of low immunity.The results are similar to other studies carried out in Iraq(Sukkar, 1985; Bray *et al*., 1985),and in other countries (Navin *et al*., 1995 ; Tanner, 1996; Takai, 2002). A large number of cases (56.8%) were from rural, the disease is not limited to certain focus. The rural areas provided suitable conditions for the vector and reservoir, and this was agreed with other studies in Babylon and Thi-Qar (Al-Maamori, 2004 ; Al-Kassar, 2005). There had been a prolonged period before hospitalization in majority of cases resulting in significant complications, this could be due to poor education of the general population, failure on the side of general practitioners in early detection and diagnosis of Kala-azar.

The monthly distribution of cases showed that the highest cases registered during winter mainly January due to the growth and propagation of vector and declined in July. These results are consistent with other previous studies done in Iraq, the peak incidence of cases was during December and January (Sukkar, 1985; Korzeniewski, 2004), and in other countries (Maggi *et al*., 2004).

About 54% of seropositive individuals were males and 46 % females. No statistically significant difference ($P < 0.05$) was observed between them. Cross-sectional IFA and DAT serological surveys of VLin endemic foci of the Iraq showed that males are exposed and become infected at least as much as females. In most rural areas the rate of active Kala-azar cases in males may be higher than females (Bora, 1999 ; Sreenivas *et al*., 2002).

Therefore,it does seem that VL affects males more than females. It has been suggested that there may be a hormonal factor linked to gender (Costa *et al*., 1999). It was found that male: female ratio was 1.2 : 1; this may indicate that both males and females are equally exposed to the risk of transmission of the disease. These results are consistent with other studies done in Iraq (Sukkar, 1985; Al-Kassar, 2005), and in other regions out side (Edrissian and Darabian, 1999). Although one study done in Pakistan (Zijlstra *et al*., 2001), showed that male : female ratio was 3:1.

All of the patients had fever (100%) , spleenomegaly (96.7%), hepato-megaly (83.3%), weight loss (51.7%), anemia (50%), paleness (35.3%), diarrhoea (22%), leukopenia (16.7%), and abdominal pain (15.3%) ; these findings reflect the delay in consultation. Fever, splenomegaly and hepato-megaly were the predominant clinical features. These signs and symptoms are the same as those found in other clinical studies in Iraq (Korzeniewski, 2004; Al-Kassar,2005) and in other countries (Delgado *et al*.,1998; Maggi *et al*., 2004).

Conclusions and

Recommendations

Conclusions and Recommendations

Conclusions

1-The PCR assay has high sensitivity and specificity by capability of detecting low levels of parasitemia from the peripheral blood.

2- The comparison between serological methods was showed that DAT had higher sensitivity than rK39 dipstick, ELISA and IFAT, respectively.

3-The rK39 is a sensitive and specific test with high diagnostic accuracy. It is a cost effective test, as it does not require any sophisticated equipments. It is also a simple test and can be carried out as well as interpreted even by unskilled paramedical staff.

4- The PCR assay has higher diagnostic accuracy than serological tests.

5- Kala-azar is febrile disease affecting childrens particularly those less than one year of age in Baghdad and Wasit governorates.

6- The Kala-azar infection was higher in Wasit than Baghdad governorates.

Recommendations

1- The molecular approach of this study can be used for other parts of the country to predict and map VL.

2-Should closely monitor all information regarding ecological and clinical studies of *L. donovani* being conducted in Iraq.Therefore, the researchers should be queried concerning any advances in diagnostic techniques for further identifying *L. donovani*.

3-Appropriate laboratory studies of interactions between *L. donovani* and *L. tropica* should be conducted.

4- The severity of infection may lead to the presence of different strains, and later needs further investigations of DNA of sandfly by PCR method.

5- Adaptation to climate change is vital for increasing the adaptive capacity of sandfly vector.

6-The potential for long-term survival of *Leishmania* in the sandyfly vector and in human host should be conducted.

References

References

Adhya, S. Chatterjee, M. Hassan, M. Mukherjee, S. and Sen, S. (1995). Detection of *Leishmania* in blood of early kala-azar patients with aid of the polymerase chain reaction.Trans R Soc Trop Med Hyg, 89: 622-624.

Aguilar, CM. Fernandez, E. Cannova, DC. Ferrer, E. and Coutinho,SG. (1998).Urban visceral leishmaniasis in Venezuela.Memŕias do Instituto Oswaldo Cruz, 93:15-16.

Ahluwalia,IB. Bern, C. Costa, C. Akter,T. and Ali, M. (2003).Visceral leishmaniasis. Am. J. Trop. Med. Hyg., 69:624–8.

Ahluwalia, S. Lawn, S. Kanagalingam, J. and Lockwood, D. (2004). Mucocutaneous leishmaniasis: an imported infection among travelers to central and south America. BMJ., 329 : 842-4.

Ajdary, S. Alimohammadian,M. Eslami,M. Kemp, K. and Kharazmi, A. (2000). Comparison of the immune profile of non-healing cutaneous leishmaniasis patients with those with active lesions and those who have recovered from infection. Infection and Immunity, 68: 1760-1764.

Alberts, B. Johnson, A. Lewis, J. Raff, M. Roberts, K. and Walter, P. (2002). Cell communication, Molecular Biology of The Cell, 4th ed. Garland Science,New York. P: 831-905.

Ali, A. and Ashford, RW. (1994).Visceral leishmaniasis in Ethiopia.The magnitude and annual incidence of infection,as measured by serology in an endemic area. Ann. Trop. Med. Parasitol., 88(1): 43-7.

Aljeboori, TI. and Evans DA. (1980). *Leishmania ssp.* in Iraq. Trans. R. Soc. Trop. Med. Hyg. 74(2), 169-177

Al-Kassar, NR. (2005). Study in epidemiology of visceral leishmaniasis inThi-Qar city.MSc thesis,College of Vet.Medicine,Baghdad Un.,p: 14-20.

Allen, L. and Aderem, A.(1995). Protein kinase C regulates MARCKS cycling between the plasma membrane and lysosomes in fibroblasts. EMBO. J., 14:1109-1121.

Al-Maamori, MA. (2004). Study in the endemicity of Kala-azar in Babylon governorate for period 1992-2003. Higher Diploma in public health thesis. Baghdad, p: 9-12.

Alvar,J. Canavate,C. Gutierrez-Solar,B. Lopez-Velez, R. Molina, R. and Moreno, J. (1997). *Leishmania* and human immuno - deficiency virus coinfection:The first 10 years.Clinical Microbiology Reviews,10:298-319.

Amaral, V. Pirmez, C. Goncalves, A. and Grimaldi, GR. (2000). Cell populations inlesions of cutaneous leishmaniasis of *L.amazonensis*-infected rhesus macaques,Macaca. Mem. Inst. Oswaldo. Cruz., 95: 209-16.

Amorim, A. Carrington, M. Miles, M. Barker, D. de Almeida, M. (1996). Identification of the C-terminal region of 70 kDa heat shock protein from *L.*(*Viania*) *braziliensis* as a target for the humoral immune response.Cell Stress Chaperones, 1:177-187.

Andre, R. Brumpt, L. Dreytus, B. Puseleg, A. and Jacob, S. (1958). Leishmaniose Cutanee.Leishmaniose cutanee-ganglionnare et kala-azar transfusional. Trop. Dis. Bull., 55 : 379-81.

Andresen, K. Ibrahim, M. Theander, T. and Kharazmi, A. (1996). Random amplified polymorphic DNA for the differentiation of *L. donovani* isolates from Sudan. Int. J. Parasitol., 18:445-452.

Anez, N. Tang, Y. Rojas, A. Killick-Kendrick, M. and Killick-Kendrick, R.(2003). Detection of amastigote-like forms in the valve of *Ph.papatasi* infected with *Leishmania major*. Mem. Inst. Oswaldo Cruz., 98 : 495-8.

Antoine, J. Prina, E. Courret, N. and Lang, T. (2004). *Leishmania* spp.: on the interactions they establish with antigen-presenting cells of their mammalian hosts. Advances in Parasitology, 58:1-68.

Aoun, K. Bouratbine, A. Chahed, MK. and Ben Ismail, R. (2000). Role of direct aggultination test in the diagnosis of visceral leishmaniasis in Tunisia. Tunis. Med.,78 (12):719-722.

Arias, JR. and Naiff, RD. (1981). The principal reservoir host of cutaneous leishmaniasis in the urban areas of Manaus, Central Amazon of Brazil. Memórias do Instituto Oswaldo Cruz, 76:279-286.

Arias, JR. Monteiro, PS. and Zicker, F. (1996). The re-emergence of visceral leishmaniasis in Brazil.Emerging Infectious Diseases,2:145-146.

Ashford, RW. (2000). The leishmaniases as emerging and re-emerging zoonoses. Intern. J. Parasitol., 30:1269-1281.

Ashford, R. Rioux, J. Jalouk L, Khiami, A. and Dye C.(1993). Evidence for longterm increase in the incidence of *L.tropica in Aleppo*.87,247-249.

Ashford, RW. and Bates, P.(1998). Leishmaniasis in the Old World. In:

Cox FEG., Kreier JP., Wakelin D., editors. Topley and Wilson's microbiology and microbial infections. Vol.5. Parasitology. 9th ed.New York, USA: Oxford University Press, p. 215-40.

Attar, ZJ. Chance, ML. el-Safi, S. Carney, J. and El-Hadi, M. (2001). Latex agglutination test for the detection of urinary antigens in visceral leishmaniasis. Acta. Trop., 78 : 11-6.

Awadalla, P. (2003). The evolutionary genomics of pathogen recombination. Nature Reviews: Genetics, 4: 50-60.

Axton, R. and Hanson, I.(1998). Conformation based mutation.Elsevier Trends. J. Tech. Tips. Online, 13/5/98; P01390.

Badaro, R. (1995). Changing epidemiology of leishmaniasis in Brazil. In: Tropical Diseases, Society and the Environment , SAREC Documentation, Conference reports, 2:97-101.

Badaro, R. Benson D. and Eulalio, MC.(1996).rK39: a clone antigen of *L.chagasi* that predicts active visceral leishmaniasis. J.Inf. Dis.,173:758-7.

Ban˜ uls, A. Dujardin, J. Guerrini, F. De Doncker, S. and Tibayrenc, M. (2000). Is *Leishmania* (*Viannia*) peruviana a distinct species? A MLEE/ RAPD evolutionary genetics answer. J. Eu. Microbiol., 47: 197–207.

