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Genetic Diversity of Sudanese Leishmania Parasite and Possible Correlation to Clinical Signs

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DEDICATION

To the soul of my mother who would have been proud of this degree.

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ABSTRACT

Introduction: Leishmaniasis forced itself upon medical attention as an increasingly significant problem over the last decade hence it is associated with poverty in developing countries and considered as imported or re-emerging diseases in Europe. Leishmaniasis which is a globally widespread group of diseases is caused by obligatory, Intracellular pathogen of the immune system targeting macrophages and dendritic cells. In humans the infection is transmitted through the bites of female sand flies. The symptoms ranging from disfiguring cutaneous and muco-cutaneous lesions that can cause widespread destruction of mucous membranes to fatal visceral disease affecting the haemopoetic organs. It is endemic in the tropical and subtropical regions of 88 countries, sixteen are developed countries, 72 are developing countries, 13 of them are among the least developed. Sudan is one of the greatest foci worldwide where Leishmaniasis represents a serious health problem causing high number of fatalities. *Leishmania donovani complex* and *L.major* are known to be the etiologic agents, however, the *Leishmania donovani complex* parasites that causes different clinical forms, i.e. visceral (VL), cutaneous (CL), muco-cutaneous (ML) and post kala-azar dermal Leishmaniasis (PKDL) are genetically considered as a homogenous group.

Objectives: To understand the correlation between the genetic diversity and the clinical manifestations.

Specific Objectives 1) To genetically characterize isolates from Sudanese patients diagnosed with different types of leishmaniasis to understand the correlation between different clinical manifestations and the genetic profile of Leishmania.**2)** Investigate presence or absence of mixed infections and/ or hybrids. **3)** To build up a reliable picture of the various phylogenetic subdivisions with relation to virulence, using the Internal Trascribed Spacer (ITS) gene, the Lyshmanolysin (GP63) and the cytochrome Oxidase sub- unit II gene.

Materials and Methods: Comparative genome based approach was used to analyse the three genes, Cytochrome Oxidase II (COII) gene, the Internal Transcribed Spacer (ITS) and the GP63 (both gp63 3/4 and MSPC). 111 samples from 72 Sudanese patients suspected of different Leishmaniasis were used. Firstly a Real Time PCR was developed to pick up positive samples. Secondly, to detect the interspecific polymorphism at species level, Cytochrome Oxidase II (COII) gene was amplified for all Real Time PCR positive samples. The methodology encompasses the use of direct sequencing and cloning techniques. Thirdly, to detect the intraspecific polymorphism at strain level and the under types of some positive isolates, (ITS) and the GP63 (both gp63 3/4 and MSPC) genes were amplified , The methodology encompasses the use of cloning techniques.

Results and Discussion: 40 patients out of 72 (55.6%) and 58 samples out of 111 (52.3%) were confirmed by the Real Time PCR as Leishmania positive. Five CL samples from five patients have been examined, three of them have been concomitantly infected with both L.donovani and L.major as confirmed by the cloned COII and ITS genes. The ITS sequence data analysis of *L. major* related colonies in the three CL samples revealed the presence of more than one strain /hybrid of *L. major* as different polymorphic sites were detected within and between patients. The other two CL samples were found to be caused merely by L.donovani, but different sequence patterns have been detected by the ITS gene.

One patient possessed *L. infantum* similar COII sequence, the GP63 sequence data analysis of the same patient confirmed some colonies as *L. infantum*, while two out of the six identified polymorphic sites in the ITS gene were similar to those characteristic for L.infantum different zymodemes.

The number of PKDL and ML samples was not sufficient to draw a conclusion., However, COII and ITS data pointed L.donovani as the causative agent. ITS data showed considerable different sequence patterns between colonies from the two samples. The same results were found regarding other samples attributed to VL.

Gp63 sequence data analysis of some colonies attributed to VL samples (namely 10 HBM, 3HBM and 11HBM) has identified two genetic groups, both within the *L. donovani complex* but different from *L. infantum* and *L. donovani* .

Conclusions: The study concludes that **1)** affirmed the presence of mixed infection with *L. donovani* and *L. major* from a single cutaneous aspirate in CL patients. To the best of our knowledge this is the first report of *L. donovani* and *L. major* mixed-infection in Sudan. Additionally no other cases of such mixed-infection from the same cutaneous sample were reported elsewhere. **2)** the study assessed the circulation of more than one strain/hybrid of *L. major* and *L. donovani* between Sudanese patients. **3)** the presence of *L. infantum* as an etiological agent of VL in Sudan has been affirmed by CO II and ITS genes. **4)** *Leishmania* isolates with mixed species or strains might be subjected to selective pressure upon parasite culture, so direct extraction from biological materials is important for characterization.

These findings have important implications regarding the diagnosis, the choice of the most appropriate therapy and the possibility of developing drug resistance and at the same time set the stage for future studies as the prevalence of different species and vectors and the competence of *Phlebotomus spp*. in transmitting different *Leishmania*.

ABSTRACT

Introduzione: Negli ultimi anni Leishmaniosi si imposta all'attenzione medica come un problema sempre più rilevante. Le malattie sono associate con la povertà nei paesi in via di sviluppo e considerate importati o riemergenti in Europa. Leishmaniosi è un gruppo di malattie globalmente diffusa causata da un patogeno intracellulare del sistema immunitario targeting i macrofagi e le cellule dendritiche. Nell'uomo l'infezione si trasmette attraverso le punture dei flebotomi. I sintomi variano dalle lesioni cutanea e muco-cutanea che possono causare vasta distruzione delle membrane mucose alle malattie mortale viscerale che colpisce gli organi haemopoetici. leishmaniosi è endemica nelle regioni tropicali e subtropicali di 88 paesi, sedici sono paesi sviluppati, 72 sono in via di sviluppo, 13 di loro sono tra i meno sviluppati.

Il Sudan è conosciuto fra i più grandi focolai a livello mondiale dove Leishmaniosi rappresenta un grave problema di salute causando elevato numero di vittime. *Leishmania donovani complex* e *Leishmania major* sono noti per essere gli agenti eziologici, tuttavia, i parassiti della *Leishmania donovani comlex* che causano diverse forme cliniche, i.e viscerale (VL), cutanee (CL), muco-cutanea (ML) e post kala-azar Leishmaniosi (PKDL) sono considerati geneticamente un gruppo omogeneo.

Obiettivi: Per comprendere la correlazione tra la diversità genetica e le manifestazioni cliniche. **Obiettivi specifici: 1)** geneticamente caratterizzare isolati da pazienti Sudanesi diagnosati di diversi tipi di leishmaniosi per capire la correlazione tra diverse manifestazioni cliniche e il profilo genetico di Leishmania.**2)** Indagare sulla presenza o assenza di infezioni miste e / o ibridi. **3)** Per costruire un quadro attendibile delle varie suddivisioni filogenetici in relazione alla virulenza, utilizzando i gene Internal Transcribed Spacer (ITS) Lyshmanolysin (GP63) e il citocromo ossidasi II

Materiali e metodi: è stato utilizzato un'approccio basato sul genoma comparativo per analizzare i tre geni, citocromo ossidasi II (COII), Internal Transcribed Spacer (ITS) e il GP63 (sia gp63 3/4 che il MSPC). Sono stati utilizzati 111 campioni provenienti da 72 pazienti sudanese sospettati di diversi Leishmaniosi.In primo luogo, un real time PCR è stato sviluppato per raccogliere campioni positivi. In secondo luogo, per rilevare il polimorfismo interspecifico al livello di specie, citocromo ossidasi II (COII) gene è stato amplficato per tutti i campioni positivi nel real time PCR. Il metodo comprende l'uso di sequenziamento diretto e tecniche di clonazione. In terzo ed ultimo luogo, per rilevare il polimorfismo intraspecifico al livello del ceppo e dei sotto tipi di alcuni isolati positivi, (ITS) e il GP63 (entrambi gp63 3/4 e MSPC) geni sono stati amplificati. Il metodo comprende l'uso di tecniche di clonazione

Risultati e Discussione: 40 pazienti su 72 (55,6%) e 58 campioni su 111 (52,3%) sono state confermate dal Real Time PCR come *Leishmania* positivi. Cinque campioni cutanei provenienti da cinque pazienti sono stati esaminati, tre di loro sono stati infettati in concomitanza sia con *L.donovani* che con *L.major* come è confermato dal clonaggio del COII e ITS geni.

L' analisi dei dati ITS delle colonie correlate a *L. major* nel tre campioni cutanee ha rivelato la presenza di più di un ceppo / ibrido di *L.major* come è stato dimostrato dai siti polimorfici rilevati all'interno dei pazienti e tra di loro. Gli altri due campioni cutanei sono stati causati soltanto da *L.donovani*, ma differenti tipi di sequenza sono stati rilevati dal ITS gene.

Un paziente possedeva sequenza simile di *L. infantum* COII, il GP63 analisi di sequenza dallo stesso paziente ha confermato alcune colonie come *L. infantum*, mentre, due dei sei siti polimorfici individuati nel ITS gene erano simili a quelli caratteristici per *L.infantum* diversi zymodemi.

Il numero di campioni PKDL e ML non era sufficiente per trarre una conclusione. Tuttavia, COII e ITS dati hanno indicato *L.donovani* come l'agente causale, ITS dati ha mostrato notevoli tipi di diversi sequenze tra le colonie dei due campioni. Gli stessi risultati sono stati trovati per quanto riguarda altri campioni attribuiti al VL.

Gp63 analisi dei dati di sequenza di alcune colonie attribuite ai campioni VL (ossia 10 HBM, 3HBM e 11HBM) ha individuato due gruppi genetici all'interno della *L. donovani complex*, ma diversi da *L. infantum* e *L. donovani.*

Conclusioni: Lo studio conclude che **1)** ha affermato la presenza di infezione mista con L. donovani e L. major da un unico aspirato cutaneo. a nostra conoscenza questo è il primo rapporto di L. donovani e L. maggiore mista-infezione in Sudan. In aggiunta altri casi di tale infezione mista dallo stesso campione cutanea non sono stati riportati altrove. **2)** lo studio ha valutato la circolazione di più di un ceppo / ibrido di *L. major* e *L. donovani* tra pazienti sudanesi. **3)** la presenza di *L. infantum* come agente eziologico di VL in Sudan è stato affermato da CO II e ITS geni. **4)** *Leishmania* isolati con le specie o ceppi misti potrebbero essere sottoposti a pressione selettiva durante la cultura, l'estrazione diretta da materiali biologici è importante per la caratterizzazione.

Questi risultati hanno implicazioni importanti per quanto riguarda la diagnosi, la scelta della terapia più appropriata e la possibilità di sviluppare resistenza ai farmaci e allo stesso tempo, porre le basi per futuri studi come la prevalenza delle diverse specie e vettori e la competenza del *Phlebotomus spp*. nel trasmettere diversi *Leishmania.*

LIST OF ABBREVIATIONS AND ACRONYMS

Abbreviation	Significance
AIDS	Acquired Immuno Deficiency Syndrome
CL	Cutaneous Leishmaniasis
DALY	Disability- Adjusted Life-years
DCL	Diffused Cutaneous Leishmanias
FCS	Fetal Calf Serum
HAART	Highly Active Antiretroviral Therapy
HEPES	hydroxyl-ethyl piperazine-ethane sulfonic acid
HIV	Human immunodeficiency virus
IFN	Interferon
IL	Interleukin
LmjF	L.major strain Friedlin
LPG	Lipophosphoglycan
MCL	Muco-Cutaneous Leishmaniasis
MCL	Marcov Cluster algorithm
NO	Nitric Oxide
PBS	Phosphate Buffer Saline
PCR	Polymerase chain Reaction
SDS	Sodium Dodecyl Sulfate
SML	Sudanese Mucosal Leishmaniasis
SSG	Sodium Stibogluconate
TAMRA	tetra-methyl carboxyrhodamine
TEMED	N,N,N′,N′-Tetramethylethylenediamine
Th	T helper cells
TNF	Tumor Necrosis Factor
WHO	World Health Organization
ZCL	Zoonotic Cutaneous Leishmaniasis
ZVL	Zoonotic Visceral Leishmaniasis

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CHAPTER ONE

INTRODUCTION

INTRODUCTION

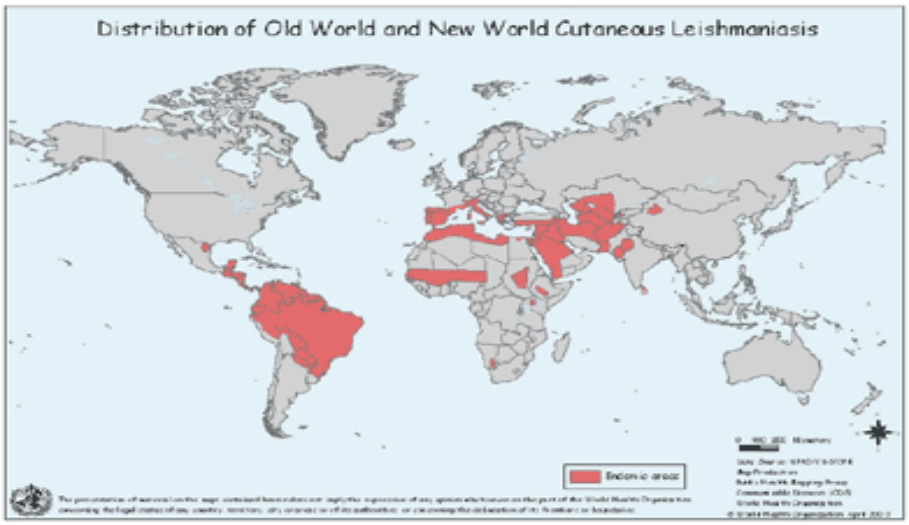
1.1 Leishmania Epidemiology

The Leishmaniases are a globally widespread group of diseases caused by obligatory, intracellular haemoflagellate protozoan parasites of the genus *Leishmania* (family *Trypanosomatidae*). The disease is transmitted generally by at least 30 species of sand flies (either *Phlebotomus* or *Lutzomyia* genera) and rarely by congenital and parenteral routes (Magill, 1995; Herwaldt, 2001). The protozoan was first discovered by William Leishman in 1901 in India, and confirmed in 1903 by Charles Donovan.

The disease is endemic in the tropical and subtropical regions of 88 countries, including 16 developed countries and 72 developing countries, 13 of them among the least developed but its public health impact remains neglected (Desjeux, 2004). Population displacements and increasing cases of *Leishmania/HIV* co-infection brought new dramatic concerns to the disease (Alvar *et al.,* 2008). Leishmaniasis affected individuals are estimated to be around 12-13 millions worldwide. Annually, 0.7-1.3 millions new cases of cutaneous leishmaniasis (CL) occur, about 95% of CL cases occur in the Americas, the Mediterranean basin, and the Middle East and Central Asia. Over two-third of CL new cases occur in six countries: Afghanistan, Algeria, Brazil, Colombia, Iran and Syria.

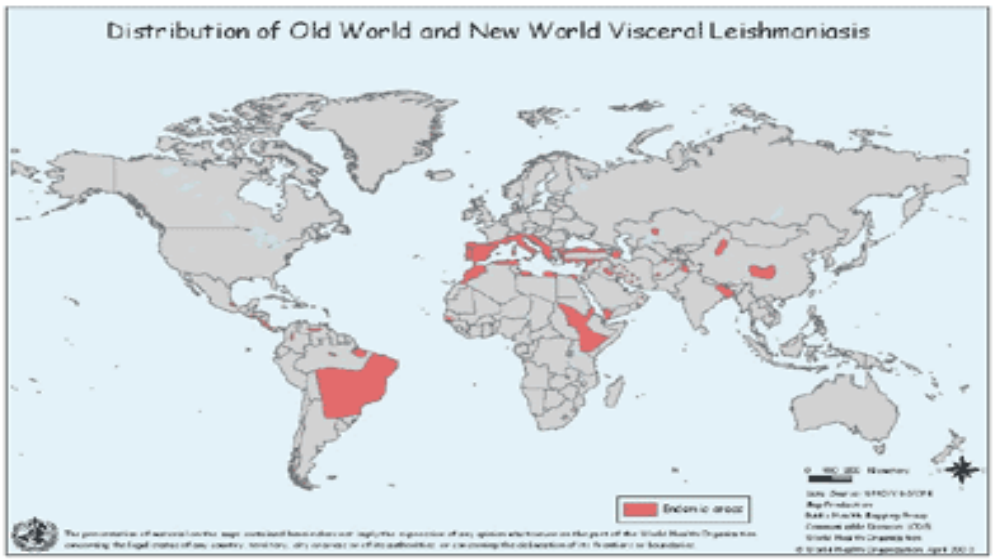
Regarding Visceral Leishmaniasis (VL), there are 200,000-400,000 new cases occurring annually, 90% of these cases are reported from six countries: Bangladesh, Brazil, Ethiopia, India, South Sudan and Sudan (WHO, 2014) and about 90% of Mucocutaneous Leishmaniasis (ML) form is reported in Bolivia, Brazil, Ethiopia and Peru (WHO, 2014). However, the real burden of leishmaniasis is greatly underestimated because substantial number of cases remains unrecorded and misdiagnosed (Guerin *et al.*, 2002; Murray *et al.*, 2005). The burden of leishmaniasis expressed in disability-adjusted life years (DALYs) is estimated to be from 946,000 to 2,357,000 for males and 1,410,000 for females (Desjeux, 2004).

Map 1: The Distribution of Old and New World Cutaneous Leishmaniasis



WHO 2003, Public Health Mapping Group. Communicable Diseases (CDS)

Map 2: Distribution of Old and New World Visceral Leishmaniasis



WHO 2003, Public Health Mapping Group. Communicable Diseases (CDS)

1.2 Diagnosis of Leishmania Infections

Correct clinical diagnosis is vital before starting treatment for prompt initiation of proper therapy and to shorten the treatment schedule. Diagnosis of *leishmania* infections is based on:

1. **Direct demonstration of the parasite on stained smears** (mainly Giemsa or *Leishmania* stain) has been considered as gold standard for diagnosis. Smears can be made from lymphoid aspiration, bone marrow aspirate, splenic aspirate (Siddig *et al.*, 1988; Zijlstra *et al.*, 1992), blood (Martinez *et al.*, 1993) cutaneous skin aspirates and skin biopsies (Ghosh *et al.*, 1995).
2. **Culture-based diagnosis** is also a tool for parasite demonstration (Lightner *et al*., 1983; Sinha *et al*., 1993). Micro-culture method (MCM) which utilized micro capillary has also been introduced for diagnosis of CL (Allahverdiyev *et al.*, 2004).
3. **Immunodiagnosis**

   (i)Detection of parasite antigen in tissue, blood, or urine samples such as latex agglutination test (KATEX) (De Colmenares *et al.*, 1995; Attar *et al*., 2001)

   (ii) Detection of non-specific or specific anti-leishmanial antibodies (immunoglobulin) (Vinayak *et al.*, 1994; De Colmenares *et al.*, 1995). Other Conventional methods for antibody detection included Gel Diffusion, Complement Fixation, Indirect Hemagglutination Test, IFA , and Countercurrent Immunoelectrophoresis Test (Haldar *et al*., 1981; Hockmeyer *et al.*, 1984; Sinha and Sehgal, 1994; Sundar and Rai, 2002). Serodiagnostic method such as (ELISA) is also widely used (Bray *et al.*, 1973; Sundar and Rai, 2002). Other widely used methods under field condition include the Direct Agglutination Test (DAT) (el Harith *et al.*, 1988) and the user-friendly Immune-Chromatographic (ICT) rk39 strip test which is used in antibodies detection against VL (rk39 antibodies are absent in CL and ML). rk39 strip test has shown to be less reliable in East Africa compared to the Indian subcontinent (Ritmeijer *et al.*, 2006; Srivastava *et al.*, 2011). However, newly developed assay based on the detection of the k28 fusion protein has been evaluated in studies performed in Sudan and Bangladesh. This method could be implemented as a first-line point-of-care test after its confirmation in large-scale field evaluations (Pattabhi *et al.*, 2010). No data has been gathered and made available thereafter.

4.**Cellular Immunity Test**

(i) Intradermal skin test: it measures delayed type hypersensitivity in reaction to intradermal injection of *Leishmania* antigen. It relies on the strong T-cell responses usually accompanying CL, ML or cured VL (Haldar *et al.*, 1983; Sundar and Rai, 2002).