Ban˜ uls, A. Hide, M. and Tibayrenc, M. (2002). Evolutionary genetics and molecular diagnosis of *Leishmania* species. Trans. Roy.Soc. Trop. Med. Hyg. 96 (suppl. 1),S9–S13.

Bard, E. (1989). Molecular biology of *Leishmania*. Biochemistry and Cell Biology, 67: 516-524.

Barker, D.(1989). Molecular approaches to DNA diagnosis.Parasitology, 99: S125-S146.

Basu, MK. and Ray, M. (2005). Macrophage and *Leishmania*:an unacceptable coexistence. Critical Reviews in Microbiology, 31: 145-154.

Bates, PA. (1993). Axenic culture of *Leishmania* amastigotes. Parasitol. Today,9 : 143-6.

Bates,PA.(1994).Complete developmental cycle of *Leishmania mexicana* in axenic culture. Parasitology,108 : 1-9.

Bates, PA. and Tetley, L. (1993). *Leishmania mexicana* ; induction of metacyclogenesis by cultivation at acidic *p*H. Exp. Parasitol.,76: 412-23.

Bates, PA. and Rogers, ME. (2004). New insights into the developmental biology and transmission mechanisms of *Leishmania*. Current Molecular Medicine, 4: 601–609.

Belli, A. García, D. Palacios, X. and Harris, E.(1999). Widespread atypical cutaneous leishmaniasis caused by *L. chagasi* in Nicaragua. Am. J. Trop. Med. Hyg. 61(3), 380-385.

Ben Salah, A. Ben Ismail, R. Amri, F. Chlif, S. and Delagi, K. (2000). Investigation of the spread of human visceral leishmaniasis in central Tunisia.Trans. R. Soc. Trop. Med. Hyg., 94:382-386.

Berman, J. and Neva, F. (1981). Effect of temperature on multiplication of *Leishmania* amastigotes within human monocyte-derived macrophages in vitro. Am. J. Trop. Med.Hyg., 30:318-321.

Berzunza -Cruz, M. Bricaire, G. Perez-Becker, R. and Becker, I. (2000). *Leishmania mexicana mexicana*: genetic heterogeneity of Mexican isola-

tes revealed by restriction length polymorphism analysis of kinetoplast DNA. Experimental Parasitology, 95: 277-284.

Beverley, S. Asmach, R. and McMahon Pratt, D. (1987). Evolution of the genus *Leishmania* as revealed by comparison of nuclear DNA restriction fragment patterns. Proc. Natl. Acad. Sci., 84: 484-488.

Beverley, S. Akopyants,N. and Wang, Y. (2002). Putting the *Leishmania* genome to work: functional genomics by transposon trapping and expression profiling. Ph. Trans. Roy. Soc. London. Sciences, 357: 47-53.

Bhattacharyya, R. Singh, R. Hazra, T. and Majumder, H. (1993). Application of polymerase chain reaction with specific and arbitrary primers to identification and differentiation of *Leishmania* parasites.FEMS Microbiol Lett, (15); 114: 99-104.

Blaineau, C. Bastien, P. Rioux, J. Roizes, G. and Page`s, M. (1991).Long-range restriction maps of size - variable homologous chromosomes in *L.infantum*. Molecular and Biochemical Parasitology, 46: 292-302.

Boelaert, M. EI Safi, S. de Muynck, A. Vander Stuyft, P. and Le Ray, D. (1999).Operational validation of the direct aggultination test for diagnosis of visceral leishmaniasis. Am. J. Trop. Med. Hyg., 60(1):129-134.

Boelaert, M. Rijal, S. and Regmi, S.(2004). Comparative study of the effectiveness of diagnostic tests for visceral leishmaniasis. Am. J. Trop. Med. Hyg. 70 : 72-7.

Bogdan, C. and Rollinghoff, M. (1999). How do protozoan parasites survive inside macrophages? Parasitol. Today, 15:22-8.

Bora, D. (1999). Epidemiology of visceral leishmaniasis in India. Natl. Med. J. India, 12 : 62-8.

Boulanger, N. Lowenberger, C. Volf, P. and Bulet, P. (2004).Characterization of a defensin from the sand fly *Ph.duboscqi* induced by challenge with protozoan parasite *L.major*. Infection and Immunity, 72: 7140-7146.

Bray, R. Ashford, A. Mukherjee, M. and Sengupta, P. (1973). Studies on the immunology and serology of leishmaniasis. Trans. R. Soc. Trop. Med. Hyg., 67:125-129.

Bray, R. (1974).*Leishmania*.Annual Review of Microbiology,28:189-217.

Bray, R. Rahim, G. and TaJ-Eldeen, S. (1985). Leishmaniasis in Iraq. J. Helmin. Protozo., 2: 171-186.

Brenie` re, S. Telleria, J. Bossenq, M and Barker, D. (1999). Polymerase chain reaction - based identification of New World *Leishmania* species complexes by specific kDNA probes. Acta. Tropica., 73: 283-293.

Brewster, S. and Barker, D. (2002). Analysis of the minicircle classes in *Leishmania*(*Viannia*) species.Trans. Roy. Soc.Trop. Med. Hyg., 96(supl.1), S55-S63.

Brisse, S. Dujardin, J. and Tibayrenc, M. (2000). Identification of six *T. cruzi* lineages by sequence - characterised amplified region markers. Molecular and Biochemical Parasitology, 111: 95-105.

-Britten, RJ. (1986). Rates of DNA sequence evolution differ between taxonomic groups. Science, 231:1393-1398.

Britto, C. Ravel, C. Bastien, P. Blaineau, C. Page`s, M. and Wincker, P. (1998). Conserved linkage groups associated with large-scale chromosomal rearrangements between Old World and New World *Leishmania* genomes. Gene, 222: 107-117.

Brumfield, R. Beerli, P. Nickerson,D. and Edwards, S.(2003). The utility of single nucleotide polymorphisms in inferences of population history. Trends in Ecology and Evolution, 18: 249-256.

Burns, J. Shreffler, W. Benson, D. Ghalib, H. Badaro, R. and Reed, S. (1993). Molecular characterization of a kinesin - related antigen of *Leishmania chagasi* that detects specific antibody in African and American visceral leishmaniasis. Proc. Natl. Acad. Sci., 90:775–779.

Caetano-Anollés, G. Bassam,B. and Gresshoff, P.(1991). DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. Bio/Technology, 9: 553-557.

Campos-Ponce, M. Ponce,C. and Maingon, R.(2005). *L.chagasi/infantum*: further investigations on *Leishmania* tropisms in atypical cutaneous and visceral leishmaniasis foci in Central America. Experimental Parasitology, 109: 209-219.

Carlton, J. Angiuoli, S. Suh, B. and Carucci, D.(2002). Genome sequence and comparative analysis of the model rodent malaria parasite *P. yoelii yoelii*. Nature, 419: 512-519.

Carlton, J.. (2003). Genome sequencing and comparative genomics of tropical disease pathogens. Cellular Microbiology, 5: 861-873.

Carreira, P. Maingon,R. Ward, R. Zeledon,R. and Sousa, O.(1995). Molecular techniques in the characterization of *Leishmania* isolates from Central America. Ann. Trop. Med. Parasitol., 89 Suppl 1: 31-36.

Carroll, MC.(1998).The role of complement and complement receptors in induction and regulation of immunity. Annu. Rev. Immunol.,16: 545-568.

Carvalho,SF. Lemos, EM. Corey, R. and Dietze, R. (2003). Performance of recombinant K39 antigen in diagnosis of Brazilian visceral leishmaniasis. Am. J. Trop. Med. Hyg., 68: 321-4.

Cascio, A. Calattini, S. Colomba, C. and Antinori, S. (2002). Polymerase chain reaction in the diagnosis and prognosis of Mediterranean visceral leishmaniasis in immunocompetent children. Pediatrics, 10: 27.

Chang, KP. Akman, L. and Nielsen, J. (1999). *Leishmania* virulence and genetic heterogeneity. Clinics in Dermatology, 17: 269-273.

Chang, KP. and McGwire, BS. (2002). Molecular determinants and regulation of *Leishmania* virulence. Kinetoplastid Biology and Disease, 1:1-7.

Chang, KP. Reed, SG. McGwire, BS. and Soong, L. (2003). *Leishmania* model for microbial virulence: the relevance of parasite multiplication and pathoantigenicity. Acta Tropica, 85: 375-390.

Chulay, JD. and Bryceson, AD.(1983). Quantitation of amastigotes of *Leishmania donovani* in smears of splenic aspirates from patients with visceral leishmaniasis. Am. J. Trop. Med. Hyg., 32 : 475-9.

Chung, HL. Chow KK. and Lu, JP.(1948). The first two cases of transfusion kala-azar. Chin. Med. J., 66 : 325-6.

Clayton, CE.(1999). Genetic manipulation of Kinetoplastida.Parasitology Today, 15: 372-378.

Cohen,C. Corazza, F. Moll, PD. and Brasseur, D. (1991). Leishmaniasis acquired in Belgium. Lancet, 338 : 128.

-Cortes, S. Rolão, N. Ramada, J. and Campino, L. (2004). PCR as a rapid and sensitive tool in the diagnosis of human and canine leishmaniasis using *L. donovani* - specific kinetoplastid primers. Trans. R. Soc. Trop. Med. Hyg., 98: 12-17.

Costa, C. (1998). Urbanisation and kala azar in Brazil : kala - azar in Teresina.In: Brandao Filho,SP,ed.Research and Control of leishmaniasis in Brazil. Proceedings of a Workshop, Recife, Cpq. Fiocruz.

Costa,C. Pereira,HF. Pereira,F. Tavares,JP. Araujo, MV. and Gonçalves MJ. (1999).Is the household dog a risk factor for American visceral leishmaniasis in Brazil ? Trans. Roy. Soc. Trop. Med. Hyg., 93:464.

Costa, C. Stewart, J. Gomes, R. and Maguire, J. (2002). Asymptomatic human carriers of *L. chagasi*. Am. J. Trop. Med. Hyg., 66: 334-337.

Courtenay, O. Santana,E. Johnson P. Vasconcelos I. and Vasconcelos A. (1996).Visceral leishmaniasis in the hoary zorro *Dusicyon vetulus*: a case of mistaken identity. Trans. R. Soc. Trop. Med. Hyg., 90: 498-502.

Cruz,I. Morales,MA. Noguer,I. Rodriguez, AI. and Alvar, J. (2002). *Leishmania* in discarded syringes from intravenous drug users. Lancet, 9312:1124-1125.

Cruz, I. Chicharro,C. Nieto,J. Bailo, B. Canavate,C. and Alvar, J.(2006). Comparison of new diagnostic tools for management of pediatric mediterranean visceral leishmaniasis. J. Clin. Microbiol., 44: 2343-2347.

Cuervo, P. Cupolillo, E. Hernandez, V. Saravia, N. and Fernandes, O. (2004). *Leishmania* (*Viannia*) : genetic analysis of cutaneous and mucosal strains isolated from the same patient.Experimental Parasitology108, 59-66.

Cummins, D. Amin, S. Halil,O. Hewitt, P. and Radley-Smith, R.(1995). Visceral leishmaniasis after cardiac surgery. Arch. Dis. Child, 72: 235-6.

Cupolillo, E. Grimaldi, Junior, G. Momen, H. and Beverly, S. (1995). Intergenic region typing (IRT): a rapid molecular approach to the characterization and evolution of *Leishmania*. Mol. Biochem. Parasitol. 73(1-2):145-155.

Cupolillo, E. Brahim, LR. Toaldo, CB. and Grimaldi, G.(2003).Genetic polymorphism and molecular epidemiology of *Leishmania* (*Viannia*) *braziliensis* from different hosts and geographic areas in Brazil. J.Clin. Mic., 41: 3126–3132.

Da Silva, E. Gontijo, CM. Pacheco, S. and Brazil, R. (2005). Diagnosis of human visceral leishmaniasis by PCR using blood samples spotted on filter paper. Genet. Mol. Res., 3 : 251-7.

Daulaire, N. (1999). Globalization and Health. Development, 4:22-24.

De Castro, E. Luz,E. Telles,F. Pandey,A. Biseto, A. and Soccol,V.(2005). Ecoepidemiological survey of *Leishmania*(*Viannia*)*braziliensis* American cutaneous and mucocutaneous leishmaniasis in Ribeira Valley River, Parana State, Brazil. Acta. Tropica., 93: 141-149.

De Colmenares, M. Portus, M. Riera,C. Torras, S. and Munoz, C. (1995). Short report: detection of 72-75-kD and 123-kD fractions of *Leishmania*

antigen in urine of patients with visceral leishmaniasis.Am. J. Trop. Med. Hyg., 52: 427-428.

Dedet, JP. (1993). *Leishmania* et leishmanioses du continent ame´ricain. Annales de l'Institut Pasteur, 4: 3-25.

Delgado, J. Pineda, J. Macías, J. Regordán,C. Gallardo, JA. and Leal, M. (1998). Low sensitivity of peripheral blood smear for diagnosis of sub-clinical visceral leishmaniasis in human immunodeficiency virus type1-infected patients. J. Clin. Microbiol., 36: 315-6.

Dennert, G. (1974). Evidence for non-identity of T killer and T helper cells sensitised to allogeneic cell antigens. Nature, 249: 358-360.

De Oliveira, C. Bafica, A. Oliveira, F. Correa,T. and Freitas, L. (2003). Clinical utility of PCR-based detection of *Leishmania* in the diagnosis of American cutaneous leishmaniasis.Clin. Infect. Dis., 37: 149-53.

Dereure, J. Rioux, J. Khiami, A. Pratlong,F. Perie`res, J. and Martini, A. (1991). Ecoe´ pide´ mıologie des leishmanioses en Syrie. 2- Pre´ sence, chez le chien, de *L. infantum* Nicolle et *L. tropica* Wright . Annales de Parasitologie Humaine et Compare´e, 66: 252–255.

Desjardins, M. (2003). ER-mediated phagocytosis: a new membrane for new functions. Nat. Rev. Immunol., 3:280-91.

Desjeux, P. (1996). Leishmaniasis : Public health aspects and control. Clinics in Dermatology, 14:417-423.

Desjeux, P. (2000). *Leishmania*/ HIV co-infection, Southwester Europe, 1990-1998. WHO, CDS/CSR/EDC,WHO/LEISH/2000.42.

Desjeux, P.(2001). The increase in risk factors for leishmaniasis worldwide. Trans. Roy. Soc. Trop. Med. Hyg., 95:239-243.

Desjeux, P. and Alvar, J.(2003). *Leishmania* /HIVco-infections:epidemiology in Europe.An. Trop. Med. Parasitol., 97(1):3-15.

Desjeux, P.(2004). Leishmaniasis: current situation and new perspectives. Comparative Immunology, Microb. and Inf. Dis., 27:305-318.

Edrissian, GH. and Darabian, P.(1999). A comparison of enzyme- linked immunosorbent assay and indirect fluorescent antibody test in the serodiagnosis of cutaneous and visceral leishmaniasis in Iran.Trans. R. Soc. Trop. Med. Hyg.,73:289-292.

Elamin, A. and Omer, MA. (1992). Visceral leishmaniasis in a 6 week old infant: possible congenital transmission.Trop. Doct., 22:133-5.

-El Fakhry, Y. Ouellette, M. and Papadopoulou, B. (2002). A proteomic approach to identify developmentally regulated proteins in *L. infantum*. Proteomics, 2: 1007-1017.

El-Masum, MA and Evans, DA.(1995). Characterization of *Leishmania* isolated from patients with kala azar and post kala azar dermal leishmaniasis in Bangladesh. Trans. R. Soc. Trop. Med. Hyg., 89(3): 331-2.

El-Sayed, N. Myler, P. Blandin, G. Berriman, M. and Hall, N. (2005). Comparative genomics of trypanosomatid parasitic protozoa. Science, 309: 404-409.

Engvall, E. and Perlmann P. (1972). Enzyme - linked immunosorbent assay (ELISA). J. Immunol., 109: 129-35.

Espinoza, J. Skinner, A. Davies, C. and Blackwell, J. (1995). Extensive polymorphism at the Gp63 locus in field isolates of *L. peruviana*. Molecular and Biochemical Parasitology, 72: 203-213.

Fargeas, C. Hommel, M. Maingon, R. and Mayer, R. (1996). Synthetic peptide-based enzyme-linked immunosorbent assay for serodiagnosis of visceral leishmaniasis. J. Clin. Microbiol., 34: 241-8.

Ferro, C. Morrison, A. Torres, M. Pardo, R. and Tesh, RB. (1995). Age structure, blood-feeding behavior, and *L. chagasi* infection in *Lu. Longipalpis* at an endemic focus of visceral leishmaniasis in Colombia. J.Med. Ent., 32: 618-629.

Fisa, R. Riera,C. Ribera, E. Gállego, M. and Portús,MA.(2002).Nested PCR for diagnosis and follow – up of human visceral leishmaniasis patients using blood samples.Trans. R. Soc.Trop. Med. Hyg.,1:191-194.

Franco, A. Machado, G. Moreira, C. and Grimaldi, G.(2000). Minicircle kDNA microheterogeneity in Endotrypanum indicate diversity within this genus. Memo´rias do Instituto Oswaldo Cruz, 95: 189-191.

Fraser, C. Eisen, J. Fleischmann,R. Ketchum, K. and Peterson, S. (2000). Comparative genomics and understanding of microbial biology.Emerging Infectious Diseases, 6: 505-512.

Friedman, R. Hanson, S. and Goldberg, L.(2003). Squamous cell carcinoma arising in a *Leishmania* scar. Dermatol. Surg., 29:1148-1149.

Gangneux, JP. Sulahian, A. Honore,S. Derouin, F. and Garin, Y.(2000). Evidence for determining parasitic factors in addition to host genetics

and immune status in the outcome of murine *L.infantum* visceral leishmaniasis. Parasite Immunology, 22: 515–519.

Gangneux, JP. Menotti, J. Lorenzo, F. Sarfati,C. Blanche,H. and Bui, H. (2003). Prospective value of PCR amplification and sequencing for diagnosis and typing of old world *Leishmania* infections in an area of non-endemicity. J. Clin. Microbiol., 41 : 1419-22.

Gardener, P. Chance, ML. and Peters,W.(1974). Biochemical taxonomy of *Leishmania*. Ann. Trop. Med. Parasit., 68: 317-325.

Gari – Toussaint, M. Lelievre, A. Marty, P and Le-Fichoux, Y.(1995). Contribution of serological tests to diagnosis of visceral leishmaniasis in patients infected with the human immunodeficiency virus. Trans. R. Soc. Trop. Med. Hyg., 88(3): 301-2.

Ghalib, HW. Piuvezam, M. Skeiky, Y. and Reed, S. (1993). Interleukin 10 production correlates with pathology in human *L.donovani* infections. J. Clin. Inves., 92: 324-329.

Godfrey, DG. (1979). The zymodemes of trypanosomes. In : Problems in the identification of parasites and their vectors.Proceedings of the Symposium of the British Society of Parasitology :17:31-53.

Grimaldi,G. and Tesh, R. (1993). Leishmaniasis of New World : current concepts and implications for future research.Clin. Micro. Rev. 6:230-250.

Grimm, F. Gessler,M. and Brun, R.(1996). Isolation and characterization of *Leishmania* parasites importedto Switzerland - a retrospective study, Parasitol. Res., 82: 563-565 .

Guessous - Idrissi, N. Berrag, B. Riyad, M. Sahibi, H. Bichichi, M. and

Rhalem, A. (1997). *L. tropica*: etiologic agent of a case of canine visceral leishmaniasis in northern Morocco. Am. J. Trop. Med. Hyg., 57:172–173.

Guevara, P. Rojas, L. Gonzales, N. Scorza, JV. Anez, N. and Valera, M. (1994). Presence of *L. braziliensis* in blood samples from cured patients or at different stages of immunotherapy.Clin. Diag. Lab. Imm.,1: 385-9.

Gutierrez, J. (2000). Genomics: from novel genes to new therapeutics in parasitology. Int. J. Parasitol., 30: 247-252.

Handman E. and Bullen D.V.,(2002). Interaction of *Leishmania* with the host macrophage. Trends Parasitol 18:332-4.

Harith, A. Kolk,A. Leeuwenburg, J. Muigai, R. Huigen, E. Jelsma,T. and Kager P. (1988). Improvement of DAT for field studies of visceral leishmaniasis. J. Clin. Microbiol., 26:1321-1325.

Hashim, F. Khalil, EA. Ismail, A. and El-Hassan, AM.(1995).Apparently successful treatment of two cases of post kala azar dermal leishmaniasis with liposomal amphotericin B.Trans.R.Soc.Trop. Med.Hyg., 89(4): 440.