(ii) Lymphocyte proliferation test: in this assay, patient's peripheral blood lymphocytes are cultured in presence of *Leishmania* antigen for five days, proliferation can be recorded either by the diagnosis of radioactive thymidine incorporation into the DNA of dividing cells, or by the measurement of the cytokine levels produced by the proliferating lymphocytes and comparisons with cytokines level from cultures where no antigen is added. This test is used in patients with ML, CL, sub-clinical or cured VL (Reed *et al.*, 1990). The application of this assay in routine diagnosis is limited.

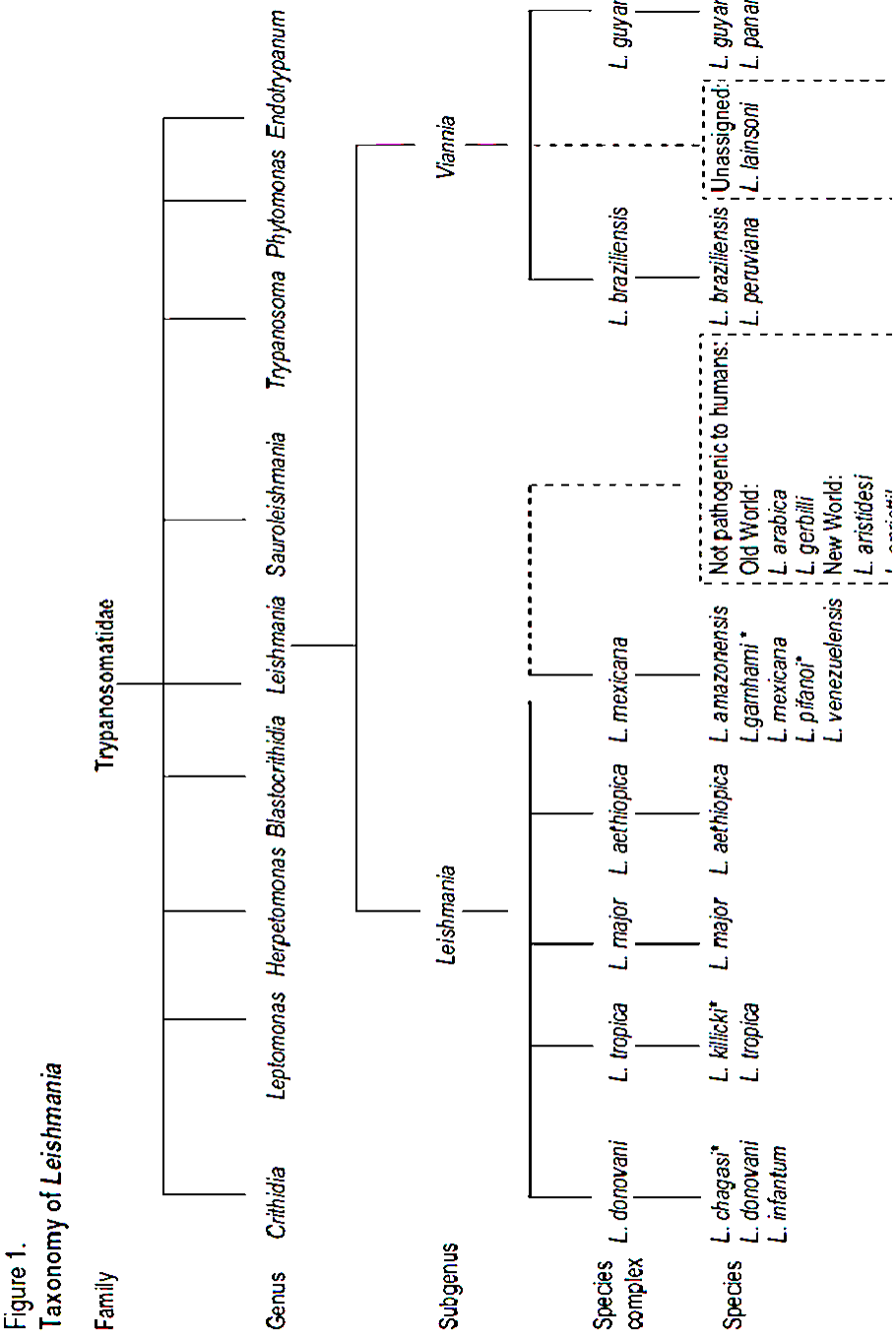
5.**Nucleic acid based detection**: PCR amplification of parasite DNA and Real-time PCR techniques are widely used for diagnosis and species identification (Andresen *et al.*, 1997; Osman *et al*., 1997; Lachaud *et al*., 2001).

1.3 Leishmania Taxonomy

Classification of *Leishmania* organism was initially based on ecobiological criteria such as geographical and epidemiological distribution, vector, tropism and clinical manefestations (Bray, 1974; Lainson *et al.*, 1987) and later on biochemical data (Arnot DE and Barker DC., 1981; Miles *et al.*, 1981) and immunological data (de Ibarra *et al.*, 1982; Wirth and Pratt, 1982). Presently, classification is based on the isoenzyme characterization (Rioux *et al.*, 1990). However, the isoenzyme classification has been questioned after the wide *Leishmania* genetic evolution. Revision of the taxonomic status of *Leishmania* species has been proposed by different researchers (Schonian *et al*., 2010; Van der Auwera *et al.*, 2011).

The genus *Leishmania* belongs to the family T*rypanosomatidae*, order *Kinetplastida*, class *Zoomastigophora*, Subphylum *Mastigophora*, Phylum *Sarcomastigophora*, Subkingdom *Protozoa*, Kingdom *Protista*. Depending on the site of parasite development within the female sandfly vector, the genus *Leishmania* is divided into two sub genera, *Leishmania* (*Leishmania*) and *Leishmania* (*vianna*) (Lainson *et al*., 1987). The two subgenera are divided into complexes and species as shown in Figure 1

Figure 1.
Taxonomy of *Leishmania*



Source: (WHO, 2010)

1.4 Leishmania Biology

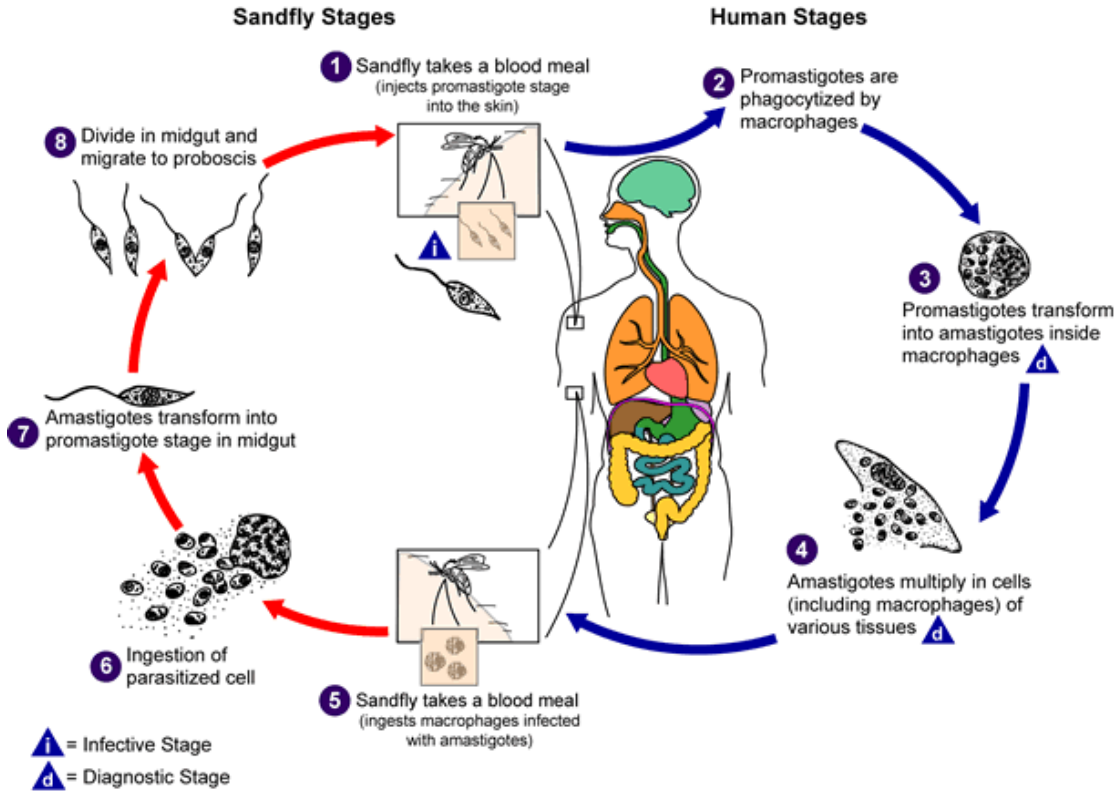
Leishmania parasite has a digenic life cycle that alternates between intracellular a mastigote in a vertebrate host and extracellular promastigote in the vector sandfly host.

The life cycle

When an infective sand fly feeds in a vertebrate host, metacyclic promastigotes enter the vertebrate host via the insect's proboscis. The promastigotes are then phagocytosed by host macrophages where they metamorphose into amastigote forms after they exposed to the acidic pH of the phagolysosomes (Chang and Dwyer, 1976; Berman *et al*., 1979); they then reproduce by binary fission and increase in number until the cell eventually bursts and then infects other phagocytic cell (Figure 2).

When female sand fly takes a blood meal from an infected vertebrate host, the ingested blood that contains amastigotes passes through the food canal to the midgut. Parasites of the subgenus *Vianna* establish an initial infection in the hindgut, where those of the subgenus *Leishmania* develop in the midgut (Lainson, 1984).

Figure 2: The Life Cycle of the Leishmania Parasite



Source: Centre of Disease Control and prevention (CDC)

Sand fly midgut cells secrete peritrophic matrix (PM) that completely envelops the fresh blood meal in protein and glycoprotein sac and at the same time protect the midgut epithelial cells from damages by blood contents (Sacks, 1997).

In the sand fly midgut, two developmental stages of promastigotes were recognized, the procyclic and the metacyclic stages (Sacks and Perkins, 1985). The procyclic attachs to the epithelial cells of the midgut via binding to the lipophosphoglycan (LPG) (Sacks and Kamhawi, 2001). Metacyclic parasites undergo changes in the LPG structure reducing the ability of binding to midgut and allow the release of the parasite and its migration to the salivary glands of the vector and thus the sand fly becomes competent to transmit the infection (Sacks *et al.*, 2000).

1.5 Leishmania Species and Clinical Manifestations

In Human, both children and adults, leishmaniasis disease is caused by nearly two-dozen distinct *Leishmania* species. It manifests itself in several diverse clinical forms including ulcerative skin lesions, destructive mucosal inflammation, and disseminated visceral infection (Kala azar) (Herwaldt, 1999; Desjeux, 2001; Desjeux, 2004). Diseases outcome are similarly diverse. Variable disease expressions have also been shown in naturally infected animals (Lainson *et al*., 2002) and, especially, experimentally infected ones (Alvar *et al.*, 2004; Murray *et al.*, 2005). Disease expression and manifestations vary depending on parasite species, endemic region, host factors and host responses (Weigle and Saravia, 1996; Murray *et al.*, 2005).

Subclinical Infection

Viscerotropic *Leishmania* species can cause asymptomatic infection (Badaro *et al.*, 1986; Follador *et al.*, 2002; Gama *et al.*, 2004; Riera *et al.*, 2004). Diverse predisposing factors have been reported and attributed to the sub-clinical infection such as host immunogenetic polymorphisms (Davies and Mazloumi Gavgani, 1999; Blackwell *et al*., 2004) and the host nutritional status (Cerf *et al.*, 1987).

Cutaneous Leishmaniasis

The causative organisms of CL are *L. tropica, L. major, L. aethiopica, L. donovani* and *L. infantum* in the Old World. In the New World CL is caused by multiple species of both the *Leishmania* subgenera *[L.(Leishmania): L. amazonensis, L. infantum, L. mexicana, L.*

venezuelensis] and the *Viannia* subgenera *[L.(Viannia): L. braziliensis, L. guyanensis, L. panamensis, L. peruviana*] (Monge-Maillo and Lopez-Velez, 2013a). CL is frequently self-healing unless secondary bacterial infection develops. Usually, it affects exposed parts of the body with incubation period that varies from 1 week to several months depending mainly on the *Leishmania* species involved and the size of the inoculum. (Grevelink and Lerner, 1996; Salman *et al.*, 1999). The fully mature lesion remains stable for several weeks before healing starts (Kubba *et al.*, 1987). Differential diagnosis should be made to exclude other diseases.

Diffused Cutaneous Leishmaniasis (DCL) is another form of the CL which is caused either by *L. aethiopica* or *L. amazonensis* (Salman *et al*., 1999). It is characterized by lack of *Leishmania*-specific cellular immune response and thus limited inflammatory reaction (Bañuls *et al.*, 2007). DCL usually starts with a primary lesion that progresses to numerous ulcerated nodules and plaques that disseminate to other skin area (Salman *et al*., 1999). Infections persisting for more than 1–2 years are regarded as chronic CL (Dowlati., 1996).

Leishmania Recidiva Cutis is a rare form associated with *L. tropica* in the Old World and *L. braziliensis* in the New World. This form of the disease refers to the development of new lesions within the scar of a healed acute lesion (Salman *et al*., 1999), incubation period ranges 1–15 years and in some cases different *Leishmania* strains from the primary eziology were found suggesting either change in the immune status of the host or an exogenous reinfection (Saravia *et al.*, 1990).

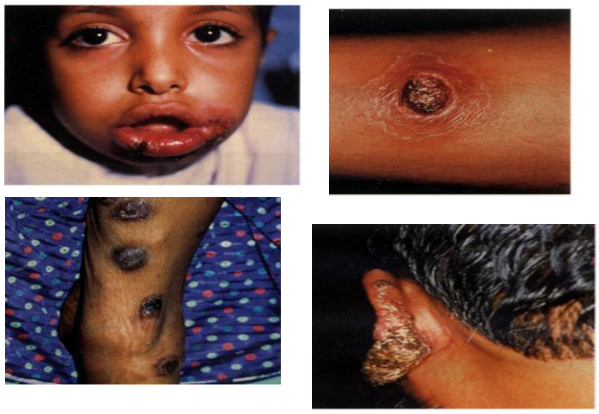


Figure 3: *CL patients*

Affected patients have a vigorous cellular immune response but low antibody titer (Salman *et al*., 1999).

Mucocutaneous Leishmaniasis

MCL involves the mucous membranes of the upper respiratory tract. It occurs when cutaneous lesions expand to the mucosal region or through metastasis. It is not self-healing and if left untreated it develops to mutilation or tissue destruction (Reithinger *et al.*, 2007; Strazzulla *et al.*, 2013). Treatment failure and relapses are common (Amato *et al.*, 2008; David and Craft, 2009). MCL it is a typical Leishmaniasis disease in the New World. Ninety per cent of the cases occur in Brazil, Bolivia, ethiopia , and Peru (WHO., 2014) It is mostly related to *Leishmania* species of the New World such as *L. braziliensis*,*L. panamensis* and *L. guyanensis* (Guerra *et al.*, 2011). Nonetheless, mucosal lesions have been reported in the Old World due to *L. donovani* (el-Hassan *et al.*, 1994; el-Hassan *et al.*, 1995; Pulimood *et al.*, 2012), to *L. major* (Alborzi *et al.*, 2013) and to *L. infantum* in immunosuppressed patients (Ehlert *et al.*, 2013).

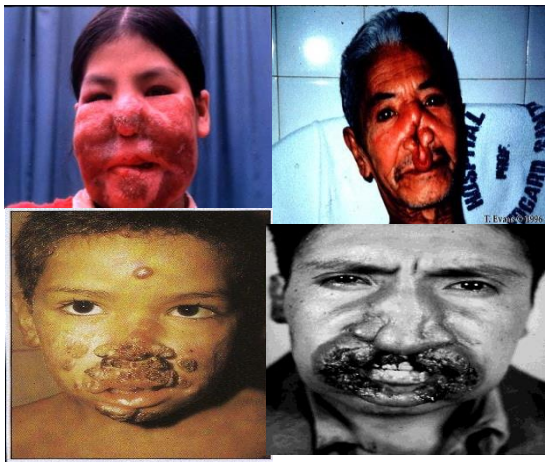


Figure 4: *ML patients*

Unlike South American ML, Sudanese Mucosal Leishmaniasis (SML) is not preceded or accompanied by a cutaneous lesion (el-Hassan *et al.*, 1995). Few sporadic cases of SML have been reported in Sudan, most of which were from endemic areas of VL. Isoenzyme characterization identified that *L. donovani zymodeme MON 18* isolated from ML patients is the same that causes VL in the western Upper Nile Province and among the Misairriya tribe in Kordofan (el-Hassan *et al.*, 1994; el-Hassan *et al.*, 1995)

Visceral Leishmaniasis

Also known in Asia as ' black fever' or 'kala-azar', VL is the most severe form of the disease. The parasite invades internal organs (spleen, liver and bone marrow) and the consequences are usually with an almost 100% mortality rate if left untreated (Desjeux, 2004). It is characterized by irregular fever, loss of weight, splenomegaly, hepatomegaly and/or lymphadenopathy and anaemia (Zijlstra and el-Hassan, 2001a). Among the 200,000 to 400,000 new cases of VL, which occur annually, 90% are concentrated in 6 countries, Bangladesh, Brazil, India, Ethiopia, South Sudan and Sudan (WHO., 2014).

Figure 5: *VL patient*

VL is caused by *L. donovani* in the Indian subcontinent and in East Africa, by *L. infantum* in the Mediterranean region and by *L. chagasi*, which is closely related or undistinguishable from *L. infantum* in the New World, mainly in Brazil, Peru and Paraguay (Berman, 1997; Herwaldt, 1999). However, *L. tropica* and *L. major* have been isolated from bone marrow cultures of patients with systemic leishmanial infection in Kenya, Iraq, the Middle East and India (Mebrahtu *et al.*, 1991; Al-Diwany *et al.*, 1995; Magill, 1995). Occasionally, *L. amazonensis* in South America can produce VL (van Griensven and Diro, 2012).

Post Kalazar Dermal Leishmaniasis

Post-kala-azar Dermal Leishmaniasis (PKDL) is a dermatosis that usually occurs in VL patients who have been successfully treated and rarely, in individuals who have no previous history of symptomatic VL (Gasim *et al.*, 2000; Zijlstra *et al.*, 2003). The disease is mainly caused by *L. donovani* in Indian sub continent and East Africa, however, few cases have been reported to be caused by *L. infantum*, *L. chagasi*, or *L. tropica.*(Sacks *et al.*, 1995; Singh *et al* ., 2011). Disease pathogenesis is thought to be immunologically mediated (Singh *et al.*, 2011).

It has been estimated that after successful treatment of VL, about 10–20% cases in India and 50–60% cases in Sudan develop PKDL (Ramesh and Mukherjee, 1995; Zijlstra and el-Hassan, 2001b). PKDL patients are thought to act as human reservoir for the parasite (El Hassan and Khalil, 2001).

PKDL usually develops within months to several year (2–7 years) after apparent cure of kala-azar.

Leishmania parasites were detected in lymph node and/or bone marrow aspirates of the PKDL cases. Clinically, the PKDL is characterized by the appearance of macules, papules or nodules in the skin especially on the face but other parts of the body may also be infected (Zijlstra and el-Hassan, 2001b). The disease may develop particularly severe and generalized skin lesions especially in children (el Hassan et al., 1992; Zijlstra et al., 2003).

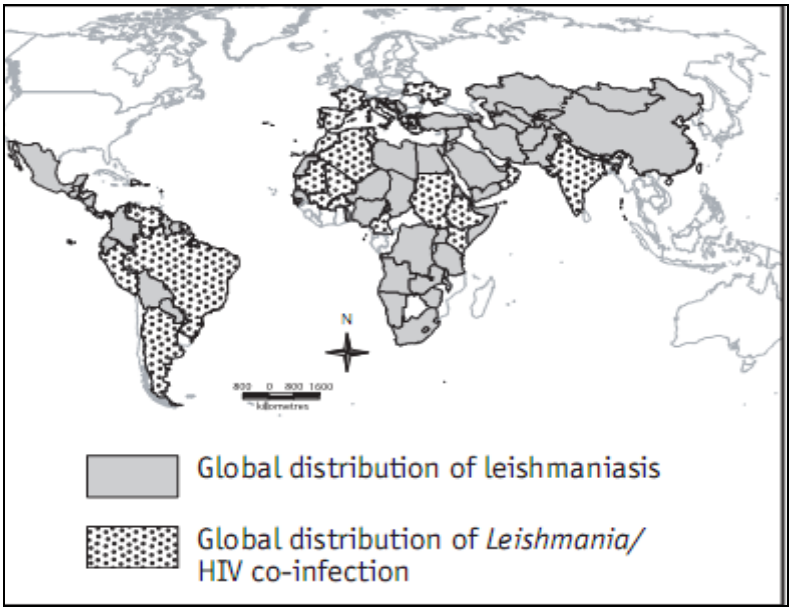
Figure 6: *PKDL patients*

Leishmania-HIV/AIDS Co-infection

The first case of leishmaniasis associated with *HIV* infection was reported in 1985, and the numbers of cases have subsequently increased rapidly in Southern Europe. Thirty-five countries have since then reported cases of co-infection. After the introduction of the HAART (Highly Active Anti-Retroviral Treatment), the numbers of co-infected cases in European countries where the disease is endemic fell sharply. However, the problem has expanded to other major foci of leishmaniasis in the world due to the increased overlap of the two diseases (Tremblay *et al*., 1996; Alvar *et al.*, 2008).

Both *Leishmania* and *HIV* disease exert a synergistic detrimental effect on the cellular immune response because they target similar immune cells (Tremblay *et al*., 1996). *HIV* increases the risk of developing VL leishmaniasis by 100 to 2,320 times in areas of endemicity, reduces the likelihood of a therapeutic response and greatly increases the probability of relapse (Pintado *et al.*, 2001; Alvar *et al.*, 2008) and at the same time VL promotes the clinical progression of *HIV* disease and the development of the AIDS- defining conditions. In South Europe, France, Italy, Portugal and Spain were mainly involved in *Leishmania HIV* co-infection, while in South Asia the co-infection was found in Bangladesh, India and Nepal. Brazil is the epicentre of the *HIV/AIDS* epidemic in South America, and in sub Saharan Africa the disease is well reported in Burkina Faso, Ethiopia, Somalia, Sudan and Kenya. (Alvar *et al.*, 2008).

Map 3: Global distribution of leishmaniasis and leishmania/HIV co-infection



Report of the Scientific Working Group on Leishmaniasis WHO, 2004

1.6 Treatment

Cutaneous disease is usually self-healing (Murray *et al.*, 2005), the treatment options are used to accelerate cure, reduce scarring especially at cosmetic sites and to prevent dissemination or relapse (Murray *et al.*, 2005). Drug efficacy is based on the *Leishmania* species involved, the geographical regions and the clinical presentations. CL treatment can follow topical or systemic regime (Monge-Maillo and Lopez-Velez, 2013a). Local therapies offer less systemic toxicity, and include thermotherapy, cryotherapy, paromomycin ointment and local infiltration with antimonials, whilst systemic treatment with azole drugs, miltefosine, antimonials or amphotericin B formulations are reserved for complex cases (Monge-Maillo and Lopez-Velez, 2013a). Combinations of local therapies and parenteral pentavalent antimonials are also common and showed to have an additional therapeutic effect (Monge-Maillo and Lopez-Velez, 2013a).

Mucosal diseases must be treated as it can produce potentially life threatening inflammatory disease. Parenteral antimony is the drug of choice. Amphotericin B can be used as rescue therapy (Murray *et al.*, 2005).

PKDL disease is not routinely treated hence the majority of cases heal spontaneously within one year. Treatment is limited to patients with severe, disfiguring or long persisting lesions and to

young children with oral lesions that interfere with their feeding. Sodium stibogluconate (SSG) and liposomal amphotericin B are the drugs of choice (Murray *et al.*, 2005).

Visceral disease expression varies from sub-clinical to fatal systemic. Active VL may also represent relapse or late reactivation of subclinical or previously treated infection (Murray *et al.*, 2005). Factors as development of drug resistance, malnourishment, geographical location and concomitant infections should be considered upon choice of the proper treatment (Monge-Maillo and Lopez-Velez, 2013b).

Since the late 1940s, the traditional treatment for VL has been the use of pentavalent antimonials. However, in the1980s, conventional amphotericin B deoxycholate was introduced, followed by lipid formulations of amphotericin B (liposomal amphotericin B) (Monge-Maillo and Lopez-Velez, 2013b), which is considered to be the drug of choice in high-income countries (Desjeux, 1996), the intramuscular Paromomycin and later, the oral miltefosine were developed as drug option for VL. In East Africa, the combination of sodium stibogluconate and paromomycin is common (Murray *et al.*, 2005).

1.7 Vectors of Leishmania

Phelobtomine sand flies are the only known natural vector of *Leishmania* (Ready, 2013), however, recently other haematophagous arthropods such as ticks (*Rhipicephalus sanguineus*) (Dantas-Torres *et al.*, 2010; Dantas-Torres, 2011) and the day-feeding midges (*Diptera: Ceratopogonidae*) (Dougall *et al.*, 2011) have been incriminated. Table 1 shows proven and suspected vectors of *Leishmania.*

Vector Taxonomy

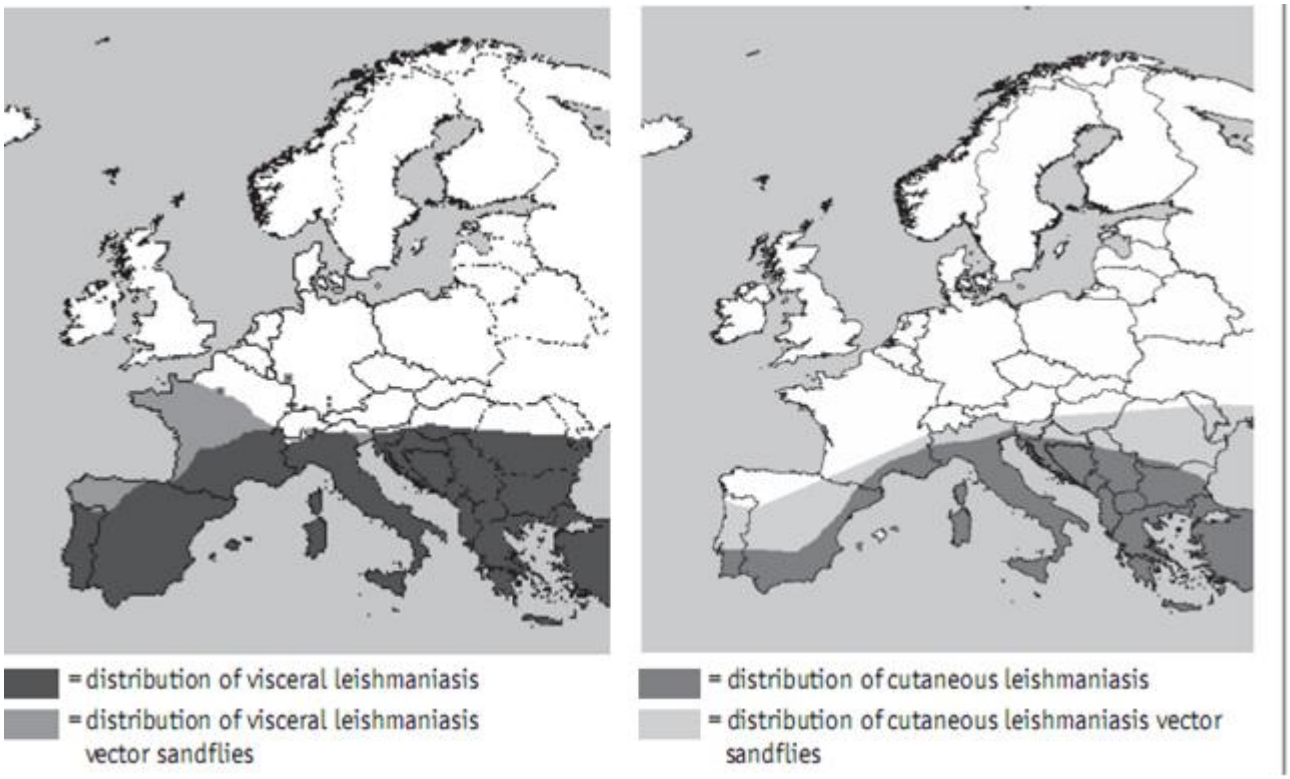
There is no universal agreement on the generic and higher classification of sand-flies. *Phlebotomus, Sergentomyia* and *Chinius* are the three Old World genera recognized and widely accepted, however, others have been named but they are of unknown medical importance (WHO, 2010). For Neotropical sand-flies, three genera, 15 subgenera and 11 species groups have been recognized for decades, but this classification has been strongly criticized because it failed in revealing the evolutionary relations between specie belonging to the genus *Lutzomyia* (WHO, 2010). Several revisions have been proposed, but none has been universally accepted (WHO, 2010). A most recent and comprehensive taxonomy classifies the 464 species of Neotropical sand-flies into 22 genera, 20 subgenera, 3 species groups and 28 series but yet it has not been fully accepted and further changes are expected (WHO, 2010).

The phelobtomine sand flies that are known as natural vector of *Leishmania* belong to the Family *Psychodidae*, Subfamily *Phlebotominae* of the genus *Phlebotomus* (Old World), and *Lutzomyia* (New World) (Killick-Kendrick, 1990). Sand-flies transmit many zoonotic diseases (*Arboviruses, Bartonelloses* and especially *Leishmaniases*) of importance to human health. Among over 800 species of sand-fly recorded, 98 are proven or suspected vectors of human Leishmaniases (42 *Phlebotomus* species in the Old World and 56 *Lutzomyia* species in the New World) (Maroli *et al.*, 2013).

Vector Distribution

Altitudinal distribution of sand-flies extends from below sea level to 3300 m above sea level in Afghanistan (Lane and Crosskey, 1993; Maroli *et al.*, 2013). Usually they are limited to areas where daytime temperature is above 15.6 C° for at least three months of the year (Lane and Crosskey, 1993) in Asia, Africa, Australia, Southern Europe and the Americas (Killick-Kendrick, 1999). Sand-fly population densities change in areas characterizes with seasonal fluctuations in temperature. They are absent from New Zealand and the Pacific islands (Lane and Crosskey, 1993). Population density reaches its peak towards the end of the rainy season in arid and semi-arid zones and reflow at the end of the dry season. Adults of some species disappear during the driest and / or coldest seasons (Sacks, 2001). Maps 4 and 5 demostrate the approximate distribution in Europe of VL and its vector compared to CL and its vector.

Map 4 and Map 5: Approximate distribution in Europe of Visceral Leishmaniasis and its vector compared to Cutaneous leishmaniasis and its vector



WHO Report of the Scientific Working Group on Leishmaniasis 2004

Vector Biology

The biology of each species of sand-fly is unique and complex and the preferences of vectors for different vertebrates vary according to species and to the availability of hosts (WHO, 2010). The time from an infecting blood-meal until a female can transmit leishmaniasis by bite is probably 1–3 weeks, although the precise period is not known for any species of *Leishmania* and undoubtedly varies according to the gonotrophic cycles of the different vector species, the ambient temperature and, perhaps, the sugars taken by the females (WHO 2010). Phlebotomine sand-flies undergo four developmental stages: egg, larva, pupa, and adult (WHO, 2010). The immature stages need high relatively moist and warm habitats. The eggs are laid by adult females in sheltered habitat rich in organic content, such as animal excreta and soil. Eggs (0.3–0.5 mm in length) are initially white or light grey in colour but often turn dark brown or black within a few hours of oviposition (Maroli *et al.*, 2013). Egg hatching is highly temperature-dependent while larval development is generally slow. First-instar larvae emerge in 12–19 days, pupae in 25–59 days, and adults in 35–69

days (Volf and Volfova, 2011). Both male and female require carbohydrate for energy, supplied by sugar secretions from plants and from honeydew (Killick-Kendrick and Killick-Kendrick, 1987; Molyneux *et al.*, 1991). With the exception of few species, female phlebotomine sand-fly requires at least one blood meal in order to complete development of egg batches. Unlike mosquitoes, their attack on the host is silent. Adults are usually dormant during the day resting in cool humid sites that vary according to species such as animal burrows, bird nests, human dwellings and caves (Killick-Kendrick, 1999; Maroli *et al.*, 2013). Adults are mainly active in the evening, at night and in the early morning, although they can bite during the day if disturbed. The flight range of adult sand-fly is very short (about 300 m) and thus adult activities are usually restricted to the vicinity of larval breeding sites. However, peridomestic species disperse at longer distances than forest one (WHO, 2010). The flight speed is less than that of mosquitoes and in the order of 1 m/s (WHO, 2010) and they are unable to fly at wind speeds above this rate, which limits their distance of dispersal (WHO, 2010).

Species-restricted Vector competence

Species-restricted vector competence comes from laboratory based studies. Vectors can be divided into specific vectors, which support the growth of only one species of *Leishmania*, and permissive vectors, which support the growth of more than one species (WHO, 2010).

Different factors determine the ability of a given sand-fly species to act as specific or permissive vectors, these factors include:

(i)The ability of the *Leishmania* parasite species to resist the digestive action of the enzymes produced in the midgut of the fly.

(ii)The lipophosphoglycan-binding sites on the inner surface of the fly gut should match the lipophosphoglycan on the surface of the promastigotes.

(iii) Successful establishment, growth, differentiation, migration and transmission of the parasite to the vertebrate host.

Despite its wide distribution, *P. papatas*is is known as specific vector of *L. major* in natural transmission, however, an experimental evidence of *P. papatasi* infection with hybrid strains of *L. infantum / L. major* causing visceral disease in *HIV*-positive patients (Volf *et al.*, 2007) has been documented. Based on these results *P. papatasi* has been suggested as vector of this hybrid in nature (Volf *et al.*, 2007)

Leishmania species	Proven or suspected vector sand fly species	Place of known or suspected transmission
L.donovani	*P. (E)argentipes*	India,Bangladesh, Nepal
	P.(P.).alexandri	West China and north Africa
	P.(La.)orientalis	Sudan and Ethiopia
	P.(S.)martini	Kenya, Ethipoia
	P.(S.)celiae	Kenya, Ethipoia
L. infantum	*P.(La) perniciosus*	
	P.(La) ariasi	Mediterranean
	P.(La)perfiliewi	
	P P.(La)neglectus.	
	alexandri	Iran and Middle East
	P.(A.).brevis	Iran
L.major	*P.(Ph.)papatasi*	Norht Africa and middle East
	P.(Ph.)duboscqi	Sahelian Africa
L.tropica	*P.(La.)acleatus*	Kenya
	P.(La.)guggisbergi	Kenya
	P.(P.)sergenti	Asia, Middle East and north Africa
L.aethiopica	*P.(La.)longipes*	Kenya, Ethipoia
	and P.(La.)pedifer	Kenya, Ethipoia
L.braziliensis	*NewWorld species*	
	Lu.(Ps.)carrerrai	Western Amazon Basin
	Lu.(Ps.)compexa Lu.(N.)intermedia	
	Lu.(Ps.)wellcomei Lu.(N.)Whitmani	Brazil
	Lu.ovallesi	Venezuela, Guatemala
L.chagasi	*Lu.(lu.)longipalpis*	Centeral America, Brazil, Venezuela
L.colombiensis	*Lu.(H.) hartmanni*	Colombia
	Lu.(N.) anduzei	Northern, South America, Brazil
L.guyanensis	*Lu.(N.) umbratilis*	Amazon Basin, Brazil, French Guiana
L.lainsoni	*Lu.(T.) ubiquitalis*	Brazil, Peru
	Lu.(D) anthophora	Texas USA
	Lu.(lu) diabolica	
L.mexicana	*Lu.(H.) ayacchensis*	Ecuador, Peru
	Lu.(N) olmeca	
	Lu.(V) ovallesi	Panama, Venezuela
L.panamensis	*Lu.(N.) trapidoi*	Central America, Colombia
L.peruviana	*Lu.(H.) peruensis*	Peru
	Lu.(V) verrucarum	
L.venezuelensis	*Lu.(N) olmeca*	Northern South America

Table 1: *Proven and suspected sand-fly vector species* (*The Biology of Leishmania - Sandfly Interaction- book. Page 20)*

1.8 The Reservoir

In zoonotic leishmaniasis the relationship between the parasite, the sand-fly, and the mammalian host(s) is complex and considered as a self-sustaining, independent system (Ashford, 1996). Ashford *et al* established three types of reservoir host:

(i)The primary reservoir hosts which is responsible for the long-term maintenance of the infectious agents and are basically mammals.

(ii) The incidental hosts which are irrelevant to the long-term persistence of the disease.

(iii)Liaison hosts which bring the infectious agents to the vicinity of man and facilitate their transmission.

Various species of animals may become infected with *Leishmania* parasites and develop lesions but their roles as reservoirs need to be determined. A reservoir host needs an intense–fly contact, long-relatively non-pathogenic course of infection and an identical genotype of the parasite as that in human (WHO, 2010).

Humans as Reservoir Hosts

The two *Leishmania* species that are thought normally to be transmitted between humans are *L. donovani* and *L. tropica,* both tend to occur in unstable epidemics and may depend on people displacements from endemic foci where most people are immune, to new foci (Ashford, 1996). PKDL infected individuals are thought to be source of infection (El Hassan and Khalil, 2001) , whilst *HIV*-coinfected patients are known to be highly infectious to sand flies, the role of asymptomatically infected individuals in the transmission cycle is currently unknown (WHO, 2010). Humans have also thought to play a reservoir role in some outbreaks caused by *L. braziliensis*, *L. guyanensis* and *L. panamensis* (WHO, 2010).

Reservoir of Zoonotic Cutaneous and Mucocutaneous Leishmaniasis

Humans play minimal role in the transmission of ZCL caused by *L. major* (Schlein and Jacobson, 1996). In arid regions the main reservoir host of *L. majoris* is the great gerbil *Rhombomysopimus spp.* (Ashford, 1996; Yaghoobi-Ershadi and Javadian, 1996), while in Middle East and North Africa, the sand fat jird (*Psammomysobesus*), *Merionesshawi, Merionessacramenti* and *Gerbilluspyramidum* are the primary reservoirs (Ashford *et al.*, 1977; Schlein *et al.*, 1984;

Morsy *et al.*, 1993; Saliba *et al.*, 1994; Fichet-Calvet *et al.*, 2003). *Merioneslibycus, M. crassus ,Mastomysnatalensis* and *Arvicanthusspp* are suspected reservoir rodents for *L. major* (Saliba and Oumeish, 1999). *Proechimysguyanensis* and *Proechimyscuvieri* are primary reservoir hosts of *L. amazonensi*, while *Oryzomys, Nectomys* and *Dasyprocta* are secondary hosts. Many species of ground rodents are involved in the transmission cycle of *L. mexicana* (WHO, 2010).

Two species of hyrax, *Procaviacapensis* and *Heterohyraxbrucei*, are the reservoir hosts of *L. aethiopica* in East Africa (WHO, 2010).

Several species of sloth are important reservoir hosts of various *Leishmania* species in New World. *Choloepusdidactylus* is a major reservoir host of *L. guyanensis* in Brazil and *L. shawi* in the Amazon region (WHO, 2010). While the *Choloepushoffmani* and *Bradypusgriseus* are primary reservoir host of *L. panamensis. B. infuscatus* has also been reported to be infected by *L. panamensis*, but its importance in the zoonotic cycle is not clear (WHO, 2010). The Lesser anteater *(Tamanduatetradactyla)* is the principal reservoir of *L. guyanensis* in Brazil. The *Opossums (Didelphismarsupialis)* is considered as a secondary reservoir host for *L. guyanensis* and *L. brazielienses* (Arias *et al.*, 1981).

Reservoir of Zoonotic Visceral Leishmaniasis

The reservoir of *L. donovani* is mainly humans (El Hassan and Khalil, 2001; Zijlstra and el-Hassan, 2001b). However, in Sudan other species are incriminated as the *Arvicanthis niloticus* (el-Hassan *et al.*, 1995).