Herwaldt, BL. and Juranek, DD.(1993). Laboratory - acquired malaria, leishmaniasis, trypanosomiasis, and toxoplasmosis. Am. J. Trop. Med. Hyg., 48 : 313-23.

Herwaldt, BL. (1999). Leishmaniasis. Lancet,354 : 1191-9.

Hewitt, S. Rowburn, H. Ashford, R. and Rowland, M. (1999). Anthroponotic cutaneous leishmaniasis in Kabul,Afghanistan : vertical distribution of cases in apartment blocks.Trans. Roy. Soc. Trop. Med. Hyg., 92: 273-274.

Hiss, R. Norris, D. Dietrich, C. Whitcomb, R. and Black, W. (1994). Molecular taxonomy using single-strand conformation polymorphism (SSCP) analysis of mitochondrial ribosomal DNA genes. Insect Molecular Biology, 3: 171-182.

Hommel, M. Peters, W. Ranque, J. and Lanotte, G. (1988). The micro-ELISA technique in the serodiagnosis of visceral leishmaniasis. Ann. Trop. Med. Parsitol.,72:213-218.

Hommel, M. Attar, Z. Fargeas,C. Mayer, R. and Chance, M.(1997).The direct agglutination test: a non-specific test specific for the diagnosis of visceral leishmaniasis? Ann. Trop. Med. Parasitol. 91. 795- 802.

Houghton,R. Petrescu,M. and Benson, D. (1998).A cloned antigen(rK39) of *L.chagasi* diagnostic for VL in human. J. Inf. Dis., 177:1339–1344.

Howard, M. Kelly,J. Lane,R. and Miles, M.(1991).A sensitive repetitive DNA probe that is specific to the *L. donovani* complex and its use as epidemiological and diagnostic reagent.Mol. Biochem. Parasitol., 44: 63-72.

Indiani de Oliveira, C. Teixeira, M. Teixeira, C. and Barral, A. (2004). *L. braziliensis* isolates differing at the genome level display distinctive features in BALB/c mice. Microbes and Infection, 6: 977-984.

International Mouse Genome Sequencing Consortium (2002). Initial sequencing and comparative analysis of the mouse genome.Nature,420: 520-562.

Islam, M. Itoh,M. Shamsuzzaman, S. and Kimura, E. (2002). Diagnosis of Visceral Leishmaniasis by enzymelinked immunosorbent assay using urine samples.Clin. Diagn. Lab Immunol., 9: 789-794.

Itagaki,T. Tsutsumi, K. Sakamoto,T. Tsutsumi,Y. and Itagaki, H.(1995). Characterisation of genetic divergence among species within the genus *Fasciola* by PCR-SSCP. Jpn. J Parasitol., 44: 244-247.

Ivens, AC. Peacock, C. Worthey, E. and Myler, PJ. (2005). The genome of the kinetoplastid parasite, *L. major*. Science, 309:436-442.

Jaffe, C. and McMahon – Pratt, D. (1987). Serodiagnostic assay for visceral leishmaniasis employing monoclonal antibodies. Trans. R. Soc. Trop. Med. Hyg., 81: 587-94.

Jelinek,T. Eichenlaub,S. and Loscher,T.(1999).Sensitivity and specificity of a rapid immunochromatographic test for diagnosis of visceral leishmaniasis. Eur. J. Clin. Microbiol. Infect. Dis.,18 : 669-70.

Jensen, AT. Gasim, S. Moller, T. Ismail, A. Gaafar, A. and Kemp, M. (1999). Serodiagnosis of *Leishmania donovani* infections.Trans. R. Soc. Trop. Med. Hyg., 93 : 157-60.

Kamhawi, S. Modi, G. Pimenta, PF. Rowton, E. and Sacks, DL. (2000). The vectorial competence of *Ph. sergenti* is specific for *L.tropica* and is controlled by species-specific,lipophosphoglycan-mediated midgut attachment. Parasitology, 121: 25–33.

Kapoor, G. Arora, S. and Sehgal, S. (1998). Genetic polymorphism of *Leishmania* species using kinetoplast DNA restriction fragment length polymorphism and cDNA probe of *L. donovani*. Medical Microbiology and Immunology (Berlin), 186:209-214.

Kaul, P. Malla, N. Mahajan, R. and Ganguly, N. (2000). Evaluation of 200-kDa amastigote-specific antigen of *L.donovani* by enzyme-linked immunosorbent assay(ELISA) for the diagnosis of visceral leishmaniasis. Trans. R. Soc. Trop. Med. Hyg., 94: 173-175.

Kemp,M. Hey, A. Kurtzhals, J. Ismail, A. Kharazmi, A. and Theander,T. (1994). Dichotomy of the human T cell response to *Leishmania* antigens. Clinical and Experimental Immunology, 96:410-415.

Kemp, K. (2000). Cytokine-producing Tcell subsets in human leishmaniasis. Archivum Immunologiae et Therapiae Experimentalis, 48:173-176.

Kenney, RT. Sacks, DL. Gam, AA. Murray, HW. and Sundar, S. (1998). Splenic cytokine responses in Indian kala-azar before and after treatment. J. Inf. Dis., 177: 815-818.

Khalil, E A. Ayed, N. Musa, AM. and El-Hassan, A. (2005). Dichotomy of protective cellular immune responses to human visceral leishmaniasis. Clinical and Experimental Immunology, 140: 349-353.

Killick-Kendrick, R.(1990). Phlebotomine vectors of the leishmaniases: a review. Med. Vet. Entomol.,4 : 1-24.

Killick-Kendrick, R.(1999). Leishmaniasis: an English disease of the future? Bulletin of Tropical Medicine and International Health, 3:5.

Kisielow, P. Hirst, JA. Shiku, H. Beverley, PC. and Oettgen, HF. (1975). Ly antigens as markers for functionally distinct subpopulations of thymus-derived lymphocytes of the mouse. Nature, 253: 219-220.

Klein, J. and Sato, A.(2000). The HLA system.First of two parts. N. Eng. J. Med., 343: 702-709.

Kopf, M. Brombacher, F. Kohler, G. Kienzle, G. and Solbach, W. (1996). IL-4-deficient Balb/c mice resist infection with *L.major*. J. Exp. Med. 184: 1127-1136.

Korzeniewski, K. (2004). Iraq Warszawa : Wydawnictwo Akademickie DIALOG.,73(2): 54-56.

Korzeniewski, K.(2005). Health hazards in Iraq, Lekarz Wojskowy, 81 (3): 176-180.

Kostman, R. Barr, M. Bengtson, E. Garnham, PCC. and Hult, G. (1963). Kala-azar transferred by exchange blood transfusion in two Swedish infants. In:Proceedings of the seventh international congress of tropical medicine and malaria. Geneva, Switzerland: WHO ; p.384.

Kreutzer, R. Yemma, J. Grogl, M. and Martin, T. (1994). Evidence of sexual reproduction in the protozoan parasite *Leishmania*. Am. J. Trop. Med. Hyg., 51: 301-307.

Kropf, P. Etges, R. Schopf, L. Chung, C. Sypek, J. and Müller, I.(1997). Characterization of T cell-mediated responses in nonhealing and healing *Leishmania major* infections in the absence of endogenous interleukin-4. J. Immunol., 159: 3434-3443.

Kropf, P. Brunson, K. Etges, R. and Müller, I.(1998). The Leishmaniasis Model. IN : Immunology of Infection Series: Methods in Microbiology, Vol. 25: p419-458. Academic Press Ltd, London.

Kropf, P. Schopf, L. Chung, C. Xu, D. Liew, F. Sypek, J. and Müller, I. (1999). Expression of Th2 cytokines and stable Th2 marker ST2L in the absence of IL-4 during *L.major* infection. Eur. J. Immunol.,29:3621-3628.

Kumar, V. Kishore, K. Palit, A. Keshari, S. Sharma, MC. and Das, VN. (2001).Vectorial efficacy of *Ph. argentipes* in Kala-azar endemic foci of Bihar under natural and artificial conditions. J. Comm. Dis., 33: 102- 9.

Kwok, P.(2001).Methods for genotyping single nucleotide polymorphisms. An. Rev. Gen. Hum. Gen., 2: 235-258.

Lachaud, L. Chabert,E. Reynes,J. Lamothe, J. and Bastien,P.(2001).Comparison of various sample preparation methods for PCR diagnosis of visceral leishmaniasis using peripheral blood. J. Clin. Microbiol., 39 (2): 613-617.

Lachaud, L. Chabbert, E. Dereure,J. Dedet, J. and Bastien, P. (2002). Comparison of Six PCR Methods Using Peripheral Blood for Detection of Canine Visceral Leishmaniasis. J. Clin. Microbiol., 40: 210-215.

Lainson, R. and Shaw, J.(1987). Evolution, classification and geographical distribution .In: Peters W, Killick -Kendrick R , editors. The leishmaniasis in biology and medicine. London : Academic Press; p.1-120.

Lainson, R. and Shaw, J. (1998). New World leishmaniasis - Neotropical *Leishmania* species.Parasitology. 9th ed.New York: Oxford University Press, p. 241-82.

Lainson, R. Shaw, J. Silveira, FT. de Souza, A. Braga, R. and Ishikawa, E. (1994).The dermal leishmaniases of Brazil,with special reference to the eco-

epidemiology of the disease in Amazonia. Memo´rias do Instituto Oswaldo Cruz, 89: 435–443.

Lambrechts,A. Van Troys,M. and Ampe, C.(2004).The actin cytoskeleton in normal and pathological cell motility.Int. J. Bio. Cell.Biol.,36:1890-909.

Lambson, B. Smyth, A. and Barker, DC.(2000). *Leishmania donovani:* Development and characterization of a kinetoplast DNA probe and its use in the detection of parasites. Exp. Parasitol., 94:15-22.

Lang,T. Warburg,A. Sacks, D. Croft, S. and Blackwell. JM.(1991).Transmission and scanning EM immunogold labeling of *L.major* lipophosphoglycan in the sand fly *Ph. papatasi*. Eur. J. Cell. Biol., 55 :362-72.

Lanotte, G. Rioux, J. Maazoun, R. and Lepart, J. (1981). Application de la méthode numérique à la taxonomie du genre *Leishmania* Ross,1903. [Application of numerical method to taxonomy of the genus *Leishmania* Ross, 1903]. Annls. Parasitol. Hum. Comp., 56: 575-592.