The reservoir of *L. infantum*/*L. chagasi* is usually the dog. Naturally infected asymptomatic dogs are believed to play an important role in the transmission cycle as they are easily infective to sand-flies under experimental conditions (WHO, 2010). However, wild *Canidae* such as fox (*Vulpes spp*.), jackal (*Canisaureus*), wolf (*Canis lupus*) and the raccoon dog (*Nyctereutes procyonoides*) have been found to be infected with *L. infantum* in both Old World and New World, but their role as reservoir has not been fully established (WHO, 2010). Domestic cat (*Felis catus*) may act as secondary reservoir for *L .infantum /chagasi* (Poli *et al.*, 2002; Savani *et al.*, 2004).

1.9 Immunopathology of Leishmania

In humans, different patterns of immunological response are observed according to the clinical manifestation and exposure to different *Leishmania* species. Nevertheless, how different *Leishmania spp* cause human diseases and why the clinical symptoms are so variable remain enigmatic (Bañuls *et al*., 2007).

The majority of *Leishmania* species are adapted to a large range of hosts. In human and other less adapted animals *Leishmania* infections can produce a wide range of diversified

pathologies ranging from asymptomatic carriers to benign cutaneous lesions to more serious life threatening disease such as the visceral form. In well-adapted animals infections remain asymptomatic (Lainson *et al.*, 1987). However, the rate of asymptomatic carriers is not accurately known, but it has been suggested that it is higher than expected (Sharma *et al.*, 2000; Gama *et al.*, 2004; Riera *et al.*, 2004).

1.9.1 Immune Response of Leishmaniasis Patients

In asymptomatic individuals, the natural immune response can eliminate or control the parasites. Macrophages are key cells in the host immune defense, they are the first host cells to contact and be parasitized by *Leishmania* (Basu and Ray, 2005). Together with dendritic cells, macrophages present the parasite antigens to T cell receptors via the major histocompatibility complex (MHC) molecules (Zinkernagel and Hengartner, 2001) forming acquired immune response which could be influenced by the cytokine context and the *Leishmania* peptides presented (Bañuls *et al*.,2007).

During infection, *Leishmania* parasites resist the T cell mediated immune responses through complex and various strategies and mechanisms (Antoine *et al.*, 2004). In humans, different patterns of immunological response are observed according to the clinical manifestation and exposure to different *Leishmania* species. As example: Th1 response is present in patients with self-healing cutaneous lesions and absent in those with diffuse cutaneous Leishmaniasis (Kemp *et al.*, 1994; Ajdary *et al.*, 2000), while in patients with Mucocutaneous Leishmaniasis a mixed Th1/Th2 response with high interferon-γ (IFN-γ) levels were present (Carvalho *et al.*, 1985; Bacellar *et al.*, 2002). In VL mixed Th1/Th2 response is observed with production of IFN-γ with interleukin-10 (IL-10)(Ghalib *et al.*, 1993) and in individuals with asymptomatic or subclinical infections of visceralizing species of *Leishmania*, peripheral blood mono nuclear cell (PBMC) proliferation and production of IL-2, IFN-g and IL-12 were observed.

In cured patients, both Th1 and Th2 clones producing IFN-γ and IL-4 have been isolated (Kemp *et al.*, 1993).

1.9.2 Factors Involved in Pathogenicity and Virulence

Parasite factors

Although *Leishmania* virulence may be modulated by the environmental and genetic factors of their mammalian hosts and sand fly vectors, molecular determinants of parasite remains the key elements hence there is no Leishmaniasis without intact living *Leishmania* parasite (Chang and McGwire, 2002).

Chang et al (2002) identified two different groups of parasite molecules that thought to be involved in leishmanial mechanisms of virulence, these are:

(i) Invasive/Evasive determinants:

Infection-related parasite cell surface and secreted molecules allow to establish successful intracellular parasitism in phagolysosomes or parasitophorous vacuoles of macrophages (Chang and McGwire, 2002). They enhance evasion of humoral factors, attachment of parasites to macrophages followed by the intracellular entry into phagocytes, the intracellular survival of the endocytized parasites, differentiation of promastigotes to amastigote and amastigote replication (Chang and McGwire, 2002).They protect not only the parasite its self, but also the infected host cell from premature cytolysis. These determinants are crucial for infection but they produce no pathology of the host (Chang and McGwire, 2002). Invasive/evasive determinants include among others phosphoglycans (PG), lipophosphoglycans (LPG), glycosylphosphatidylinositol (GP1), proteophosphoglycan (PPG), Leishmanolysin (GP63) and cysteine protease (CPs) (Chang and McGwire, 2002).

(ii) Pathoantigens

Are all molecules that are capable of inducing host immunopathology as the principal cause of clinical symptoms, are conserved structural or soluble cytoplasmic proteins which are often complexed with other molecules to form sub-cellular particles (Chang and McGwire, 2002). They produce high antibodies titers and include among others Cytoskeleton (as kinesin and tubulins), ribosomes (PO, P2a and b), nucleosomes (as H2A/B, H3 and H4) and glycosomes such as TPI. The molecules listed have been found to contain immunogenic B-cell pitopes (Chang and McGwire, 2002).

Host Factors

Host genetic variation and susceptibility to Leishmania infection:

Evidence from animal models and humans indicates that host factors play an important role in determining both disease severity and resolution (Cabello *et al.*, 1995; Mohamed *et al.*, 2003). Despite the differences between the human disease and the mouse models, much of the knowledge gained from studies in mice has found to be applicable to human *Leishmaniasis* (Sakthianandeswaren *et al.*, 2009). Candidate genes identified in mice as important in host response to infection, showed to be useful in identifying genetic polymorphisms involved in the human response to Leishmaniasis (Sakthianandeswaren *et al.*, 2009). Researches using candidate gene association analysis and genome-wide linkage studies in selected families from Sudan, Brazil and

India, identified number of genes/regions related not only to environmental risk factors but also to genes responsible for determining type 1vs type 2 cellular immune responses (Blackwell *et al.*, 2009). Moreover, VL development has been demonstrated to be associated with two loci on chromosomes 22q12 and 2q23-q24 among Aringa ethnic group in Sudan (Bucheton *et al.*, 2003), also IL-2 receptor b (IL2RB) was identified as the candidate gene for the 22q12 locus (Bucheton *et al.*, 2007).

In Iranian CL patients, it has been suggested that the risk of developing CL could be influenced by functional genetic variants in the IL-4 promoter while the polymorphism in the first intron of the IFN-gamma gene might influence the progression of disease towards Chronic CL (Kamali-Sarvestani *et al.*, 2006). Similar role of L-10 has been found in humans infected with *L. braziliensis* (Salhi *et al.*, 2008).

Vector Factors

Sand-fly saliva consists of roughly thirty different salivary proteins, some are linked to blood feeding facilitation and several have been shown to be immunogenic (Gomes and Oliveira, 2012). The saliva modulates both the innate and the acquired host immune responses (Rohousova and Volf, 2006) and induce positive macrophage chemotaxis (Zer *et al.*, 2001)

Hosts including humans repeatedly exposed to uninfected sand fly bites develop antibodies against the sand fly saliva as well as cellular response (Barral *et al.*, 2000; Rohousova *et al.*, 2005; Gomes and Oliveira 2012). High levels of anti-saliva IgG, specific to the local sand fly species has been documented in people living in endemic areas of Leishmaniasis in Brazil and Turkey. However, the frequency and intensity of salivary proteins recognitions varied among individuals (Rohousova *et al.*, 2005). The presence of antibodies to salivary proteins from sand fly vectors of VL has been correlated with protection in humans (Gomes *et al.*, 2008; Aquino *et al.*, 2010) and in dogs (Vlkova *et al.*, 2011).

Two sand fly species have been studied for their salivary immunomodulatory properties, the *Lutzomyia longipalpis* and *Phlebotomus papatasi* (Rohousova and Volf, 2006). Studies demonstrated that these two genera could stimulate common as well as specific host immune responses (Rohousova and Volf, 2006). *L. longipalpis* saliva / maxadilan (vasodilator peptide) modulates the secretion of various cytokines and alters the expression of co-stimulatory molecules. In human it decreases the secretion of TNF-α and IL-10 and increases the levels of IL-6, IL-8, and IL-12p40 (Rogers and Titus, 2003; Costa *et al.*, 2004). Changes in the expression of co-stimulatory molecules can alter the T-cell activation and response (Qureshi *et al.*, 1996; Costa *et al.*, 2004), however, the intensity of the stimulatory and inhibitory effects varies depending on the individuals (Costa *et al.*, 2004). Similarly to *L. longipalpis*, the saliva of *P. papatasi* has an inhibitory effect on

lymphocyte proliferation. It suppresses early production of IL-2, IL-4, and IFN-γ (Rohousova and Volf, 2006) and increases production of IL-6 (Rogers and Titus, 2003) and differently from *L. longipalpis, P. papatasi* saliva shows three important species-specific modulations: it has no effect on TNF-α production by LPS-stimulated human monocytes (Rogers and Titus, 2003), it down-regulates the expression of inducible NO synthase (iNOS) and subsequent production of NO in LPS- or IFN-γ-activated murine macrophages and thirdly, it up-regulates the expression of the Th2 cytokine IL-4 (Mbow *et al.*, 1998),thus preventing the development of inflammatory response.

PART TWO

Molecular Genetic approach in Leishmania

2.1 Leishmania Genome

The *Leishmania* genome is composed of the nuclear genome and the kDNA, which is the mitochondrial DNA of the *Kinetoplastida*. kDNA constitutes 10–20% of the total DNA of *Leishmania* and can be divided into two classes: the heterogeneous minicircles and the homogenous maxicircles. The minicircles have been shown to encode guide RNA (gRNA) molecules involved in the RNA editing of maxicircle cryptogenes (Simpson, 1987; Sturm and Simpson, 1990).

The *L.major* nuclear genome which sequenced in 2005 (Ivens *et al.*, 2005), has provided a framework for future comparative genomic studies of other two *Leishmania* species, *L.braziliensis* and *L.infantum*, in 2007 (Peacock *et al.*, 2007). Comparative genomic studies of these three species identified more than 8,300 protein-coding genes and 900 RNA genes (Peacock *et al.*, 2007). Many genes remain unclassified (approximately 69%), with 40% of these being potentially *Leishmania*- (or *kinetoplastid*) specific (Myler and Stuart, 2000). Summary of the three *Leishmania* species genomes shown in table 2

Leishmania conserves about 6,200 trypanosomatid genes and has more than 1,000 *Leishmania*-specific genes, most of them uncharacterized (Peacock *et al.*, 2007). Unlike *Trypanosomes, Leishmania* chromosomes have not extended subtelomeric regions containing species-specific genes (Peacock *et al.*, 2007).

2.2 Gene Organization and Genome Content

Genome plasticity, variation in chromosomal content and of chromosome size even between homologous chromosomes is characteristic for each *Leishmania* species (Blaineau *et al.*, 1991; Sterkers *et al.*, 2012).

Leishmania is considered to be a diploid organism (Iovannisci *et al.*, 1984), however, evidences suggest that all *Leishmania* species are naturally aneuploidy (Rogers *et al.*, 2011; Mannaert *et al.*, 2012). Aneuploidy is often associated with drug pressure and with parasite adaptability to the host environment (Mannaert *et al.*, 2012). Drug pressure due to the treatment of

infected patients between whom the parasites are transmitted may be the cause of the extreme chromosome copy number variations in *L. donovani* (Mannaert *et al.*, 2012).

Genes are organized into directional clusters, which are either polycistrons (PGCs) or groups of genes sharing uncharacterized regulatory elements (Peacock *et al.*, 2007). Old World *Leishmania* species *(L. donovani/infantum, L. major* and *L. tarentolae*) have 36 chromosomes. Due to unique chromosome fusion events, *L. braziliensis* has 35 chromosomes *and L. mexicana* has only 34 chromosomes (Table 2).

	L. major	***L. infantum***	***L. braziliensis***
Chromosome number	36	36	35
Size (bp)	32,816,678	32,134,935	32,005,207
Contigs	36	562	1,041
Overall G+C content (%)	59,7	59,30	57.76
Coding genes	8,298	8,154	8,153
Pseudogenes	97	41	161
Coding G+C content (%)	62,5	62,45	60,38

Table 2: *Summary of the L. major, L. infantum and L. braziliensis genomes (Peacock et al., 2007).*

2.3 Genes and Leishmania Parasite Tropism and Virulence

The comparative genomic studies of the three *leishmania* species (*L.major*, *L.braziliensis* and *L.infantum*) revealed that only few species-specific parasite genes are important in pathogenesis and that parasite gene expression levels differ considerably between species This may be attributed to the variation in gene copy number (Peacock *et al.*, 2007). Beside the two groups of parasite molecules that are thought to be involved in Leishmanial mechanisms of virulence, i.e the invasive/ evasive determinant and the pathoantigens (Chang *et al* 2002) which have been mentioned previously in section 1.9.2, other genes and loci associated with clinical polymorphism but not directly involved in virulence or pathogenicity were explored and known as the Indirect Genetic Markers (Bañuls *et al.,* 2007). Few of the Indirect Genetic Markers were found to be associated with clinical phenotypes at the intraspecific level, however, they are able to distinguish the different species and reveal intraspecific polymorphism (Bañuls *et a.,l* 2007).

Gene Families

Trible "Marcov Cluster algorithm (MCL) of the *L.major* strain Friedlin (LmjF) predicted protein sequences "which is an algorithm used to assignment of proteins into families basing on pre-computed sequences similarity information" (Enright, *et al.,* 2002) revealed 662 gene families

containing 3,083 sequence (Ivens *et al.*, 2005). Smaller gene families consist of tandem arrays of one to three genes including large number of gene duplications particularly for ribosomal proteins and translation initiation factors. Members of the large gene families may be found in tandem arrays as well as dispersed throughout the genome. The largest protein families include among others 198 kinesin-related protiens, 60 protien kinases, 46 amastins, 32 GP64 and 29 RNA helicases (Myler P J, Fasel N, 2008).

RNA Genes

In LmjF genome 1,000 genes encoding structural RNAs were identified. The presence of approximately 24 copies of the rRNA unit per diploid genome in LmjF has been suggested (Martinez-Calvillo *et al.*, 2001). There is a tandem array of 18S, 5.8S and 28S ribosomal RNA genes on chromosome 27 while the 5.8S rRNA are found as dispersed single copies in different loci on different chromosomes. LmjF genome contains 83 tRNA genes and six snRNAs. The rRNA genes of *L. donovani* are organized on chromosome 27 as tandem repeats (Yan *et al.*, 1999).

Repetitive Sequences

Unlike other *trypanosomatids*, *Leishmania* genome contains relatively little repetitive sequences (< 10%) composed of tandem arrays of protein-coding and RNA genes (Myler P J, Fasel N, 2008).

Species- Specific Differences

Genome wide comparison studies of the three *Leishmania* species *(L.major, L.braziliensis* and *L.infantum*) revealed that sequence preservation between species is high (Peacock *et al.*, 2007). Only few species-specific parasite genes are found, 5 in *L.major*, 26 in *L.infantum* and 47 in *L.braziliensis.* Another 32, 6 and 98 genes respectively are absent from *L.major, L.infantum* and *L.braziliensis*, in the last case species- specific differences attributable to pseudogene formation (Myler P J, Fasel N, 2008).

Leishmaniasis in Sudan

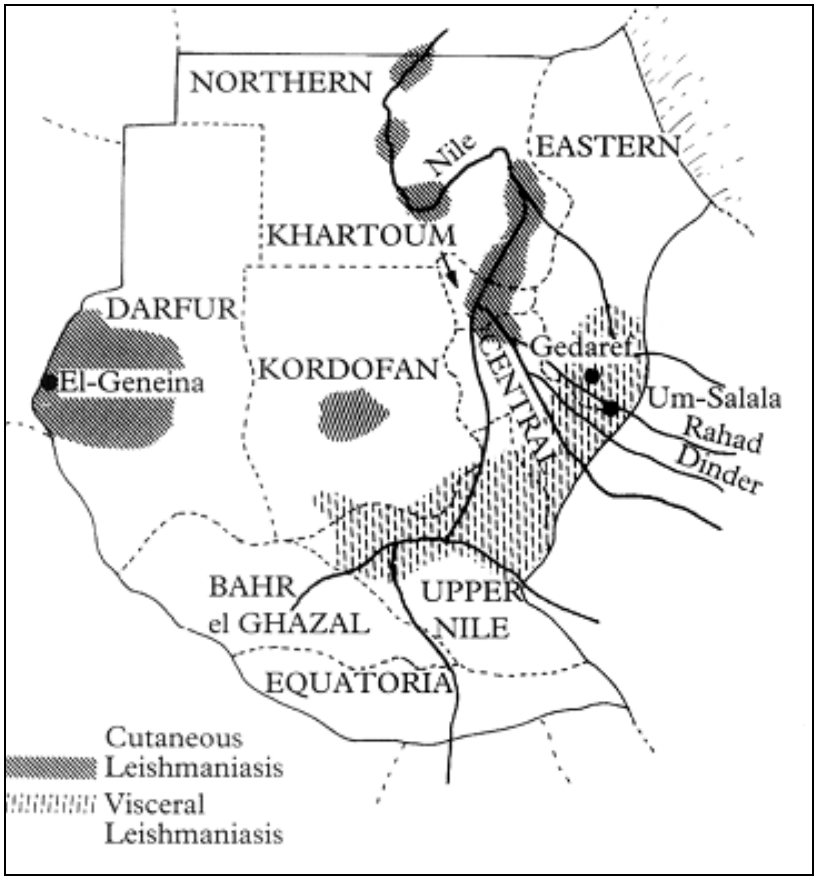
3.1 Introduction

Sudan is known as one of the most endemic areas of leishmaniasis in the world. The disease represents a serious health problem and outbreaks occur periodically causing high number of victims (Zijlstra *et al.*, 1994; Osman *et al.*, 2000; Khalil *et al.*, 2008). Worth to mention that Sudan has splitted into two independent states which are the Republic of Sudan (RoS) and the Republic of South Sudan (RoSS). Leishmaniasis is endemic in both states. The RoSS witnesses ongoing VL outbreak.

Many different clinical forms of leishmaniasis co-exist. The most serious VL is caused by *Leishmania donovani complex* and transmitted by *Phlebotomus orientalis* (Elnaiem *et al.*, 1998a; Zijlstra and el-Hassan, 2001a). VL is endemic in the Eastern part of the country (el-Hassan *et al.*, 1995; Khalil *et al.*, 2002) (map 9), however scattered cases have been reported from areas not known to be endemic in the Southern, Northern and Western parts (Osman *et al.*, 2000). Both anthroponotic and zoonotic transmission are thought to occur (Zijlstra *et al.*, 1994; Zijlstra, el-Hassan, Ismael, 1995; Elnaiem *et al.*, 2001; Dereure *et al.*, 2003; Hassan *et al.*, 2004).

CL in Sudan is caused by *L.major* and transmitted by *P.papatasi.* However, CL due to *L. donovani* has been well documented (Elamin *et al.*, 2008). The disease is endemic in the central and northern part (el-Safi and Peters, 1991; el-Hassan and Zijlstra, 2001) (map 10). Generally, the CL in Sudan is believed to be zoonotic as in other endemic areas in the world where the CL is due to *L. major.* The Nile rat *Arvicanthis niloticus* has been suggested as reservoir (Salah Eldin., 1997). Knowledge on the sandflies fauna and the animal reservoir host of CL due to *L.donovani* is still lacking.

Map 6: *Distribution of visceral and cutaneous leishmaniasis in Sudan and South Sudan (Zijlstra and el-Hassan, 2001)*



Map 7: *Distribution of CL due to L.major in Sudan: (el-Hassan and Zijlstra, 2001)*

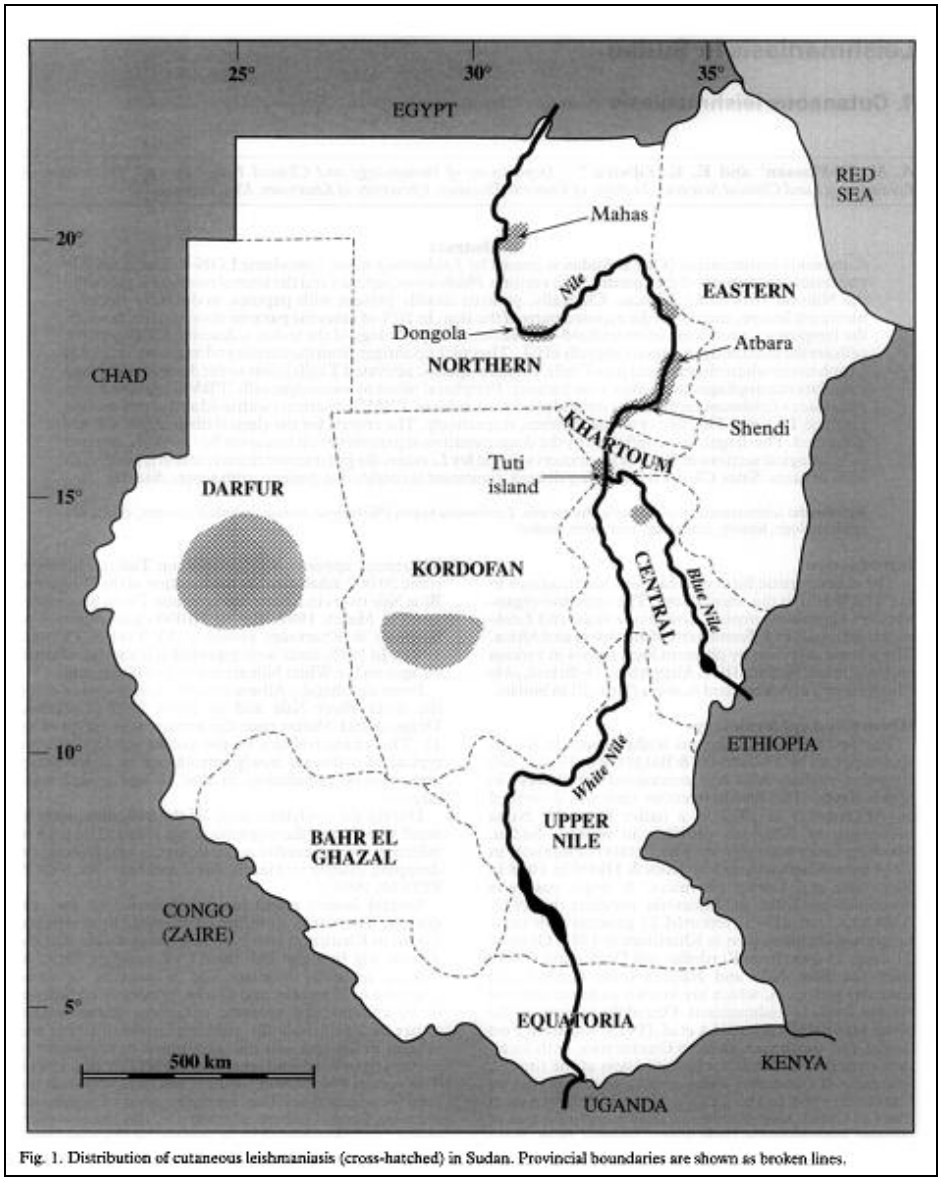
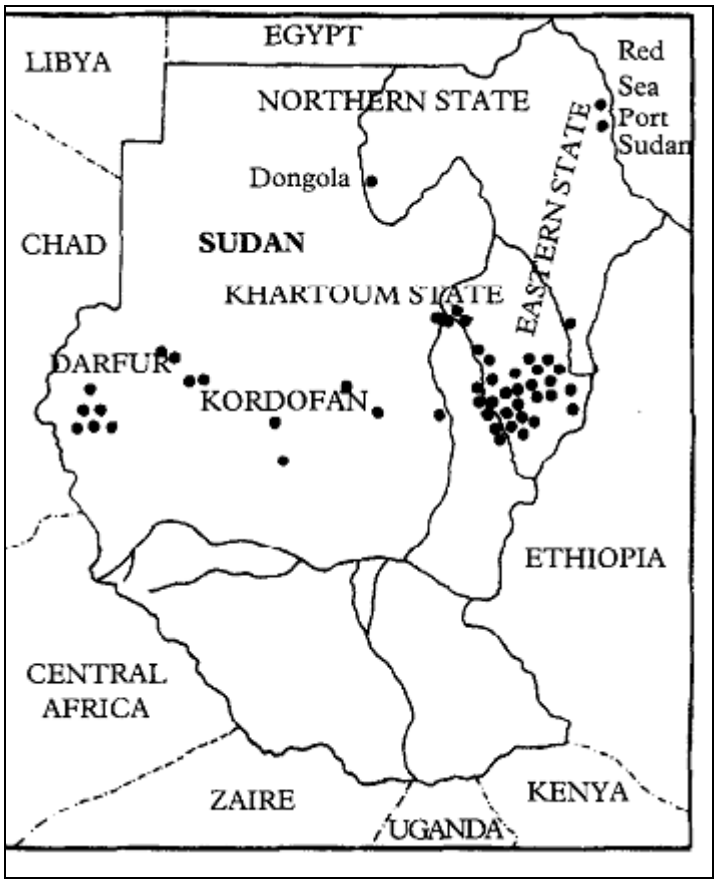


Fig. 1. Distribution of cutaneous leishmaniasis (cross-hatched) in Sudan. Provincial boundaries are shown as broken lines.

PKDL occurs in high rate during or shortly after treatment. At least 50% of VL patients develop PKDL, and this percentage is higher than in any other VL endemic area (WHO, 2010) (Zijlstra *et al.*, 1995; Zijlstra *et al.*, 2003). PKDL patients are thought to act as human reservoir for the parasite (El Hassan and Khalil, 2001).

Sudanese Mucosal Leishmaniasis (SML) is a rare and particular form of ML. Unlike ML, SML starts usually as primary mucosal disease, without being preceded or accompanied by cutaneous lesions. SML occurs in areas endemic for VL (el-Hassan *et al.*, 1995). Few sporadic cases of ML have been reported in Sudan (map 11). Most of these cases were from endemic areas of VL. Isoenzyme characterization identified that *L.donovani* zymodeme MON 18 isolated from ML patients is the same that causes VL in the Western Upper Nile Province and among the Misairriya tribe in Kordofan (el-Hassan *et al.*, 1994; el-Hassan *et al.*, 1995).

Map 8: *Distribution of known cases of mucosal leishmaniasis in Sudan (el-Hassan et al., 1995).*



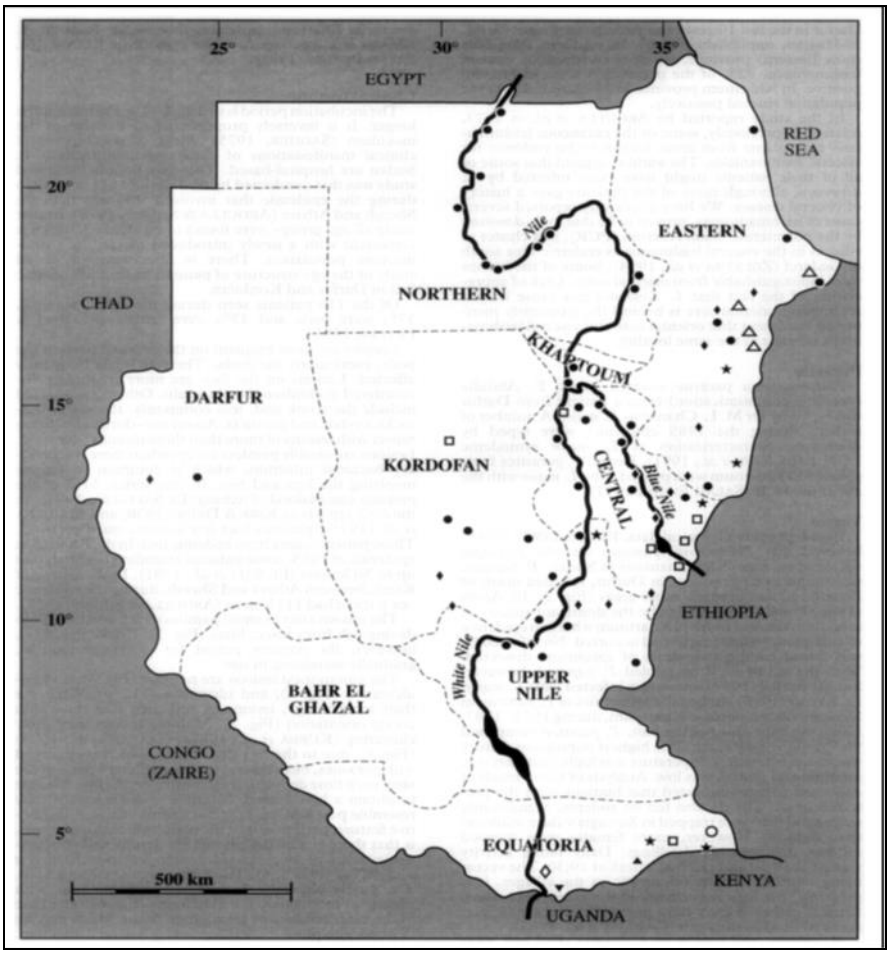
Leishmania/*HIV* co-infection is a growing concern particularly in the East (Alvar *et al.*, 2008). The first case of AIDS was reported in 1986, and in December 2006 the adult national prevalence rate was 1.6 %, indicating that Sudan is in a generalized epidemic (Alvar *et al.*, 2008). The transmission is mainly heterosexual, however vertical transmission from mother to child also play a role.

The most affected areas are Khartoum and the Eastern and Southern states, likely due to population movements (refugees and internally displaced populations) (Alvar et al., 2008).

3.2 Sand Flies in Sudan and South Sudan

Nine *Phlebotomus* species and 37 *Sergentomyia* species have been identified (map 12). *Phlebotomus* sandflies in the Sudan include *P. orientalis, P. papatasi, P. martini, P. rodhaini, P. pedifer, P. alexandri, P. saevus, P. bergeroti* and *P. duboscqi* (Elnaiem, Hassan, Ward, 1997; Lambert *et al.*, 2002).

Map 9: *Sand Flies in Sudan and South Sudan (el-Hassan and Zijlstra, 2001)*



Out of these species only two are thought to play a major role in disease transmission in Sudan, *P. orientalis*, the proven vector of VL (Elnaiem *et al.*, 1998b; Jamjoom *et al.*, 2004), and *P. papatasi*, the proven vector of CL (Desjeux P, 1991; El-Sayed S M. 1990). *P. martini* has been incriminated as a vector of VL in Southern Sudan (Ashford *et al.*, 1992). *P. rodhaini* is not known as a vector, but it was found infected with *L. donovani* in woodland of Dindir National Park. Later, this sandfly has been found in the Northern part of Sudan (Hassan *et al.*, 2007) and was thought to play a role in maintaining the zoonotic cycle among reservoirs (Hassan *et al.*, 2007).

P. orientalis and *P. papatasi* are known as seasonal species (Hoogstraal *et al.*,1962; Hoogstraal and Dietlein., 1964). The two species start to appear immediately after the rainy season (late October) and disappear during the rainy season (July-October) (Elnaiem *et al.*, 1998b; Elnaiem *et al.*, 2003). *P. orientalis* is mainly associated with *Acacia seyal / Balanites aegyptiaca*, vegetation that grows on black cracking clay soil in Eastern and Southern Sudan (Elnaiem *et al.*, 1998b). Unexpectedly, it was found in semi-arid areas in Northern Sudan (Hassan *et al.*, 2007). Its distribution is influenced by certain climatic and ecological determinants (Elnaiem *et al.*, 1998b). *P. orientalis* was found to be attracted more to dog baited traps than Mongoose, Genet and Nile rat (Hassan *et al.*, 2009). In endemic areas in the Eastern Sudan, in-village transmission is highly suggested because infected *P. orientalis* were reported from these villages (Hassan *et al.*, 2004).

P.papatasi occupies arid areas of semi-desert, desert and Savannah region. It was collected up to the north. *P. papatasi* is collected from woodland of Dindir National Park, 45 km from a nearest village. Also, it was collected from Savannah area in the south and eastern Sudan (El-Sayed S M. 1990).

3.3 The Leishmania Reservoir in Sudan

The Nile rat *Arvicanthis niloticus* is the probable reservoir of CL (el-Hassan and Zijlstra, 2001), while rodents and dogs were initially selected as the most probable reservoir hosts of a zoonotic *L. donovani* cycle (Elnaiem *et al.*, 2001; Hassan *et al.*, 2009). Rodents were particularly suspected, as they are the most abundant and widely dispersed animals of the local mammal fauna (Hoogstraal and Dietlein.,1964). Few further studies and researches have been conducted to update the state, but incrimination of rodents remains inconclusive.

Infected carnivores were found, i.e. the Senegal genet (*Genetta g. senegalensis*) and the Sudanese serval (*Felis serval phillipsi*) of the cat family (*Felidae*) (Hoogstraal and Dietlein., 1964). The wild jackal *Canis adustus* was the most common wild carnivore in the area, but these animals were not found infected. Infection of wild carnivores may have been acquired by consuming infected rodents or through bites of sand flies (Hoogstraal and Dietlein., 1964).

Domestic dogs are abundant, Dereure *et al* suggested that dog may play the role of the reservoir of VL in Sudan or it may acts as a bridge host between a possible sylvatic cycle and the anthroponotic cycle (Dereure *et al.*, 2000; Dereure *et al.*, 2003). More extensive studies are needed to assess the transmission cycle of VL in Sudan.

The zoonotic transmission of both CL and VL remain inconclusive, however different animals were suggested to act as reservoir such as the Nile rat (*Arvicanthis niloticus*), the common african rat (*Mastomys natalensis),* the egyptian mongoose (*Herpestes ichneumon*), the domestic dog (*Canis familiaris*), the genet (*Genetta genetta*), the jackal and serval cat (Hoogstraal and Dietlein., 1964; Elnaiem *et al.*, 2001; Hassan *et al.*, 2009).

3.4 Control, Diagnosis and Treatment of Leishmaniasis in Sudan

Since 2001, the leishmaniasis control program has been merged with the *Malaria* and *Schistosomiasis* control program under the National Malaria, Schistosomiasis and Leishmaniasis administration within the Division of the Endemic Disease Control created by the Federal Ministry of Health. The organization *Médecins Sans Frontières* Holland (MSF-H) has been involved in the VL control program since its first response to an outbreak in Gedarif state in eastern Sudan in 1996. From 2001 to 2004, MSF–H supported five hospitals in Gedarif state and two hospitals in Sennar State to improve diagnosis and treatment and to introduce a surveillance system for HIV/VL co-infection. In 2010 *Médecins Sans Frontières* Suisse (MSF CH) has started to support Tabarak Allah hospital in Gedarif state through diagnosis, treatment and medical follow up (WHO, 2010).

The national diagnosis guidelines recommend the use of rK39 and DAT for VL diagnosis in the field. Parasitological confirmation via a lymph node or bone marrow aspirate is recommended. MSF CH uses the rK39 and DAT for diagnosis of primary VL, and lymph node aspirates in cases of relapse and for test of cure (WHO, 2010).

The first line treatment of VL is the SSG for 30 days or Glucantime (meglumine antimoniate) for 21 days. Second line treatment is AmBisome (liposomal amphotericin B) (WHO, 2010). Combination therapy of SSG/ paromomycin has also been used as an alternative second line treatment (WHO, 2010). Amphotericin B is not recommended due to possible side effects while Miltefosine is planned to be included (WHO, 2010). SSG is used for treatment of long persisting PKDL (more than 6 months) or sever cases (WHO, 2010).

3.5 Rationale of the Study

The genus *Leishmania* includes many pathogenic species which have the ability to adapt and survive in very diverse environments encountering strong selection pressures (biotic as well as environmental) and infecting a large range of mammalian hosts and sand fly species (Bañuls et al., 2007). *Leishmania* species revealed a high degree of genetic diversity resulting in different phenotypes and pathogenic potentiality (Cupolillo *et a*l., 1998; Herwaldt, 1999; Cunningham, 2002). Paradoxically, different types of diseases are sometimes caused by the same *Leishmania* species group while the same phenotype of the disease may be caused by different *Leishmania* species. The mechanisms by which *Leishmania* parasite cause different pathologies are largely unknown.

Experimental studies demonstrated that different strains of different *Leishmania* species are attributable to differences in disease expression and immune responses (Bañuls et al., 2007) and that differential degrees of polymorphism exist within a single species among geographical location or host (vector or reservoir) (Bañuls *et al*., 2007). Moreover, the considerable overlap in clinical manifestations and the uncommon disease phenotypes (Elamin et al., 2005), the mixed infection with different strains or species of *Leishmania* and its impact on disease diagnosis, prognosis and the consequent drug resistance (Antoniou *et al*., 2004) highlight the need to unravel the genetic diversity at the inter- and intra-specific level and strain typing. In parasitic diseases, strain typing becomes a basic tool for taxonomic, epidemiologic as well as medical studies (Nadler, 1995).

To build up a reliable picture of the various phylogenetic subdivisions with relation to virulence, genes such as the ribosomal ribonucleic acid genes, the Lyshmanolysin (GP63) and the cytochrome Oxidase become indispensable.

3.6 Hypotheses

Genetic polymorphism at the Inter and intra specific level might be associated with disease phenotype.

3.7 Objectives of the Study

General Objectives

- To understand the correlation between the genetic diversity and the clinical manifestations.

Specific Objectives

- To genetically characterize isolates from Sudanese patients diagnosed with different types of leishmaniasis to understand the correlation between different clinical manifestations and the genetic profile of Leishmania.
- Investigate presence or absence of mixed infections and/ or hybrids.
- To build up a reliable picture of the various phylogenetic subdivisions with relation to virulence, using the Internal Trascribed Spacer (ITS) gene, the Lyshmanolysin (GP63) and the cytochrome Oxidase sub- unit II gene.

CHAPTER TWO

MATERIALS AND METHODS

Materials and Methods

2.1 Ethical Consideration

This study was approved by the ethical committee of the Institute of the Endemic Diseases, University of Khartoum and by the Ethical review Board at the Federal Ministry of Health, Sudan. The biological human samples were taken during a routine diagnostic service and no additional samples were required for this study.

2.1 Study area

This study was carried out in an endemic focus of visceral leishmaniasis (VL) in Gedarif State, Eastern Sudan (411 km from Khartoum). The study area is composed of clusters of villages where the disease is well known among inhabitants as kala-azar (Figure 7). The area is flat and composed of cracked alluvial clay soil characterized by tropical climate, i.e. a dry hot summer (March-June) with average temperature range of 28-44^{o}C, warm wet autumn (July-October) and moderately warm winter (November-February) with average temperature range of 18-34 ^{o}C. The annual rainfall is about 600mm. Large scale rain fed sorghum and sesame fields surround the villages. Inhabitants mainly work as farmers, woodcutters and shepherds. Many nomadic tribes roam the area.

Figure 7: *the Study area*

2.2 Samples Collection

One hundred and eleven samples composed of blood (HB), bone marrow (HBM), lymph node aspirates (HLN), extracted DNA and cultured parasites were obtained from 72 patients clinically suspected of Leishmaniasis. Samples collection occurred in periods between 14-25 December 2010 and 10 -20 January 2012.

VL Biological Samples

From 21 patients, HBM, HB and HLN aspirates were available. Seven HB samples and one HLN were collected from eight symptomatic VL patients at MSF-CH Leishmaniasis clinic inside the local Tabarak Allah hospital (Gedarif, Sudan). Other 20 HB and 5 HMB were collected from south Gedarif outpatient clinic (Table 3). Clinical samples were spotted on Whatman filter paper #3; each filter paper sample was stored in a separate polyethylene bag at room temperature till further analysis.

CL Samples

Eight samples were taken from eight patients presented at IEND with typical CL ulcers and attempted for culture (Table 3).

Extracted DNA

Ten extracted genomic DNA of seven VL, one CL, one ML and one PKDL were kindly provided by the IEND (Table 3).

Leishmania Reference strain

L.major MON 25, *L.tropica MON* 30 kindly provided by the *Leishmania* Reference Centre, Italy and *L.infantum MON 1* MHOM/TN/80/IPT1 kindly provided by the IZSVe were included in this study.