Laskay, T. Kiessling, R. DeWit, T. and Wirth, D. (1991). Generation of species-specific DNA probes for *Leishmania aethiopica*. Mol. Biochem. Parasitol., 44: 279-286.

Latif, B. Al Shenawi, F. and Al-Alousi,T.(1979).The indirect fluorescent antibody test for diagnosis of kala-azar infection in Iraq. Ann. Trop. Med. Parasitol., 73: 31-35.

Laurie, D. Manson, A. Rowell, F. and Seviour, J.(1989). "A Rapid Qualitiative ELISA Test for the Specific Detection of Morphine in Serum or Urine," Clinica. Chemica. Acta., 183: 183-196.

Le Blancq, SM. Cibulkis, R. and Peters W.(1986). *Leishmania* in the Old World. 5. Numerical analysis of isoenzyme data. Trans. R. Soc. Trop. Med. Hyg., 80:517-524.

Lee, S. Singh, A. and Liu, H.(1995). Identification of *Leishmania* species by a specific DNA probe that is conserved only in the maxicircle DNA of human-infective *Leishmania* parasites. J. Infect. Dis., 172: 891-894.

Leo´n, A. Shaw,J. and Tapia, F.(1996). A guide for the cutaneous leishmaniasis connoisseur. pp. 1–23. Austin.

LePont, F.(1994). Report of a mission to the leishmaniasis foci of Duzici and Sanliurfa in Turkey, IRD,Département Santé, 213 Rue Lafayette,Paris, France, pp. 18.

Lerner, E. Ribeiro, J. Nelson, R. and Lerner, M. (1991). Isolation of Maxadilan,a potent vasodilatory peptide from the glands of the sand fly *Lu. longipalpes*. J. Biol. Chem., 266 : 11234-6.

Lerner, E. and Shoemaker, C.(1992). Maxadilan , cloning and functional expression of the gene encoding this potent vasodilator. J. Biol. Chem., 267 : 1062-6.

Lewin, S. Schonian, G. El Tai, N. Bastien, P. and Presber, W. (2002). Strain typing in *L. donovani* by using sequence - confirmed amplified region analysis. Int. J. Parasitol., 32:1267-1276.

Li, Y. and Lazar, M.(2002).Differential gene regulation by PPAR gamma agonist and constitutively active PPARgamma2.Molecular Endocrinology 16: 1040-1048.

Liarte,D. Mendonca, I. Luz, F. Abreu, E. Mello,G. and Farias, T. (2001). QBC for the diagnosis of human and canine American visceral leishmaniasis : preliminary data. Rev. Soc. Bras. Med. Trop., 34: 577-81.

Lighthall, G. and Giannini, S. (1992). The chromosomes of *Leishmania*. Parasitology Today, 8:192-199.

Lindblad-Toh,K.Winchester,E. Daly, M.Wang, D. and Lander, E. (2000). Large-scale discovery and genotyping of single - nucleotide polymorphisms in the mouse. Nature Genetics, 24: 381-386.

Loke, Y.(1982). Transmission of parasites across the placenta . In: Advances in parasitology. New York: Academic Press, 21:155.

Low, GC. and Cooke, WE.(1926). A congenital infection of kala- azar. Lancet , 2: 1209-11.

Lowry,O. Rosenbrough,N. Farr, A. and Randll, R.(1951). Protein measurement with the folin phenol reagent. J. Biol. Chem., 193: 265.

Lucas, CM. Franke, E. Cachay, MI. Tejada, A. Cruz, M. and Watts, DM. (1998). Geographic distribution and clinical description of leishmaniasis cases in Peru. Am. J. Trop. Med. Hyg., 59: 312-317.

Lumsden, WHR. (1974). Biochemical taxonomy of *Leishmania*. Trans. R. Soc. Trop. Med. Hyg., 68:74-75.

Luz, K. da Silva,V. Gomes, E. Araujo, M. and Fonseca, H. (1997). Prevalence of anti-*L.donovani* antibody among Brazilian blood donors and multiply transfused hemodialysis patients. Am. J.Trop. Med. Hyg.,57:168-71.

Madeira, M. Schubach, A. Schubach, T., Pacheco, R. and Marzochi, M. (2006). Mixed infection with *L.* (*Viannia*) *braziliensis* and *L. chagasi* in a naturally infected dog from Rio de Janeiro, Brazil. Trans. Roy. Soc.

Trop. Med. Hyg., 100: 442-445.

Maggi, P. Gaudiano,V. Valente,M. Latorraca, A. and Marroni, M.(2004). Leishmaniasis in patients with chronic renal failure: A diagnostic and therapeutic challenge for the clinician. J. Nephrol.,17: 296-301.

Magill, AJ. (1995). Epidemiology of leishmaniases. Dermatol. Clin., 13: 505-23.

Mancianti, F. Grandoni, L. Pieri S. and Marconcini A. (1986). Canine leishmaniasis in the Isle of Elba, Italy. Trop. Med. Parasitol., 37: 110-2.

Manson-Bahr, P.(1987). Diagnosis. In: Peters W. Killick-Kendick, R. ed. Leishmaniasis in biology and medicine,Vol. 2.London: Acad. Press,p.703.

Markle,WH. and Makhoul,K.(2004).Cutaneous leishmaniasis:recognition and treatment. Am. Fam. Physician, 69: 1455-1460.

Marth, G. Korf, I. Yandell, M. Yeh, R. and Gish, WR. (1999). A general approach to single-nucleotide polymorphism discovery. Nature Genetics, 23: 452-456.

Martin-Sanchez, J. Pineda, J. Acedo, C. and Macias, J. (2004). Detection of *L.infantum* kineto-plast DNA in peripheral blood from asymptomatic individuals at risk for parenterally transmitted infections. Am. J. Trop. Med. Hyg.,70: 545-8.

Mathis, A. and Deplazes,P.(1995).PCR and in vitro cultivation for detection of *Leishmania* spp. in diagnostic samples from humans and dogs. J. Clin. Microbiol., 33: 1145-1149.

Mathur, P. and Samantaray, J. (2004). The first probable case of platelet

transfusion-transmitted visceral leishmaniasis.Transfus. Med.,14: 319-21.

Mauricio, IL. Gaunt, MW. Stothard, J. and Miles, MA. (2001). Genetic typing and phylogeny of the *L.donovani* complex by restriction analysis of PCR amplified gp63 intergenic regions. Parasitology, 122:393–403.

Mauricio, IL. Yeo, M. Baghaei, M. Pratlong, F. and Miles, MA. (2006). Towards multilocus sequence typing of the *L. donovani* complex: resolving genotypes and haplotypes for five polymorphic metabolic enzymes (ASAT, GPI, NH1, NH2, PGD). Int. J. Parasitol., 36: 757–769.

McCann, S. Eresh,S. and Barker, D.(1999). Kinetoplast minicircle DNA from *Leishmania* (*Viannia*) *lainsoni*. Parasitology, 118: 371-374.

McCarter, J. (2004). Genomic filtering: an approach to discovering novel antiparasitics. Trends in Parasitology, 20: 462- 468.

McMahon-Pratt, D. and Alexander, J.(2004). Does the *L.major* paradigm of pathogenesis and protection hold for New World cutaneous leishmaniases or the visceral disease? Immunological Reviews, 201: 206-224.

McMichael, A. (2000). The urban environment and health in a world of increasing globalization : issues for developing countries. Bulletin of the World Health Organization, 78:1117-1125.

Mebrahtu, Y. Hendricks, L. Oster, C. Perkins, P. and Pamba, H. (1993). *Leishmania donovani* parasites in the nasal secretions, tonsillopharyngeal mucosa,and urine centrifugates of visceral leishmaniasis patients in Kenya. Am. J. Trop. Med. Hyg.,48 : 530-5.

Meredith, S. Zijlstra,E. Schoone,G. El-Hassan, A. and Lawyer, P.(1993). Development and application of the polymerase chain reaction for the detection and identification of *Leishmania* parasites in clinical material. Arch. Ins. Pasteur. Tunis., 70:419-431.

Meredith, S. Kroon, N. Sondorp, E. Seaman, J. and Oskam, L. (1995). Leish-KIT,a stable direct agglutination test based on freeze-dried antigen for diagnosis of visceral leishmaniasis. J. Clin. Microbiol., 33:1742-1745.

Mimori,T. Matsumoto,T. Calvopina,M. Saya,H. and Katakura, K.(2002). Usefulness of sampling with cotton swab for PCR-diagnosis of cutaneous leishmaniasis in the New World. Acta. Trop.,81: 197-202.

Mosmann,T. Cherwinski, H. Bond,M. Giedlin,M. and Coffman,R.(1986). Two types of murine helper T cell clone. J. Imm., 136: 2348-2357.

Motyka,S. and Englund, P. (2004). RNA interference for analysis of gene function in trypanosomatids. Current Opinion in Microbiology,7: 362-368.

Mu, J. Awadalla,P. Duan, J. McGee, K. and Su, X.(2005). Recombination hotspots and population structure in *P. falciparum*. Public Library of Science Biology, 3: 1734-1741.

Mukhtar, M. Sharief,A. El Saffi,S. Harith, A. and Abdalla, H.(2000).Detection of antibodies *to L.donovani* in animals in kala-azar endemic region in eastern Sudan:preliminary report.Trans. R. Soc.Trop. Med. Hyg.,94: 33-36.

Müller, I. Freudenberg, M. Kropf, P. Kiderlen, A. and Galanos,C. (1997). *Leishmania major* infection in C57BL/10 mice differing at the Lps locus: a new non-healing phenotype. Med. Microbiol. Immunol., 186: 75-81.

Mundodi, V. Somanna, A. Farrell, P. and Gedamu, L. (2002). Genomic organization and functional expression of differentially regulated cysteine

protease genes of *L. donovani* complex. Gene, 282: 257-265.

Napier, L. and Gupta, CD. (1998). Indian kala - azar in a new born. Indian Med. Gazette., 62 : 199.

Nuwayri-Salti, N. Baydoun,E. el-Tawk, R. and Jaffe, C.(2000). The epidemiology of leishmaniasis in Lebanon. Trans. R. Soc. Trop. Med. Hyg., 94: 164-166.

Nuzum, E. White,F. Thakur,C. Dietze,R. Grogl, M. and Berman, J.(1995). Diagnosis of symptomatic visceral leishmaniasis by use of the polymerase chain reaction on patient blood. J. Infect. Disease. 171: 751-54.

Ogden, RC. and Adams, DA. (1987). Electrophoresis in agarose and acrylamide gels. Meth. Enzymol. 152, 61-87.

Orita, M. Iwahana, H. Kanazawa, H. Hayashi, K. and Sekiya, T. (1999). Detection of polymorphisms of human DNA by gel electrophoresis as single strand conformation polymorphisms. Proc. Natl. Acad. Sci., 86: 2766-2770.

Oskam, L. Slappendel,R. Beijer, E. van Ingen, C. and Terpstra,W.(1996). Dog-DAT: direct agglutination test using stabilized, freeze-dried antigen for the serodiagnosis of canine visceral leishmaniasis. FEMS Immunol. Med. Microbiol., 16: 235-239.

Osman, O. Oskam, L. Zijlstra, E. El-Hassan, A. and Kager, P. (1997). Evaluation of the PCR for diagnosis of visceral leishmaniasis. J. Clin. Microbiol., 35:2454-2457.

Osman, O. Oskam, L. Zijlstra, E. El Hassan, A. and Kager, P. (1998). PCR to assess the success of visceral leishmaniasis treatment. Trans. R. Soc. Trop. Med. Hyg., 92: 397-400.

Osorio, L. Castillo, C. and Ochoa, MT. (1998). Mucosal leishmaniasis due to *Leishmania* (*Viannia*) *panamensis* in Colombia : clinical characteristics. Am. J. Trop. Med. Hyg., 59: 49–52.

Ozbel,Y. Oskam,L. Alkan, M. Jaffe, C. and Ozcel, M. (2000).A survey on canine leishmaniasis in westernTurkey by parasite,DNA and antibody detection assays. Acta. Trop.,74: 1-6.

Palatnik-de-Sousa, C. Gomes, E. Palatnik, M. Luz, K. and Borojevic, R. (1995). *L. donovani*: titration of antibodies to the fucose-mannose ligand as an aid in diagnosis and prognosis of visceral leishmaniasis. Trans. R. Soc. Trop. Med. Hyg.,89: 390-3.

Panton, LJ. (1991). A test for genetic exchange in mixed infections of *L. major* in the sand fly Phlebotomus papatasi. J. Proto., 38: 224-228.

Pardi, F. Lewis,C. and Whittaker, J.(2005). SNP selection for association studies:maximizing power across SNP choice and study size.An. Hu.Gen., 69: 733-746.

Paredes,R. Munoz,J. Diaz, I. Domingo,P. Gurgui,M. and Clotet,B.(2003). *Leishmania* in HIV Infection. J. Postgrad. Med., 49 : 39-49.

Pappas, M. Hajkowski, R. and Hockmeyer,W. (1984). Standardization of the dot enzyme-linked immunosorbent assay (Dot - ELISA) for human visceral leishmaniasis. Am. J. Trop. Med. Hyg., 33: 1105-11.

Pappas, M. Hajkowski,R. Diggs,C. and Hockmeyer W.(1985).Disposable nitrocellulose filtration plates simplify the Dot-ELISA for serodiagnosis of visceral leishmaniasis. Trans. R. Soc. Trop. Med. Hyg., 79: 136.

Pearson, RD. and Sousa, AQ. (1996). Clinical spectrum of leishmaniasis. Clin. Infect. Dis., 22 : 1-13.

Peters,W.(1988).“The little sister”-a tale of Arabia. Trans. Roy. Soc.Trop. Med. Hyg., 82:179-184.

Piarroux, R.Trouvé,V. Pratlong,F. Martini,A. and Rioux, J.(1994).The use of isoelectric focusing on polyacrylamide gel for the enzymatic analysis of Old World *Leishmania* species.Trans. R. Soc. Trop. Med. Hyg.,88:475-478.

Pimenta, P. Turco, S. Perkins, P. and Sacks, D.(1992). Stage specific adhesion of *Leishmania* promastigotes to sand fly mid gut. Science, 256:1812-5.

Pimenta, P. Saraiva,E. Rowton, E. and Sacks, D. (1994). Evidence that the vectorial competence of phlebotomine sand flies for different species of *Leishmania* is controlled by structural polymorphisms in the surface lipophosphoglycan. Sciences, 91: 9155–9159.

Pizzuto, M. Piazza, M. and Senese, D. (2001). Role of PCR in diagnosis and prognosis of visceral leishmaniasis in patients co-infected with human immunodeficiency virus type1.J. Clin. Microbiol., 39:357-61.

Popovsky, MA. (1991). Transfusion-transmitted Babesiosis. Transfusion. 31 : 296-8.

Prasad,R. Kumar,R. Jaiswal, BP. and Singh, UK. (2004). Miltefosine: an oral drug for visceral leishmaniasis. Indian J. Pediatr., 71: 143-144.

Pratlong, E. Martini,A. Lambert, M. and Rioux, JA. (1994). Interét de la culture et de l'. identification isoenzymatique des *Leishmanies* dans le diagnostic et l'epidemiologie leishmanioses.Médecine et armies.22: 61-65.

Pratlong, F. Rioux, J. Marty,P. Dereure,J. Lanotte,G. and Dedet, J.(2004). Isoenzymatic analysis of 712 strains of *L. infantum* in the south of France and relationship of enzymatic polymorphism to clinical and epidemiological features. J. Clin. Microbiol., 42: 4077–4082.

-Qiao, Z. Miles, M. and Wilson, S. (1995). Detection of parasites of the *L.donovani* complex by polymerase chain reaction-solution hybridisation enzyme-linked immunoassay (PCR-SHELA). Parasitology, 110: 269-275.

Rajasekariah, G. Ryan, J. Hillier,S. Yi, L. Stiteler, J. and Cui, L. (2001). Optimisation of an ELISA for the serodiagnosis of visceral leishmaniasis using *in vitro* derived promastigote antigens. J. Imm. Meth., 252: 105-19.

Ramalho-Ortigao, J. Temporal, P. de Oliveira, S. and Traub-Cseko, Y. (2001). Characterization of constitutive and putative differentially expressed mRNAs by means of expressed sequence tags, differential display reverse transcriptase-PCR and randomly amplified polymorphic DNA-PCR from the sand fly vector *Lu. longipalpis*. Memo´rias do Instituto Oswaldo Cruz, 96: 105-111.

Ramos, A. Maslov,D. Fernandes,O. Campbell,D. and Simpson, L.(1996). Detection and identification of human pathogenic *Leishmania* and *Trypanosoma* species by hybridization of PCR-amplified mini-exon repeats. Exp. Parasitol., 82: 242-250.

Redhu, N S. Dey, A. Balooni, V. and Singh, S.(2006). *Leishmania*-HIV co-infection: An emerging problem in India. AIDS 2006 (in press).

Rioux, JA. Lanotte, G. Serres, E. and Perieres, J. (1990). Taxonomy of *Leishmania*. Use of isoenzymes. Suggestions for a new classification. Annls Parasitol hum comp; 65:111-125.

Rittig, M. and Bogdan, C. (2000). *Leishmania*-host-cell-interaction: complexities and alternative views. Parasitol. Today 16(7), 292-297.

Roberts, S. Swihart,K. Agey, M. and Donelson, J.(1997).Sequence diversity and organization of the msp gene family encoding gp63 of *L.chagasi*. Molecular and Biochemical Parasitology, 62:157-171.

Rodgers, W. and Wirth, D.(1990). Kinetoplast DNA minicircles: regions of extensive divergence. Proc. Nat. Ac. Sc., 84: 563-569.

Rodriguez,N. Aguilar,CM. Barrios,M. and Barker, DC. (1999). Detection of *Leishmania braziliensis* in naturally infected individual sand flies by polymerase chain reaction .Trans R. Soc.Trop.Med.Hyg., 93: 47-49.

Rodriguez-Gonzalez, I. Vargas, F. and Sanchez-Moreno,M. (2006). Identification and biochemical characterization of *Leishmania* strains isolated in Peru, Mexico, and Spain. Experimental Parasitology, 112: 44-51.

Rogers, D. (1998).The dynamics of vector-transmitted diseases in human communities. Ph. Trans. Roy. Soc. Science, 321(1207):513-539.

Rogers, ME, Nikolaev,AV. Ferguson, MA. and Bates, PA.(2004). Transmission of cutaneous leishmaniasis by sand flies is enhanced by regurgitation of f PPG. Nature, 430: 463–467.

Romero, G. Sampaio,R. Macêdo, V. and Marsden, P. (1999). Sensitivity of lymph node aspiration in localized cutaneous leishmaniasis due to *L. braziliensis*. Mem. Inst. Oswaldo. Cruz., 94 : 509-11.

Rosenthal, P. Marty, P. and Pesce, A. (1991). *Leishmania* in broncho-alveolar lavage. Ann. Int. Med.,114: 1064-5.

Ross, R.(1903). Note on the bodies recently described by Leishman and Donovan. Br. Med. J., 2 : 1261-2.

Rotureau, B. Ravel, C. Couppie, P. Dedet, J. and Carme, B. (2006). Use of PCR-restriction fragment length polymorphism analysis to identify the main New World *Leishmania* species and analyze their taxonomic properties and polymorphism by application of the assay to clinical samples. J. Clin. Microbiol,44:459-467.

Sacks, DL. and Perkins, PV.(1984). Identification of an infective stage of *Leishmania* promastigotes. Science. 223 : 1417-9.

Sacks, DL. Saraiva, EM. Rowton, E. Turco, SJ. and Pimenta, PF. (1994). The role of lipophosphoglycan of *Leishmania* in vector competence. Parasitology, 108 : 55-62.

Saiki, R. Gelfand, D. Stoffel, S. Scharf, S. and Erlich, H. (1988). Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. Science, 239: 487-491.

Salotra,P. Raina, A. and Negi,N.(1999). Immunoblot analysis of antibody response to antigens of *Leishmania donovani* in Indian kala-azar. Br. J. Biomed. Sci., 56: 263-267.

Salotra, P. Sreenivas , G. Lee, N. Nakhasi, H. and Ramesh, V. (2001). Development of species-specific PCR assay for detection of *L. donovani* in clinical samples from patients with kala-azar and post-kala-azar dermal leishmaniasis. J. Clin. Microbiol., 39 : 849-54.

Salotra, P. and Singh, R. (2006). Challenges in the diagnosis of post kala-azar dermal leishmaniasis. Ind. J. Med. Res., 123: 295-310.