Geographic origin	Sample Type	Number of samples	Leishmaniasis diagnosis	Clinical Manifestation
Central Gedarif city	HBM*	21	symptomatic	VL
	HB*	21	symptomatic	VL
	HLN*	18	symptomatic	VL
TabarakaAllah H	HB	7	serological	VL
	HLN	1	serological	VL
South Gedarif city	HBM	5	symptomatic	VL
	HB	20	symptomatic	VL
Khartoum-IEND	DNA	7	culture	VL
	DNA	1	culture	PKDL
	DNA	1	culture	ML
	DNA	8	culture	CL

Table 3: *Provenance, numbers and types of samples collected included in the study. HBM : Human Bone Marrow, HB: Human Blood, HLN: Human Lymph Node. .IEND: Institute of Endemic Diseases.*
**: samples from same patients*

2.3 Molecular Assay

Parasite Isolation and Culture Condition

Leishmania parasites were isolated as described by Evans (1989) using a modified biphasic NNN media consisting of solid and liquid phases. The solid phase was prepared by dissolving 2.5 gm blood agar base into 100 ml distilled water, the mixture was then boiled, autoclaved and cooled to 50 °C. Defibrinated rabbit blood was then added in a concentration of 10%. The media were melted in sterile glass tubes that laid in a slope position till the agar solidification. The tubes were incubated at 4°C for 24 hours. The liquid phase composed of 10% FCS and 90% RPMI 1640 was added just before use. Penicillin / Streptomycin mix (5 U/ ml) or Gentamycin (25ug/ml) were added to the media.

Cutaneous Isolates : A single cutaneous aspirate from the edges of the ulcer of each CL suspected patient was inoculated into the bottles containing biphasic media (NNN) and incubated at 24°C . Cultures were examined microscopically for parasite growth or contamination on the third day. DNA of successfully cultured isolates was extracted. No mass cultures was done.

Other IEND Isolates: The parasite isolation of the ten extracted genomic DNA (seven VL, one CL, one ML and one PKDL) was performed as in cutaneous isolates mentioned above but mass cultures were carried out. When parasite growth was established, the isolates were sub-cultured into a sterile RPMI 1640 containing 25 HEPES (pH 7.4), 10% heat inactivated FCS, streptomycin and penicillin at 5U/ml (complete media). Cultures were grown in sterile 25 ml flasks and incubated at 26°C. About 200 µl of growing *Leishmania* isolates were sub-cultured into 10 ml of complete

media and incubated at 26°C for one week when the stationary growth phased reached. The cultures were transferred into 15 ml sterile tubes and centrifuged at 3000 rpm for 10 minutes, the pellets were used for DNA extraction.

2.3.2 DNA Extraction

DNA extraction from culture

DNA of the cultured parasites was extracted in the IEND laboratory using phenol/chloroform method as described elsewhere (Maniatis *et al*., 1986) with few modifications. Briefly, promastigotes were collected by centrifugation at 3000 rpm for 15 minutes. The Pellets were washed three times with 1× PBS buffer, detached and incubated at 55°C overnight with 5ml lysis buffer (NaCl 50mM, EDTA 10 mM, tris HCl 50 m M), 25 µl of 10 % SDS (w/v) and 100 µl of proteinase K. On the second day, 5ml of phenol were added, mixed gently and centrifuged for 15 minutes at 3000 rpm at 4°C, the upper layer was transferred into a new clean 50 ml tube, 2.5ml phenol and 2.5 ml chloroform- isoamyl alcool were added, mixed well then centrifuged as above, again the upper layer was transferred into a new clean 50 ml tube, 100 µl RNAse (29 ug/ml) was added, mixed and then incubated for 2 hours at 37°C. 2.5ml phenol and 2.5 ml chloroform- isoamyl alcool were added, mixed and centrifuged and treated as previously (this step could be repeated until the interphase disappears), thereafter, 10 ml of absolute ethanol was added and mixed. The tubes were then incubated at -20°C for one hour, after that the tubes were centrifuged as previously and the supernatants were discarded. The volume was brought to 10 ml with 70% cold ethanol, mixed gently and centrifuged for 10 minutes. The supernatants were discarded and the tubes were allowed to dry for about 40 minutes, 300 µl of TE buffer or distilled water were added, mixed gently and incubated overnight at room temperature. The next day the DNA was transferred to a sterile 1.5 ml eppendorf tube and stored in - 20°C till transferred to the parasitology laboratory in the IZSVe premises.

DNA Extraction From Clinical Samples Spotted on Filter Papers

Extraction took place at the parasitology laboratory in the IZSVe premises. Filter papers with spotted biological material (Lymph node and bone marrow aspirates or blood) were punched out with a paper puncher. To prevent DNA contamination among samples, clean sheet of paper sprayed with MicroSol 3+ ™ solution was punched several times. DNA extraction was performed using QIAamp DNA Mini Kit according to manufacturer's instructions. Briefly, 3 to 6 punched-out circles (about 3mm diameter) from a dried biological spot were placed into a 1.5 ml micro-centrifuge tube, 180 µl of Buffer ATL was added, the mixture was then incubated at 85°C for 10

minutes then centrifuged to remove drops from inside the lid, 20 µl proteinase K stock solution was added, mixed by vortexing, and thereafter incubate at 56°C for one hour. 200 µl Buffer AL were added to the sample, mixed thoroughly and incubated at 70°C for 10 min. After that 200 µl ethanol (96–100%) were added to the sample, mixed thoroughly. The mixture thereafter was applied to the kit Mini spin column (in a 2 ml collection tube), centrifuged at 8000 rpm for one minute, the Mini spin column was then placed in a clean 2 ml collection tube while the filtrate was discarded, after that 500 µl Buffer AW1 were added, centrifuged at 8000 rpm for 1 minute, again the Mini spin column was placed in a clean 2 ml collection tube and the filtrate was discarded. 500 µl Buffer AW2 were added and the mixture was centrifuged at maximum speed for three minutes, the Mini spin column was then placed in a clean 1.5 ml micro-centrifuge tube and the collection tube containing the filtrate was discarded. 150 µl Buffer AE was added, incubated at room temperature for 1 minute, and then centrifuge at 8000 rpm for another minute.

2.3.3 Real Time PCR Assay

As an initial screening, a real time PCR was conducted to investigate the presence of *Leishmania complexes* (*L. viannia, L. mexicana, L. donovani/infantum*, and *L. major*) in all samples. The primers Lid-f and Lid-r which generate a 80bp fragment of the GPI gene (Wortmann, *et al.,* 2005) were used with the probe TaqMan MGB Lid-probe labelled with the fluorescent reporter dye FAM (6-carboxyfluorescein) at the 5'end and with the fluorescent quencher dye TAMRA (tetra-methyl carboxyrhodamine) (Primers and probe sequences are shown in Table 4). Real time PCRs were performed in a final volume of 20 µl containing 3 µl of DNA, 10 µl of FastStart TaqMan Probe Master (Rox)1X (Roche Mannheim, Germany), 0.4 µM of both primers and 0.3 µM of the probe. The thermal cycling profile consisted of an initial activation at 95°C for 10 min, followed by 45 cycles each consisting of denaturation at 95°C for 15sec and annealing/extension at 60°C for 30sec. Negative (sterile water) and positive (DNA extracted from *L. infantum* MON-1, MHOM/TN/80/IPT1, IZSVe-Italy) controls were included in each run of real time PCR reaction. Real time PCR was carried out on a 7900HT fast Real-time PCR system (Applied biosystems).

2.3.4 Polymerase Chain Reaction (PCR)

kDNA PCR

Fifty Real Time PCR positive samples were tested by two PCRs, using the two couple of primers AJS3/R1 and F2/DBY (Lambson *et al*, 2002) which amplify respectively a 620 bp and 250 bp fragments of the kDNA gene

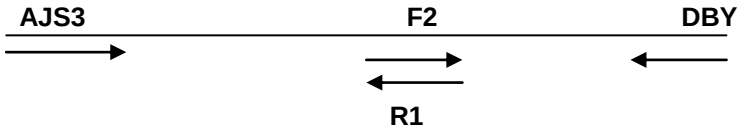


Figure 8: *The DNA segments of the selected minicircle kDNA class used for amplification*.

The two PCRs had the same amplification conditions. The reactions were performed in a final volume of 50 µl containing 5 µl of DNA, 5 µl of PCR buffer 1X (Applied Biosystems, Foster City, CA), 2 mM of $MgCl_2$ (Applied Biosystems, Foster City, CA), 0.5 µM of each primer, 0.2 mM of dNTPs (Applied Biosystems, Foster City, CA), 2 U of AmpliTaq Gold DNA polimerase (Applied Biosystems, Foster City, CA). Amplifications were carried out in a GeneAmp®PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA) with the following thermal cycling profile: denaturation for 10 min at 95°C, followed by 35 cycles each consisting of 30 sec at 94°C, 30 sec at 63°C, 30 sec at 72°C and a final extension step for 7 min at 72°C. Negative and positive controls were included in each run of PCR. PCR products were analysed on acrylamide gel, visualized and subsequently subjected to sequencing.

Cytochrome Oxidase II gene PCR

PCR was performed as described previously for the targeted Cytochrome Oxidase II (Abdalla Hassan Sharief *et al.,* 2011) for all Leishmaniasis Real Time PCR positive samples. (Primers sequences were shown in table 2). The reaction was performed in a final volume of 50 µl containing 5 µl of DNA, 5 µl of PCR buffer 1X (Applied Biosystems, Foster City, CA), 2 mM of $MgCl_2$ (Applied Biosystems, Foster City, CA), 0.4 µM of each primer, 0.2 mM of dNTPs (Applied Biosystems, Foster City, CA), 2 U of AmpliTaq Gold DNA polimerase (Applied Biosystems, Foster City, CA). Amplifications were carried out in a GeneAmp®PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA) with the following thermal cycling profile: denaturation for 10 min at 95°C, followed by 35 cycles each consisting of 30 sec at 94°C, 30 sec at 50°C, 45 sec at 72°C and a final extension step for 7 min at 72°C. Negative and positive controls were included in each run of PCR as described above. PCR products were analysed on acrylamide gel, visualized and

subsequently subjected to sequencing. Three sequences associated with cutaneous samples showed overlapping nucleotides peaks. These samples had been subjected to cloning.

Internal transcribed Spacer (ITS) PCR

The ITS PCR was performed in 10 samples. The ITS region (1044pb nucleotides) was amplified with the *Leishmania* specific primers: LITSR (El Tai *et al.,* 2000) and a new designed primer LITSRR (Table 4). The new primer was necessary to include *L. donovani complex* and *L. major*. Multiple alignments of GenBank related DNA sequences of different L. *donovani complex* and *L. major* species were gathered using the database search tool developed by the National Center for Biotechnology Information (NCBI: (http:// www.ncbi.nlm.nih.gov/). Sequences were aligned using the ClastalW alignment program implemented in Mega 5. The reverse primer was designed in a conserved region among sequences that preceded a variable region respecting the melting temperature of the existing forward primer.

The reaction was performed in a final volume of 50 µl containing 0.5 µl of DNA, 5 µl of PCR buffer 1X (Applied Biosystems, Foster City, CA), 2 mM of MgCl2 (Applied Biosystems, Foster City, CA), 0.4 µM of each primer, 0.2 mM of dNTPs (Applied Biosystems, Foster City, CA), 2.5 U of AmpliTaq Gold DNA polimerase (Applied Biosystems, Foster City, CA). Amplifications were carried out in a GeneAmp®PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA) with the following thermal cycling profile: denaturation for 10 min at 95°C, followed by 35 cycles each consisting of 30 sec at 94°C, 30 sec at 50°C, 1 minute at 72°C and a final extension step for 10 min at 72°C. Negative and positive controls were included in each run of PCR as described above.The PCR products were cloned after being analysed on acrylamide gel and visualized.

GP63 PCR

Twenty five Real Time PCR positive samples were tested using the two couple of primers gp63-1/gp63-2 and gp63-3/gp63-4 (El Tai *et al*, 2001), which amplify respectively a 442 bp and 374 bp fragments of the gp63 gene

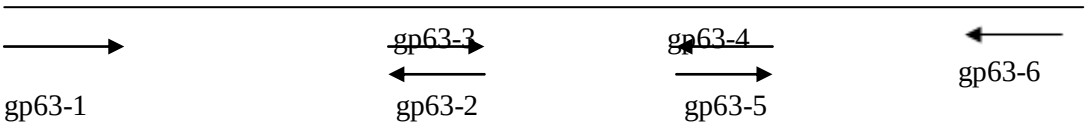


Figure 9: *The two DNA segments of the GP63 gene selected for amplification.*

The same amplification conditions were used for the two PCRs. The reactions were performed in a final volume of 50 µl containing 5 µl of DNA, 5 µl of PCR buffer 1X (Applied Biosystems, Foster City, CA), 2 mM of MgCl2 (Applied Biosystems, Foster City, CA), 0.4 µM of each primer, 0.2 mM of dNTPs (Applied Biosystems, Foster City, CA), 2.5 U of AmpliTaq Gold DNA polimerase (Applied Biosystems, Foster City, CA). Amplifications were carried out in a GeneAmp®PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA) with the following thermal cycling profile: denaturation for 10 min at 95°C, followed by 35 cycles each consisting of 30 sec at 94°C, 30 sec at 64°C, 1 min at 72°C and a final extension step for 7 min at 72°C. Negative and positive controls were included in each run of PCR, PCR products were analysed, visualized and subsequently subjected to sequencing as will be described. Some sequences showed overlapping nucleotides peaks, six were subjected to cloning. Unfortunately, gp63-5/gp63-6 PCR gave bad results, only fewer samples were amplified, these primers were later on abandoned.

MSPC PCR

PCR was performed in 4 samples. The ext region (1044pb nucleotides) (Mauricio et al, 2007) was amplified with the forward primer gp63-3 (El Tai *et al.*, 2001) and a new designed primer gp63-7 (Ttable 4). The new primer was necessary to include longer sequences in the gp63 EXT region, which is a single copy gene in L.infantum. As in the ITS reverse primer, the new primer was designed in a conserved region among different related GenBank sequences after multiple alignments using the ClastalW alignment program implemented in Mega 5. respecting the melting temperature of the existing forward primer. The reaction was performed in a final volume

of 50 µl containing 5 µl of DNA, 5 µl of PCR buffer 1X (Applied Biosystems, Foster City, CA), 2 mM of MgCl2 (Applied Biosystems, Foster City, CA), 0.4 µM of each primer, 0.2 mM of dNTPs (Applied Biosystems, Foster City, CA), 2.5 U of AmpliTaq Gold DNA polimerase (Applied Biosystems, Foster City, CA). Amplifications were carried out in a GeneAmp®PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA) with the following thermal cycling profile: denaturation for 10 min at 95°C, followed by 40 cycles each consisting of 40 sec at 94°, 45 sec at 62 °C, 2 minute at 72°C and a final extension step for 10 min at 72°C. Negative (water) and positive controls (*L.major*) were included in each run of PCR. Two PCR products that showed nucleotide overlapping were subjected to cloning.

2.3.5 PCR Product Analysis

Acrylamide Gel Electrophoresis

20 µl of APS 10% were added to 5 ml of 7% Acrylamide in 15ml clean tube, 10 µl of TEMED were added, the mix was then poured into the previously prepared vertical electrophoresis cell and a comb of 10 or 15 wells was inserted into the cell and left 15-20 minutes for polymerization at room temperature. The assembled gel was placed in the electrophoretic chamber and overlaid with the running buffer (1× TBE), the comb was then removed and wells were washed by the aid of a syringe, PCR products were loaded into wells by capillary tips after being mixed with loading dye in microtiter plate (2 -4 µl loading dye and 6-8 µl amplificate), suitable DNA marker (molecular ruler) was loaded also. The run was performed at 200V and 400mA for about 50 minutes.

Acrylamide Gel Staining and visualization

After the end of the run, the gel was stained by Sliver Nitrate. The staining process consist of adding the fixing solution (100 ml absolute ethanol and 15 ml of 65% nitric acid/1liter distilled water) for ten minutes followed by washing in distilled water for 5 minutes, after that 0.4% AgNO3 solution was added for 7 minutes and washed for 3 minutes, developing solution (Na_2CO3 activated by the addition of 750 µl of 36% formaldehyde) was added to visualize the band for maximum time of 3 minutes, after the development of the bands 5 % acetic acid solution was added for 5 minutes to stop the reaction. Gels were then washed by distilled water and closed into transparent plastic envelopes.

Sequencing Techinques and Sequence Analysis

PCR products were sequenced using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystem, Foster City, CA, USA). The products of the sequencing reactions were purified using PERFORMA DTR Ultra 96-Well kit (Edge BioSystems, Gaithersburg, MD, USA) and sequenced in a 16-capillary ABI PRISM 3130xl Genetic Analyzer (Applied Biosystem, Foster City, CA, USA). Sequence data were assembled and edited with SeqScape software v2.5 (Applied Biosystem, Foster City, CA, USA). PCR products were sequenced at the IZSVe.

The homology analysis was performed using the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/) and the software Mega 5 via alignment with sequences deposited in GenBank database (http:/ / www.ncbi.nlm.nih.gov/).

Phylogenetic analysis of the sequences was conducted using MEGA 5 software program that enables to establish the robustness of the analysis through phylogenetic bootstrap test. The bootstrap test estimates the reliability and the topology of the created phylogenetic tree, determining the proportion of all possible trees that support each node. This ratio is expressed as a percentage and are considered statistically significant values greater than 70%. neighbor-joining method with 1000 bootstrap replicates were used in the Phylogenetic analysis (Tamura *et al*., 2011).Phylogenetic analysis of the sequences was conducted by using the neighbor-joining method with 1000 bootstrap replicates implemented in MEGA 5 software (Tamura *et al*., 2011). Only sequences with more than 750 bp were used for the phylogenetic analyses.

Phylogenetic analysis of the sequences was conducted using MEGA 5 software program that enables to establish the robustness of the analysis through phylogenetic bootstrap test. The bootstrap test estimates the reliability and the topology of the created phylogenetic tree, determining the proportion of all possible trees that support each node. This ratio is expressed as a percentage and are considered statistically significant values greater than 70%. neighbor-joining method with 1000 bootstrap replicates were used in the Phylogenetic analysis (Tamura *et al*., 2011).

2.3.6 Molecular Cloning Assay

The PCR products of three COII, 10 ITS, 6 gp63 short and 4 gp63 ext genes were cloned separately into the PCR-II vector using a dual-promoter TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Neb Turbo *E. coli* competent cells and S.O.C. Medium (New England biolabs) were used in the assay for increased flexibility. At least 20 randomly selected colonies from each PCR product were sequenced using the sequencing procedure described above.

Solid LB agar medium was prepared by adding 25 LB medium capsules (MP Biomedicals, LLC, France) to 15grams Bacto™ Agar (Becton, Dickinson and Company, France) in 1 liter of distilled water, the mixture was then Autoclaved at 121 °C for 30 minutes in liquid cycle, cooled down to 60 °C and stored in 4 °C upon use, the solid medium was first liquefied in microwave for few minutes, then 50 µl (100mg /ml) ampicillin was added to 50 ml of the medium, the mixture was then divided into 3 sterile Petri plates and allowed re-solidification. 80ul of X-gal (2%) was poured into each plate and spread evenly using a spatula, the plates were allowed to dry. The LB liquid media were prepared as the solid media but without the addition of the Bacto™ Agar, they autoclaved, cooled and stored the same way. The assay was composed of the following steps:

Legation: The PCR products to be cloned were quantified by nanodrop 1000 3.7 (Thermo Fisher Scientific) to estimate the amount to be used in the ligation mix. 3 insert : 1 vector ratio was maintained. The legation mix was made by adding 1µl of salt to the due µl of insert and of vector, the volume was then brought to 6 µl by adding distilled water. The legation mix was then left 5 minutes at room temperature.

Transformation: 5 µl of the legation mix were added to an iced- thawed NEB TURBO cell, the mixture was then left in ice for 30 minutes, thereafter the thermal shock was done by immersing the tube into a hot water bath at 42 ° C for 30 seconds then again allowed 5 minutes in ice. The mixture was then added to 950 µl S.O.C contained in 15 ml tube and incubated at 37 ° C for one hour with light agitation.