Salotra, P. Duncan, R. Singh, R., Sreenivas, G. and Nakhasi, HL. (2006). Upregulation of surface proteins in *L. donovani* isolated from patients of post kala-azar dermal leishmaniasis.Microbes and Infection, 8: 637-644.

Samuelson,J. Lerner, E. Tesh, R. and Titus, R.(1991). A mouse model of *L. braziliensis* infection produced by coinjection with sandfly saliva. J. Exp. Med.,173 : 49-54.

Sandoval, C. Angulo, V. Gutierrez, R. Munoz, G. and Ferro, C. (1998). Especies de *Lutzomyia* posibles vectores de leishmaniasis en la ciudad de Bucaramanga, Santandrer, Colombia. Biomedica,18:161-168.

Sang, D. Njeru, WK. Ashford, RW. (1992). A possible animal reservoir for *L.tropica* s.l. in Kenya. Ann. Trop. Med. Parasitol . 86(3), 311-312.

Sanyal, RK.(1985). Leishmaniasis in the Indian sub-continet.New York and Oxford. p. 443-67.

Sarkari, B. Chance,M. and Hommel, M. (2002). Antigenuria in visceral leishmaniasis: detection and partial characterization of a carbohydrate antigen. Acta. Tropica., 82: 339-348.

Schallig,H. Schoone,G. Kroon,C. Hailu,A. Chappuis, F. and Veeken, H. (2001). Development and application of 'simple' diagnostic tools for visceral leishmaniasis. Med. Microbiol. Immunol., 190: 69-71.

Schallig, HD. Canto-Cavalheiro, M. and da Silva, E. (2002). Evaluation of direct agglutination test and the rK39 dipstick test for the sero-diagnosis of visceral leishmaniasis. Mem. Inst. Oswaldo. Cruz.,7:1015-8.

Schneider, P. Rosat, J. Bouvier, J. and Bordier, C. (1992). *Leishmania major*:differential regulation of the surface metalloprotease in amastigote and promastigote stages. Experimental Parasitology, 75:196-206.

Schönian,G. Akuffo,H. Lewin, S. Pratlong, F. Schnur, L. and Presber,W. (2000). Genetic variability within the species *Leishmania aethiopica* does not correlate with clinical variations of cutaneous leishmaniasis. Mol. Biochem. Parasitol.,106:239-248.

Schreiber, GB. Busch, MP. Kleinman, S. and Koreiltz, J.(1996).The risk of transfusion-transmitted viral infections.N. Engl. J. Med.,334:1685-90.

Schnur, L. and Zuckerman A. (1977). Leishmanial excreted factor (EF) serotypes in Sudan and Ethiopia. Ann. Trop. Med. Parasitol., 71: 273-94.

Sharma, R. Bahl, L. Goel, A. Upadhyay, A. Kaushik,SL. and Sharma,RL. (1996). Congenital kala-azar: a case report. J. Commun. Dis., 28: 59-61.

Sharma, VP. (1996). Ecological changes an vector - borne diseases. Tropical Ecology, 37:7-65.

Sharma, M. Gupta,A. Das, V. Verma, N. and Kar, S. (2000). *Leishmania donovani* in blood smears of asymptomatic persons. Ac.Trop.,76:195-196.

Shlomai, J. (1994). The assembly of kinetoplast DNA. Parasitol. Today,

10 : 341-6.

Sideris,V. Papadopoulou,G. Dotsika,E. and Karagouni,E.(1999).Asymptomatic canine leishmaniasis in Greater Athens area. Eu. J. Epi.,15: 271-276.

Simpson, L. (1987). The mitochondrial genome of kinetoplastid protozoa: genomic organization, transcription, replication and evolution. An. Rev. Microbiol., 41: 363-382.

-Sinagra, A. Riarte, A. Luna, C. Campanini, A. and Segura, EL. (1997). *Leishmania*(*Viannia*) *braziliensis*: biological behavior in golden hamsters of isolates from Argentine patients. Am. J. Trop. Med.Hyg., 57:115–118.

Singh, S. Gilman-Sachs, A. Chang, K. and Reed, S. (1995). Diagnostic and prognostic value of K39 recombinant antigen in Indian leishmaniasis. J. Parasitol., 81:1000-1003.

Singh, S. Chaudhry, VP. and Wali, JP.(1996) .Transfusion-transmitted kala-azar in India. Transfusion, 36 : 848-9.

Singh, N. and Rastogi, AK. (1999). Kinetoplast DNA minicircles of *L. donovani* express a protein product. Bio. et Biophy. Acta,1444: 263–268.

Singh, S. Kumari,V. and Singh, N. (2002). Predicting kala-azar disease minifestations in asymptomatic patients with latent *L.donovani* infection by detection of antibody against recombinant K39 antigen. Clin. Diag. Lab. Immunol., 9 : 568-72.

Singh,S. and Sivakumar, R.(2003). Recent advances in the diagnosis of leishmaniasis. J .Postgrad. Med., 49 : 55-60.

Singh, S.(2004).Mucosal leishmaniasis in an Indian AIDS patient.Lancet Infect. Dis., 4 : 660-1.

Singh, S. Dey, A. and Sivakumar, R. (2005). Applications of molecular methods for *Leishmania* control. Rev. Expert. Rev. Mol. Diag.,5: 251-65.

Sinha,R. and Sehgal, S.(1994).Comparative evaluation of serological test in Indian Kala-azar. J. Trop. Med. Hyg., 97: 333-340.

Singla,N. Singh,GS. Sunder, S. and Vinayak, VK. (1993). Evaluation of the direct agglutination test as an immunodiagnostic tool for Kala-azar in India. Trans. R. Soc. Trop. Med. Hyg., 87: 276-278.

Sivakumar, R. (2004). Studies on the kinesin related antigen gene of *Leishmania donovani* and its diagnostic application . All India Institute of Medical Sciences, New Delhi, India. PhD Thesis.

Sivakumar, R. Sharma, P. Chang, KP. and Singh, S. (2006). Cloning, expression and purification of recombinant antigen from *L. donovani*. Protein Expr. Purif.,46 : 156-65.

Smyth, A. Ghosh,A. Hassan,M. Adhya,S. Mallik,K. and Barker, D.(1992). Rapid and sensitive detection of *Leishmania* kinetoplast DNA from spleen and blood samples of kala-azar patients. Parasitology, 105 (Pt 2):183-192.

Soares,R. Macedo,M. Ropert,C. and Turco, S.(2002). *Leishmania chagasi*: lipophosphoglycan characterization and binding to the midgut of the sand fly vector *Lu.longipalpis*. Mol. Bioch. Parasitol., 121: 213- 224.

Sreenivas,G. Ansari, N. Singh, R. Bhatheja,R. and Negi, N. (2002). Diagnosis of visceral leishmaniasis : comparative potential of amastigote antigen, recombinant antigen and PCR. Br. J. Biomed . Sci., 59: 218-22 .

Srivastava, L. and Singh,V. (1998). Diagnosis of Indian Kala-azar by dot enzyme – linked immunosorbent assay (dot- ELISA). Ann. Trop. Med. Parasitol., 82: 331-334.

Stark, D. Pett, S. Marriott, D. and Harkness, J. (2006). Post- kala-azar dermal leishmaniasis due to *L.infantum* in human immunodeficiency virus type 1-infected patient. J. Clin. Microbiol., 44: 1178-1180.

Stears, RL. Martinsky, T. and Schena, M. (2003). Trends in microarray analysis. Nature Medicine, 9: 140-145.

Steinkraus, H. Greer, J. Stephenson, D. and Langer, P. (1993). Sequence heterogeneity and polymorphic gene arrangements of the *Leishmania guyanensis* gp63 genes. Mol. Bioch. Parasitol., 62:173-185.

Stober, C. Lange, U. Roberts, M. Gilmartin, B. and Blackwell, J. (2006). From genome to vaccines for leishmaniasis: screening 100 novel vaccine candidates against murine *L. major* infection. Vaccine, 24: 2602-2616.

Sukkar, F. (1985).Epidemiology of Kala-azar in Iraq. Bull. End. Dis.Kala-azar section. P : 11-13.

Sundar, S. Reed, S. Singh,V. and Murray, H.(1998).Rapid accurate field diagnosis of Indian visceral leishmaniasis. Lancet, 351: 563-565.

Sunder, S. Singh,G. Raj, V. Vinayak,V. and Singla,N. (2004).Immunodiagnosis of Kala-azar with special reference to DAT. New Delhi :459-462.

Takai,T. (2002).Roles of Fc receptors in autoimmunity. Nat.Rev.Immunol., 2: 580-92.

Tanner, C.(1996).Immunobiology of visceral leishmaniasis.Clin.Immunol. Immunopath., 78(2): 105-111.

Taylor, J. Geiser, D. Burt, A. and Koufopanou, V. (1999). The evolutionary biology and population genetics underlying fungal strain typing. Clin. Microbiol. Rev., 12: 126-146.

Tesh, R. (1995). Control of zoonotic visceral leishmaniasis: is it time to change strategies ? Am. J. Trop. Med. Hyg., 52:287-292.

Thakur, CP. (2000). Socio-economics of visceral leishmaniasis in Bihar (India). Trans. R. Soc. Trop. Med. Hyg. 94, 156-157.

Theodos, CM. and Titus, R. (1993). Salivary gland material from the sandfly *Lu. longipalpis* has an inhibitory effect on macrophage function *in vitro*. Parasite Immunol.,15 : 481-7.

Tibayrenc, M. (2007). Human genetic diversity and the epidemiology of parasitic and other transmissible diseases. Advances in Parasitology, 64: 377-429.

Torrico, M. De Doncker,S. Arevalo, J. Le Ray, D. and Dujardin, J.(1999). In vitro promastigote fitness of putative *L.* (*Viannia*) *braziliensis* / *L.* (*Viannia*) *peruviana* hybrids. Acta Tropica, 72: 99-110.

Traoré, K. Sawadogo, N. Traoré, A. and Guigemde, T. (2001). Etude préliminaire de la leishmaniose cutanée dans la ville de Ouagadougou de 1996 à 1998. Bulletin de la Société de pathologie Exotique, 94:52-55.

Turner, C. McLellan, S. Lindergard,L. and Mac-Leod, A. (2004). Human infectivity trait in *T. brucei*. Parasitology, 129: 445-454.

Van Eys, G. Schoone, G. Kroon,N. and Ebeling, S. (1992). Sequence analysis of small subunit ribosomal RNA genes and its use for detection and identification of *Leishmania* parasites. Mol Biochem Parasitol 51:133-142.