Plating: 100 µl, 300 µl and 600 µl of the incubated ligation mixture and the S.O.C medium were poured out onto the three prepared Petri plates containing the solid agar, X-gal and the antibiotics. The plates were incubated at 37 ° C for overnight.

Colonies selection and preservation: Plasmids with the desired inserts (white colored colonies) were selected and put into 15 ml tubes containing 3 ml liquid LB and 3 µl ampicillin (100mg/ ml), the tubes were then incubated at 37 ° C for overnight. The day after, 800 µl of the culture were preserved into 200 µl of Glycerol and stored at -80 for future use, while the rest of the culture (about 1.2ml) was transferred into 2ml Eppendorf tubes.

Extraction: Plasmids with the desired inserts were isolated from positive *Escherichia coli* colonies by using a GenElute plasmid miniprep kit (Sigma-Aldrich, St. Louis, MO). The final elution for some colonies was done with distilled water, in such cases the extracts were sent directly for sequencing. In the other colonies where the final elution was done with the kit elution solution, the extracts were first amplified by PCR and then sent for sequencing. Sequencing and sequence analysis were carried out as mentioned above.

Gene	Primer Name	Sequence
GP1	Lid F	5’CCAGATGCCGACCAAAGC-3’
	Lid R	5’CGCGCACGTGATGGATAAC-3’
	TaqMan MGB Lid-probe	5’TCGGCAGGTTCT-3’
kDNA	AJ3	5’GGGTTGGTGTAAAATAGGG-3’
	R1	5’TGCAGTATG CGCCAA CCTATA-3’
	F2	5’ATAGGTTGG CGCATA CTGCAG-3’
	DBY	5’CCAGTTTCCCGCCCCGGAG-3’
Cytochrome Oxidase II	COII F	5’GGCATAAATCCATGTAAGA-3’
	COII R	5’TGGCTTTTATATTATCATTTT-3’
ITS	LITSR (FOR)	5’CTGGATCATTTTCCGATG-3’
	LITSRR (REV)	5’AGAGTGAGGGCGCGGATA-3’
GP63	Gp63-1	5’-TCCACCGAGGACCTCACCGA-3’
	Gp63-2	5’-GTCGTACCGCGACGCAATGT-3’
	Gp63-3	5-ACATTGCGTCGCGGTACGAC-3’
	Gp63-4	5’-GTAGAAGCCGAGGTCCTGGA-3’
	Gp63-5	5’TCCAGGACCTCGGCTTCTAC-3
	Gp63-6	5’CTGGCACACCTCCACGTACG-3’
	Gp63-7	5’AGAGTGAGGGCGCGGATA-3’

Table 4: *sequences of primers used for detection and characterization of Leishmania*

CHAPTER THREE

RESULTS

RESULTS

3.1 Parasite Culture Results

Parasite culture was succeeded in five out of the eight cutaneous samples.

3.2 Real Time PCR Results

Overall, the diagnosis of leishmaniasis was confirmed by real time PCR in 40 patients out of 72 (55.6%). The real time PCR confirmed 58 samples out of 111 (52.3%) as Leishmania positive. The positivity in real time PCR was higher for HLN samples (66%) compared to HBM (57%) and HB (29%).

3.3 kDNA Results

kDNA PCR Results

The PCRs targeting the kDNA produced the expected amplificates (Figure 10) in nearly 90% of the real time PCR positive samples, only few blood samples gave negative results.

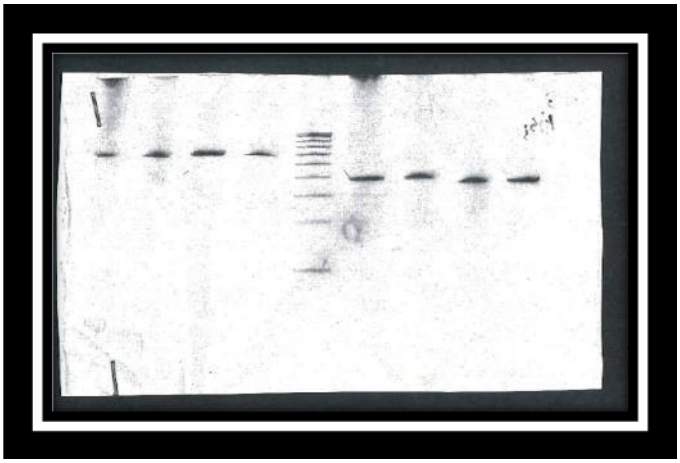


Figure 10: *four amplificates obtained by the primers AJS3/R1 to the left of the molecular ruler and primers F2/DBY to the right of the molecular ruler. Amplificates were loaded in acrylamide gel and stained by silver staining. Positive control occupy positions N° 1 and 6, molecular ruler position N° 5.*

kDNA Sequences Results

Although the gel showed an assertive band for each PCR product, kDNA sequences of CL samples (samples 108, 109 110 and 111) attributed to cutaneous cultures gave doubtful results, and were different from the other cutaneous samples (sample 107 and 111) (Figure 11). On the other

hand Sequences analysis of bone marrow samples (HBM), blood and Lymph node showed very different and unexpected results (Figure12) and as kDNA is not a single copy gene, it has been forsaken and used for confirmation purposes only.

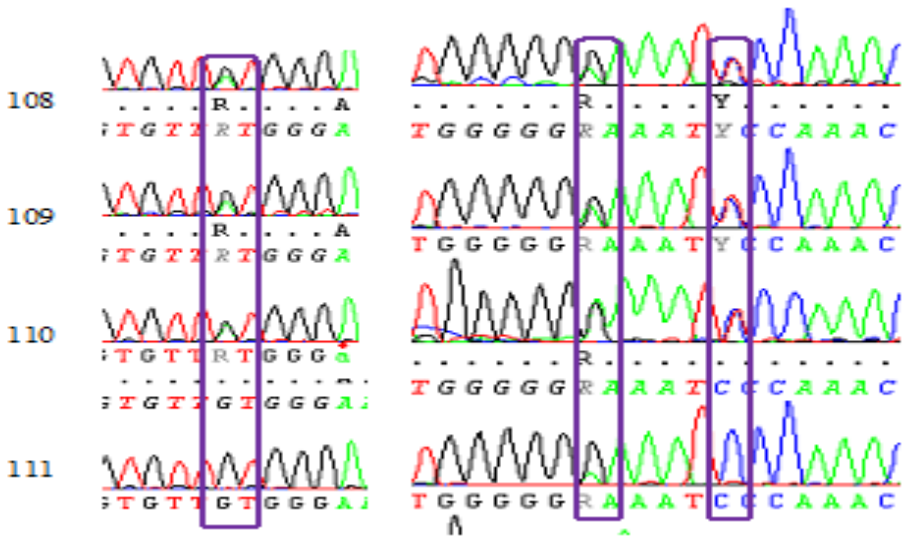


Figure 11: *schematic demonstration of kDNA sequences obtained by direct sequencing of four CL isolates (samples number 108, 109, 110 and 111) showing an example of nucleotide's overlapping in positions 633, 687 and 692, (position according to L.donovani GenBank™ accession number AF103737).*

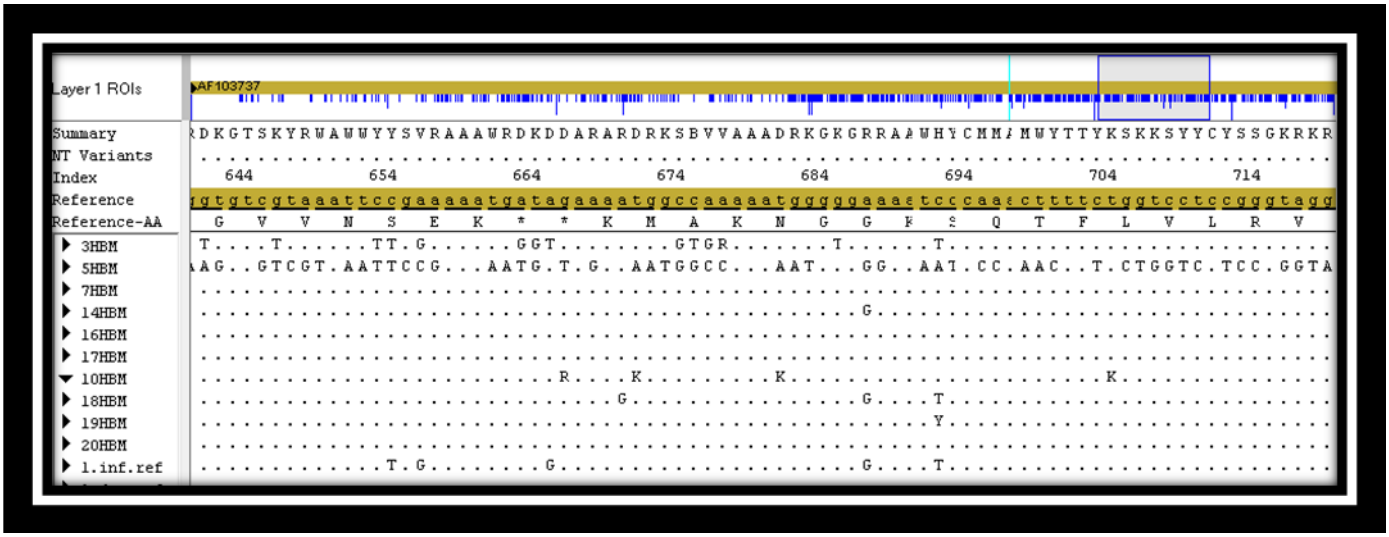


Figure 12: *schematic demonstration of kDNA sequences obtained by direct sequencing of ten HBM isolates and L.infantum reference strain used in this study.*

3.4 Cytochrome Oxidase II gene Results

COII PCR Results

The PCR targeting the COII gene produced the expected amplificates (Figure 13) in all the real time PCR positive samples.

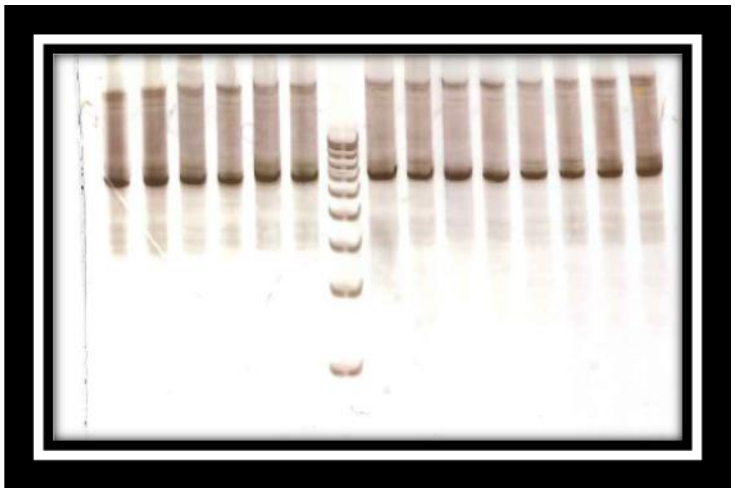


Figure 13: *showing thirteen amplificates obtained by the primers COII F and COII R. The positive control L.infantum is the last to the right. Amplificates were loaded in acrylamide gel and stained by silver staining. Molecular ruler in position N° 7.*

COII Direct Sequence Analysis Results

All samples associated with VL (except 14HBM), PKDL and ML clinical forms, together with two samples associated with CL cultures (samples 107 and 111) showed a unique sequence pattern and gave 100% genetic homology to *L. donovani* AY660023.1 reference sequences deposited in GenBankTM (Figure14 and Figure 15). Three CL sequences showed overlapping nucleotides peaks and these sequences were compared to *L.major MON 25* and to our above mentioned sequences (Figure 16).

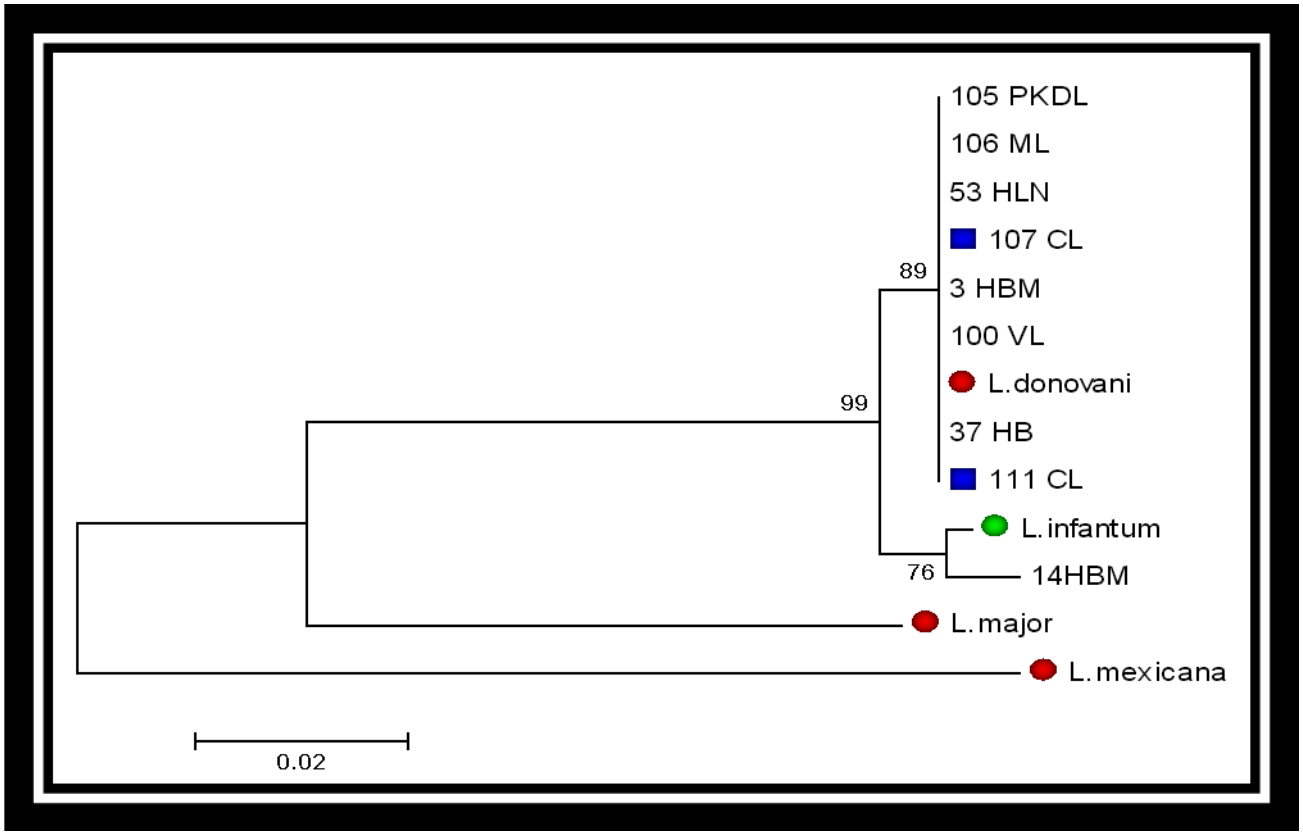


Figure 14: *Phylogenetic tree of COII sequences. Phylogenetic tree based on a fragment of 600 bp of COII gene of Leishmania parasite. Sequence dataset was analyzed using MEGA 5, the neighbour-joining (NJ) method, and bootstrap analysis (1,000 replicates) based on the ClustalW algorithm. Significant bootstrapping values (>70%) are shown on the nodes. Only representative sequences were shown.*
Number indicates the sample number, HBM indicates Human Blood Marrow origin sequence, HLN indicates lymph node origin sequence and HB indicates Human Blood origin sequence used in this study. (■)sequences number 107 and 111originated from skin culture amplificates and associated with CL patients, (●) Leishmania GenBank™ reference sequence (L.donovani GenBank™ accession number AY660023; L.major GenBank™ accession number AF63316; (■) L.major MON 25 and our positive control L.infantum MHOM/TN/80/IPT1 used in this study.

	1	2	3	4	5	6	7	8	9	10	11	12
1. 105 PKDL												
2. 106 ML	0.000											
3. 53 HLN	0.000	0.000										
4. 107 CL	0.000	0.000	0.000									
5. 3 HBM	0.000	0.000	0.000	0.000								
6. 100 VL	0.000	0.000	0.000	0.000	0.000							
7. 37 HB	0.000	0.000	0.000	0.000	0.000	0.000						
8. 111 CL	0.000	0.000	0.000	0.000	0.000	0.000	0.000					
9. 14HBM	0.019	0.019	0.019	0.019	0.019	0.019	0.019	0.019				
10. L.donovani	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.019			
11. L.major AF6331E	0.103	0.103	0.103	0.103	0.103	0.103	0.103	0.103	0.104	0.103		
12. L.infantum	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.009	0.014	0.110	

Figure15 : *Genetic differences of nine COII sequences. Genetic differences based on a 600 bp fragment of the COII gene of Leishmania parasite. Sequence dataset was analyzed using MEGA 5. Leishmania GenBank™ reference sequence used here are L.donovani GenBank ™ accession number AY660023; L.major GenBank ™ accession number EF633106; and our positive control L.infantum MHOM/TN/80/IPT1.*

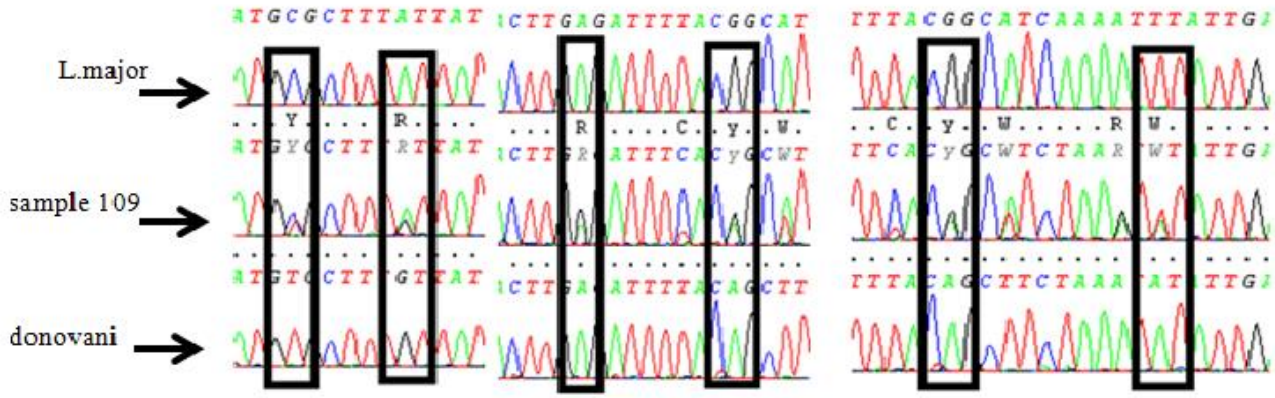


Figure 16: *schematic demonstration of COII sequence obtained by direct sequencing of one CL isolate (sample number 109) showing overlapping of nucleotides in position 93,99, 153 159, 162 and 173 (position according to L.donovani GenBank™ accession number AY660023), CL sequence compared to L.major MON 25 and to our 3HBM sequence that gave 100% identity to L.donovani (GenBank™ accession number AY660023).*

COII Cloning Results

Thirty-four, twenty nine and thirty six sequences were obtained from cloning of CL samples number 108, 109 and 110, respectively. Sequence data from colonies were assembled and edited with SeqScape software v2.5 and analyzed in MEGA 5 software as mentioned previously. Two different sequence patterns have been revealed corresponding to *L. donovani* with genetic similarity range between 99.5%-100% to *L. donovani* AY660023 GenBankTM reference sequence and to *L. major* with genetic similarity ranging between 98.9-99.3% % to *L. major* EF633106 GenBankTM reference sequence. The genetic similarity between the two GenBankTM reference sequences used here was 89.4%. (Figure17. and Figure 18)