Veeken, H. (1999). Manual for the Diagnosis and Treatment of Visceral Leishmaniasis(Kala-Azar)under Field Conditions,Médecins sans Frontières, Amsterdam, The Netherlands.

Velez, I. (2001). La leishmaniasis in Colombia : De la selva a la ciudad. Memorias XXVIII Congresso de la Sociedad Colombiana de Entomologia, Pereira, Colombia, p.51-57.

Vickerman, K. and Tetley, L.(1990).Flagellar surfaces of parasitic protozoa and their role in attachment. In : Bloodgood R.A., ed. Ciliary and Flagellar Membranes. New York and London: Plenum, p. 267-304.

Volf, P. Killick-Kendrick, R. Bates,P. and Molyneux, D. (1994). Comparison of haemagglutination activities in gut and head extracts of various species and geographical populations of phlebotomine sandflies.
An. Trop. Med. Parasitol., 88: 337-340.

Volf, P. Skarupova, S. and Man, P. (2002).Characterization of the lectin from females of *Ph. duboscqi* sand flies. Eu. J.Bioch., 269: 6294-6301.

Walsh, J.F. Molyneux, D.H. and Birley, M.H.(1993). Deforestation: effects on vector-borne disease. Parasitology,106:55-75.

Walters, LL. (1993). *Leishmania* differentiation in natural and unnatural sand fly hosts. J. Euk. Microbiol., 40 : 196-206.

Webster, P. and Russel, DG.(1993). The flageller pocket of trypanosomatids. Parasitol. Today,9 : 201-6.

Wegmann, T. Lin, H. Guilbert, L. and Mosmann,T. (1993). Bidirectional cytokine interactions in the maternal - fetal relationship : is successful pregnancy a TH2 phenomenon? Immunol Today,14 : 353-6.

Weigle, K. Valderrama, L. Arias, A. Santrich, C. and Savaria NG. (1991). Leishmanin skin test standardization and evaluation of safety,dose storage, longevity of reaction and sensitization.Am. J. Trop. Med. Hyg. 44:260-271.

Weiss, J.(1995). DNA probes and PCR for diagnosis of parasitic infections. Clin. Microbiol. Rev., 8:113-130.

Welsh, J. and McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res., 18: 7213-7218.

West, M. Prescott, AR. Eskelinen, EL. Ridley, AJ. and Watts, C. (2000). Rac is required for constitutive macropinocytosis by dendritic cells but does not control its downregulation. Curr. Biol., 10: 839-48.

WHO expert committee report (1991). Control of the leishmaniasis.Tech. Report Series 793.

WHO., (2000). Afghanistan in the 21st century: A health sector analysis, WHO Afghanistan, Afghanistan Appeal.

WHO., (2001). Conclusions and Recommendations of the IVth Joint Meeting on *Leishmania* / HIV Co - infections, Catania, Italy,17-18 May 2001,Parasite, 8:376-379.

WHO., (2003). Communicable disease profile for Iraq. 19 March 2003. http://www.who.int/infectious-disease-news.

WHO expert committee report (2006).Control of the leishmaniasis.Tech. Report Series 265.

Wickstead, B. Ersfeld, K. and Gull, K. (2003). Repetitive elements in genomes of parasitic protozoa. Microbiology and Molecular Biology Reviews, 67: 360-375.

Williams, J. Kubelik, A. Livak, K. Rafalski, J. and Tingey, S. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., 18: 6531-6535.

Williams, D. Carter, K. and Baillie, A.J.(1995). Visceral leishmaniasis in the BALB/c mouse : comparison of the in vivo activity of five non-ionic surfactant vesicle preparations of sodium stibogluconate. J. Drug. Target. 3(1): 1-7.

Wilson, S. (1995). DNA- based methods in the detection of *Leishmania* parasites: field applications and practicalities. An. Trop. Med. Parasitol., 89 (supplement 1): 95-100.

Wilson, ME. Jeronimo, S. and Pearson, R. (2005). Immunopathogenesis of infection with the visceralizing *Leishmania* species. Microbial Pathogenesis, 38: 147-160.

Wincker, P. Ravel,C. Blaineau,C. Page`s, M. Jauffret, Y. and Bastien, P. (1996). The *Leishmania* genome comprises 36 chromosomes conserved across widely divergent human pathogenic species. Nucleic Acids Research, 24: 1688-1694.

Wolday,D. Berhe, N. Akuffo, H. Desjeux, P. and Britton, S. (2001).

Emerging *Leishmania* / HIV co - infection in Africa. Med. Microbiol. Immuno. (Berlin) 190, 65–67.

Yao, C. Donelson, J. and Wilson, M. (2003). The major surface protease (MSP or GP63) of Leishmania sp. Biosynthesis, regulation of expression, and function. Molecular and Biochemical Parasitology, 132: 1-16.

Yassir, M.(2004).Leishmaniasis.PhD thesis,College of Medicine,Baghdad University , pp : 1-7.

Zhang, W. and Matlashewski, G.(1997). Loss of virulence in *L.donovani* deficient in an amastigote-specific protein, A2. Science, 94: 8807-8811.

Zijlstra, E. Ali, M. El-Hassan, A. Ghalib, H. and Kager P. (1991). Direct agglutination test for diagnosis and seroepidemiological survey of Kala-azar in Sudan. Trans. R. Soc. Trop. Med. Hyg. 85: 474-476.

Zijlstra, E. Daifalla,N. and Kager, P.(1998).rK39 enzyme-linked immuno-sorbent assay for diagnosis of *Leishmania donovani* infection. Clin.Diag. Lab. Immuno.,5:717-720.

Zijlstra, E. Nur,Y. Desjeux,P. Khalil, E. and Groen, J. (2001). Diagnosing visceral leishmaniasis with the recombinant K39 strip test:experience from the Sudan. Trop. Med. Int. Health, 6 : 108-13.

Zilberstein, D. and Shapira, M. (1994). The role of *p*H and temperature in development of *Leishmania* parasite. Annu. Rev. Microbiol.,48: 449-70.

Zinkernagel, RM. and Hengartner, H. (2001). Regulation of the immune response by antigen. Science, 293: 251-253.

Zovein, A. Edrissian,G. and Nadim, A.(1984). Application of the indirect fluorescent antibody test in serodiagnosis of cutaneous leishmaniasis in experimentally infected mice and naturally infected *Rhombomys opimus*. Trans. R. Soc. Trop. Med. Hyg., 78: 73-7.

ألخلاصة

أن ألهدف من ألدراسة ألحالية لتشخيص مرض ألكلازار في دم ألأشخاص المصابين بالطرق التشخيصية الجزيئية ومقارنة ذلك بالطرق التقليدية والمناعية.أخذت عينات ألدم من ٨٠٠ حالة أصابة و ١٠٠ عينة للمقارنة . جميع الحالات المصابة كانت تعاني من علامات و أعراض مرضية مختلفة نخص منها بالذكر : ألحمى ، تضخم الطحال والكبد ، فقدان الوزن ، فقر الدم... ألخ . أظهرت ألنتائج بأن ألأختبار ألجزيئي PCR أعطى حساسية (٩٥%) ونوعية عالية جدا (١٠٠%). أخذت عينات ألدم المصابة من المواطنين في محافظتي بغداد وواسط للفترة من شهر كانون

الثاني من عام ٢٠٠٨ ولغاية شهر حزيران من العام ٢٠٠٨ ، وتراوحت ألأصابة بين أعلى مستوى لها في شهر كانون الثاني وكانت ١٦٥ حالة أصابة (٢٠,٦ %) ألى أدنى مستوى لها في شهر تموز وكانت ٤٩ حالة أصابة (٦,١ %).

فحصت ٨٠٠ عينة مصابة بمرض الكالازار(الحمى السوداء) ووجد أن جميع المصابين يعانون

من ظهور علامات وأعراض منها الحمى (١٠٠%) ، تضخم الطحال (٩٦,٧ %) ، تضخم الكبد (٨٣,٣%) ، فقدان الوزن (٥١,٧%) ، فقر الدم (٥٠%) ، الشحوب (٣٥,٣%)، الأسهال (٢٢%)،

نقص في كريات الدم البيضاء (١١,٧%) ، ووجع البطن (١٥,٣%).

تم تقسيم المصابين على أساس الفئة العمرية الى سبع مجاميع تبدأ من فئة دون السنة وتنتهي بفئة فوق الخمسين سنة ، وكانت أعلى ألأصابات بالفئة العمرية ألأولى حيث بلغت

٥٦٠ (٧٠ %) وأدناها في الفئة العمرية ألسابعة حيث بلغت ٨ (١%). كانت نسبة أصابة ألذكور ٤٣٦ (٥٤,٥ %) أعلى مما في ألأناث ٣٦٤ (٤٥,٥ %) كذلك كانت النسبة أعلى في ألمناطق ألريفية ٤٥٤ (٦٣,٦ %) . أثبتت ألدراسة أن أستخدام الطريقة الجزيئية في تشخيص طفيلي اللشمانيا الحشوية كان ذا دقة ونوعية عاليتين (٩٥ % ، ١٠٠ %) على التوالي كما انه قادر على تحديد التراكيز القليلة من الحامض النووي للطفيلي ، مقارنة بالطرق المناعية الأربع المستخدمة وهي :

rK39 dipstick , ELISA , IFAT , DAT

التي أجريت على ٨٠٠ عينة دم ، ظهرت منها ٧١٧ (٨٩,٦ %)عينة موجبة و٨٣ (١٠,٤ %) عينة

سالبة، والتي تراوحت دقتها بين ٦٩ %- ٩٥ % والتي ممكن ان تعطي نتائج موجبة كاذبة . أقترحت الدراسة الحالية ضرورة وضع برنامج علمي وصحي متكامل و دقيق للقضاء على المرض.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة النهرين / كلية العلوم

مقارنة بين الطرق التشخيصية ألجزيئية والمصلية في تشخيص مرض الكالازار

رسالة مقدمة الى

مجلس كلية العلوم كجزء من متطلبات نيل درجة الدكتوراه في علوم التقنيات ألأحيائية

من قبل الطالب

عبدالسادة عبدالعباس راهي أل غنيمة

بكالوريوس علوم،ماجستيرعلوم/أحياء مجهرية

بأشراف

د.احسان مهدي الصفار

أستاذ

٢٠٠٨ / كانون ألأول

١٤٢٩/ في الحجة