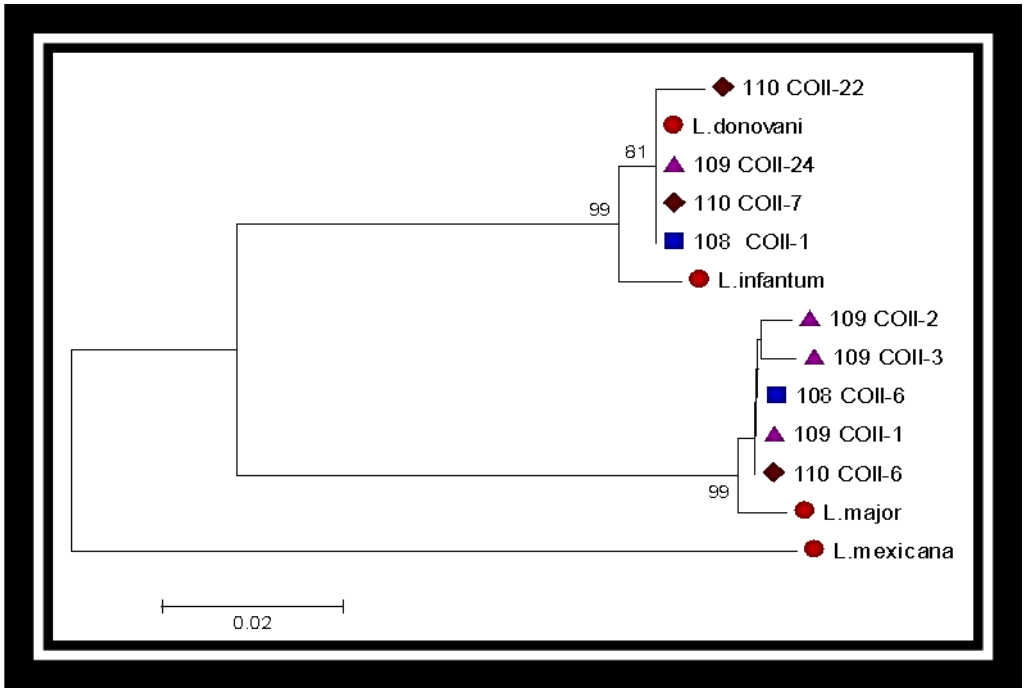


Figure 17: *Phylogenetic tree of COII cloning sequences. Phylogenetic tree based on a fragment of 600bp of COII gene of Leishmania parasite. Sequence dataset was analyzed using MEGA 5, the neighbour-joining (NJ) method, and bootstrap analysis (1,000 replicates) based on the ClustalW algorithm. Significant bootstrapping values (>70%) are shown on the nodes. Only representative sequences from each cloned sample were used, the same structure coloured icon indicates sequences from the same patient. (●) Leishmania GenBankTM reference sequence (L.donovani GenBank TM accession number AY660023; L.major GenBank TM accession number EF633106; L.mexicana GenBankTM accession number HQ586845.1 and our positive control L.infantum MHOM/TN/80/IPT1.*

	1	2	3	4	5	6	7	8	9	10	11	12
1. 110 COII-22												
2. 109 COII-24	0.003											
3. 110 COII-7	0.003	0.000										
4. 108 COII-1	0.003	0.000	0.000									
5. 109 COII-2	0.053	0.052	0.052	0.052								
6. 109 COII-3	0.053	0.052	0.052	0.052	0.004							
7. 108 COII-6	0.051	0.050	0.050	0.050	0.002	0.002						
8. 109 COII-1	0.051	0.050	0.050	0.050	0.002	0.002	0.000					
9. 110 COII-6	0.051	0.050	0.050	0.050	0.002	0.002	0.000	0.000				
10. L.donovan	0.005	0.002	0.002	0.002	0.055	0.055	0.053	0.053	0.053			
11. L.major	0.052	0.051	0.051	0.051	0.006	0.006	0.004	0.004	0.004	0.054		
12. L.infantum	0.008	0.005	0.005	0.005	0.055	0.055	0.053	0.053	0.053	0.007	0.054	

Figure 18: *Genetic differences of the three COII cloned sequences. Genetic differences based on a 600 bp fragment of the COII gene of Leishmania parasite. Sequence dataset was analyzed using MEGA 5.only representative sequences from each cloned sample were included. the Leishmania GenBank™ reference sequence used here are L.donovani GenBank ™ accession number AY660023; L.major GenBank ™ accession number EF633106; and our positive control L.infantum MHOM/TN/80/IPT1.*

3.5 Internal transcribed Spacer (ITS) Results

PCR Results

The PCR targeting the ITS gene produced the expected amplificates (Figure19) . Ten samples were subjected to cloning.

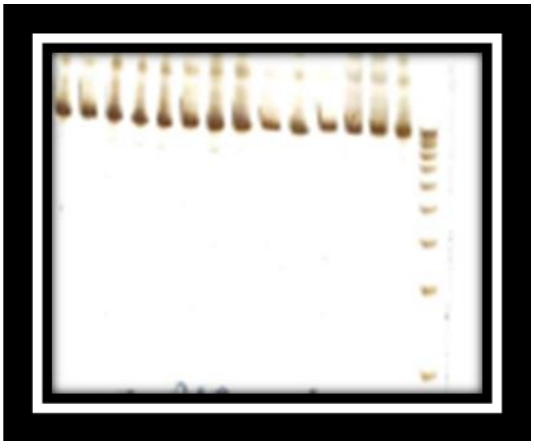


Figure 19*: showing ITS amplificates obtained by the primers LITSR and LITSRR.. Amplificates were loaded in acrylamide gel and stained by silver staining. positive control in position N°1 and molecular ruler in the last position*

Samples cloned and sequences obtained from colonies are shown in Table 5

Sample Identification	Clinical Manifestation	N° of successfully sequenced colonies	Length of sequences pb
3HBM	VL	26	930
14HBM	VL	27	830
17HBM	VL	27	843
105	PKDL	38	845
106	ML	45	845
107	CL	40	836
108	CL	23	900
109	CL	13	900
110	CL	14	751
111	CL	38	850

Table 5: *Types and number of colonies obtained from each ITS cloned sample*

ITS Cloning Results

Polymorphism of the sequences obtained from colonies is based on different microsatellite repeat numbers. We compared our polymorphic sites to those identified by Kuhls *et al* 2005, (Figure 20) who analyzed the ITS sequences of *L.donovani complex* members including 27 *L. infantum*, 2 *L. chagasi*, 18 *L. donovani* and 5 *L. archibaldi* strains of different zymodemes and geographical origin (Kuhls *et al* 2005). Our sequences lies between nucleotides position number 61 and 937 in figure (17) .we have identified two other loci after poly (G_2) named poly "CT" and poly "GT…. Poly TA".

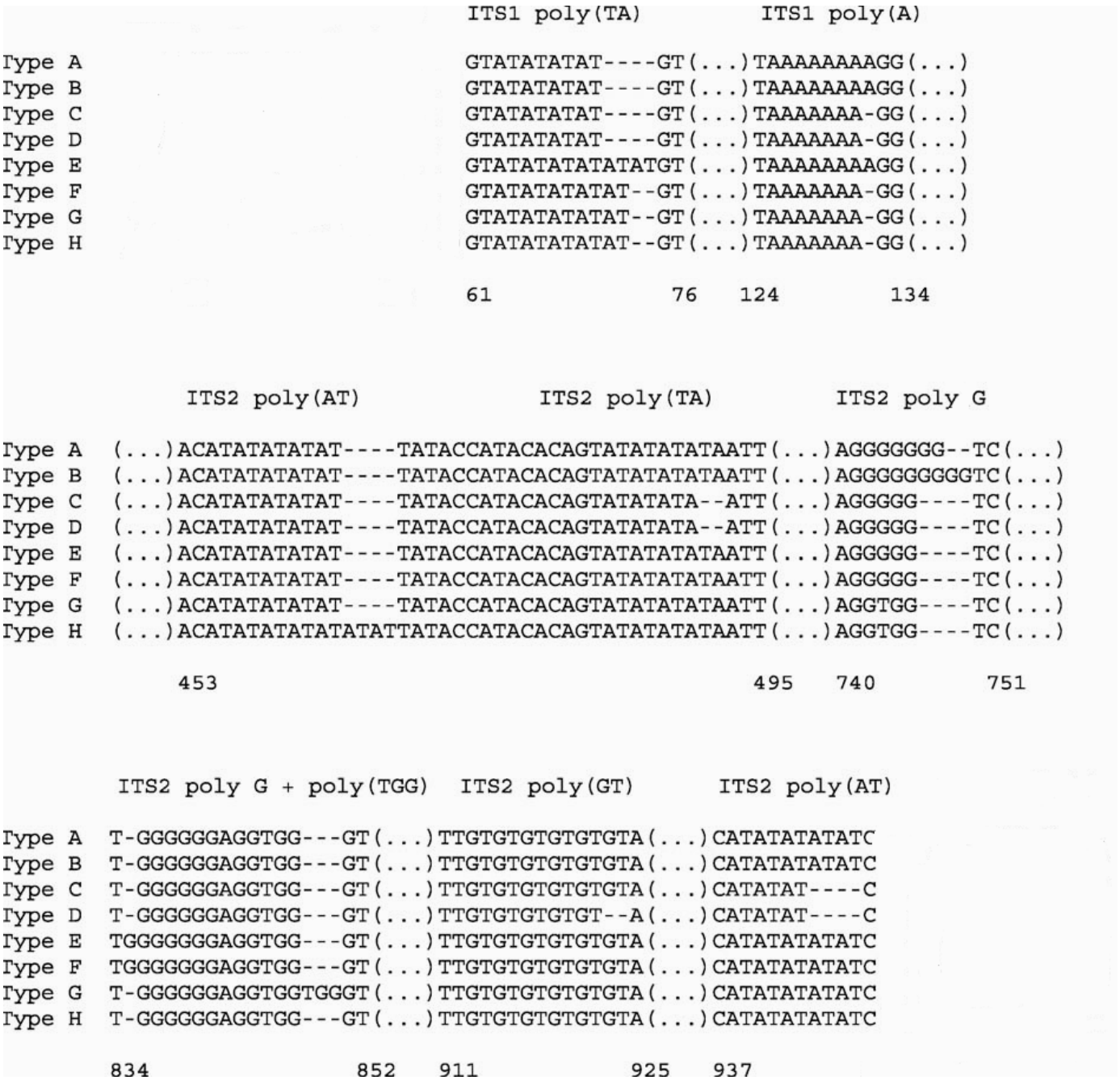


Figure 20*: ITS sequence types found for strains of L.donovani complex (Kuhls et al 2005)*

ITS 3HBM

Thirteen colonies of the 3 HBM sample were studied. Five sequences were found to be type E, one type F and five sequences type D (Figure 21). One sequence possessed mixed type E/F (at poly TA type E while at poly A type F), another sequence was found to be mixed type D/F(at poly TA type D while at the other polymorphic sites type F). In addition one modified type E sequence possess 8 G at poly $_{G2}$ instead of seven . (Figure 21) No polymorphism was detected at sites poly G1

Type	Poly TA	Poly A	Poly AT poly TA	Poly G_2	Poly GT…. Poly TA
E	TG**TATATATATATA**TGTA	GT**AAAAAAAA**GG	G**TATATATATA**ATT	T**GGGGGGG**AGG	T**TGTGTGTGTGTGT**ATGT CACA**TATATATA**TCC
Modified E	TG**TATATATATATA**T**GTA**	GT**AAAAAAAA**GG	GT**ATATATATA**ATT	T**GGGGGGG**GAGG	T**TGTGTGTGTGTGT**ATGT CACA**TATATATAT**CC
F	TG**TATATATATA**-- TGTA	GT**AAAAAAA**—GG	G**TATATATATA**ATT	T**GGGGGGG**AGG	T**TGTGTGTGTGTGT**ATGT CACA**TATATATAT**CC
Mixed E/F	TG**TATATATATATA**TGTA	GT**AAAAAAA**—GG	G**TATATATATA**ATT	T**GGGGGGG**AGG	T**TGTGTGTGTGTG**TATGT CACA**TATATATAT**CC
D	TG**TATATATA**-- TGTA	GT**AAAAAAA**—GG	G**TATATATA**ATT	T**GGGGGG**AGG	T**TGTGTGTGTGT**ATGT CACA**TATATAT**CC
Mixed D/ F	TG**TATATATA**-- TGTA	GT**AAAAAAA**—GG	G**TATATATATA**ATT	T**GGGGGGG**AGG	T**TGTGTGTGTGTG**TATGT CACA**TATATATAT**CC

Figure 21*: 3HBM ITS polymorphic sites among 930 nucleotides*

ITS 14HBM

Twenty colonies of the 14 HBM sample were studied. Four polymorphic sites similar to those of the Kuhls et al (2005) (Figure 22) were found, these sites are: poly TA, poly A, poly "AT + TA", in addition to other two new polymorphic sites which have not been identified before. The first site is poly CT which consist of 4, 5 or 6 repeats of CT, this site lies between poly "G_2 and poly TGG", in our aligned sequences of the 14 HBM it took position from 824 to 837. The second site is the poly AT which consist of 4 or 5 repeats of AT at position from 843 to 852 in our 14 HBM aligned sequences.

Type F is the dominant type, but it get modifications at some sequences, as example, two sequences of this type had 9 and 8 G at poly G2, another sequence is type F sequence but at poly G2 possess 6G which are characteristic of types A, B, C, D. Another type F sequence at poly A and Poly "AT poly TA" possessed types A, B, C or D at poly TA . mixed F/ "Cor D" sequence was found (type F

sequence that possess type C or D at "Poly AT poly TA"). Four sequences possessed mixed E/F type, at the poly TA, poly A they follow type E, while at Poly "AT poly TA" they follow type F and they showed no polymorphism at the other sites.

Type	Poly TA	Poly A	Poly AT poly TA	Poly G2	Poly CT r
Mixed E /F	TG**TATATATATATA**TGTA	GT**AAAAAAAA**GG	AC**ATATATATAT**TATAC**C**(--) G**TATATATATA**ATT	T**GGGGGGG**AGG	G**CTCTC**
F	TG**TATATATATA..**TGTA	GT**AAAAAAA**-GG	AC**ATATATATAT**TATACC(--) G**TATATATATA**ATT	T**GGGGGGG**AGG	G**CTCTC**
MixedF/E	TG**TATATATATA**..TGTA	GT**AAAAAAAA**-GG	ACATATATA—TTATA G**TATATATATA**ATT	T**GGGGGGG**AGG	G**CTCTC**
Mixed F/ C or D	TG**TATATATATA..**TGTA	G**TAAAAAAA**-GG	AC**ATATATATAT**TATACC(--) G**TATATATA**ATT	T**GGGGGGG**AGG	G**CTCTC**
ABCD	TG**TATATATAT--** GTA	GT**AAAAAAA**-GG	AC**ATATATATAT**TATACC(--) G**TATATATATA**ATT	T**GGGGGGG**AGG	G**CTCTC**
Seq N° 2	TG**TATATATATA**..TGTA	GT**AAAAAAA**-GG	AC**ATATATATAT**TATACC(--) G**TATATATATA**ATT	T**GGGGGGG**AGG	G**CTCTC**
Seq N° 45	TG**TATATATATA**..TGTA	GT**AAAAA**A-GG	AC**ATATATATAT**TATACC(--) G**TATATATATA**ATT	T**GGGGGGG**AGG	G**CTCTC**
Seq N° 54 51	TG**TATATATATA**..TGTA	GT**AAAAAAA**-GG	AC**ATATATATAT**TATACC(--) G**TATATATATA**ATT	T**GGGGGGGGG**AGG T**GGGGGGGG**AGG	G**CTCTC**
Mixed F/ABCD	TG**TATATATATA**..TGTA	GT**AAAAAAA**-GG	AC**ATATATATAT**TATACC(--) G**TATATATATA**ATT	T**GGGGGG**-AGG	G**CTCTC**

Figure 22: *14 HBM ITS polymorphic sites among 854 nucleotides*

ITS 17 HBM

Twenty six colonies of the 17HBM sample were studied. The majority of the sequences were type F. Only four sequences were found to be type E. Two sequences possessed modified type F (at Poly "AT + TA" possess type C or D and at poly G_2 possess 9 G instead of 7). Three sequences possessed mixed type F/E hence at poly TA, they possessed type F and in poly A type E. (Figure 23). No polymorphysim was found at site poly G_1.

Type	Poly TA	Poly A	Poly AT poly TA	Poly G_2
E	TG**TATATATATATA**TGTA	GT**AAAAAAAA**GG	AC**ATATATATAT**TATACC(...)	T**GGGGGGG**AGG
F	TG**TATATATATA**-- TGTA	GT**AAAAAAA**--GG	AC**ATATATATAT**TATACC(...)	T**GGGGGGG**AGG
Modified F	TG**TATATATATA--** TGTA	GT**AAAAAAA**--GG	AC**ATATATAT--**TATACC(..)	T**GGGGGGG**AGG
	TG**TATATATATA**-- TGTA	GT**AAAAAAA**--GG	AC**ATATATATAT**TATACC(...)	T**GGGGGGGGG**AGG
Mixed F/E	TG**TATATATATA**-- TGTA	GT**AAAAAAAA**GG	AC**ATATATATAT**TATACC(...)	T**GGGGGGG**AGG

Figure 23: *17 HBM polymorphic sites among* 843 nucleotides

105 PKDL

Twenty nine colonies of the 105 PKDL sample were studied. Ten sequences were found to be type E, three type F. One sequence possessed mixed types, at the poly TA and poly A it gives type E, while in poly G_2 it becomes type C. Four sequences possessed 8 G in poly G_2 , two were found to be type E and two type F in both previous polymorphic sites. Modified type E was also found in 8 other sequences that possess 10 A in the polymorphic site A and in one sequence with 9 A and 9 G respectively at the polymorphic sites A and G_2. Modified type F was found in two sequences with 10 and 9 A at the polymorphic sites A. (Figure 24) . No polymorphysim was found at sites Poly "AT + TA" nor at poly G1.

Type	Poly TA	Poly A	Poly G_2
E	TG**TATATATATATA**TGTA	GT**AAAAAAAA**GG	T**GGGGGGG**AGG
F	TG**TATATATATA**-- TGTA	GT**AAAAAAA**--GG	T**GGGGGGG**AGG
Modified E/F	TG**TATATATATATA**TGTA	GT**AAAAAAAAA**-GG	T**GGGGGGGGG**AGG
	TG**TATATATATA**-- TGTA	GT**AAAAAAAAAA**GG	T**GGGGGGG**AGG
Mixed E/C	TG**TATATATATATA**TGTA	GTAAAAAAAAGG	TGGGGGGAGG

Figure 24: *105 PKDL polymorphic sites among 843 nucleotides.*

106 ML

Forty colonies of the 106 ML sample were studied. The majority of the sequences were type E . Two sequences were found to be type F. One sequence possessed mixed type E and F (at poly TA seemed type E and in poly A seemed type F) (Figure 25).

For all sequences, no polymorphism was found at sites Poly "AT +TA", poly G_1 and poly G_2. (Figure 25)

Figure 25

Type	Poly TA	Poly A
E	TGT**ATATATATATA**TGTA	GT**AAAAAAAA**GG
F	TG**TATATATATA**-- TGTA	GT**AAAAAAA**GG
Mixed E/F	TGT**ATATATATATA**TGTA	GT**AAAAAAA**GG

Figure 25*: 106ML polymorphic sites.*

The new identified polymorphic site "CT" showed different patterns with 4, 5 or 6 repeats, figure 26 explains Poly CT .

Figure 26

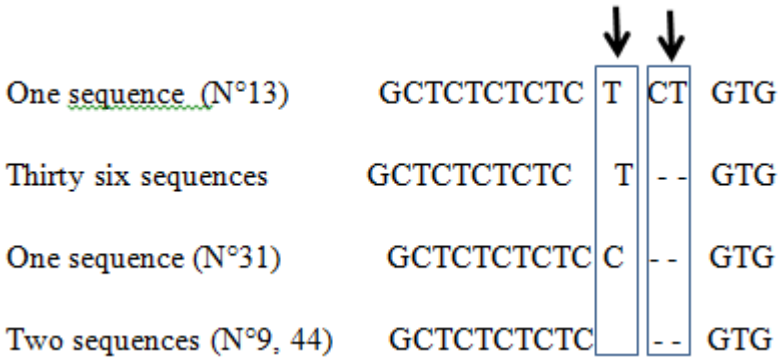


Figure 26: *106 ML, poly CT, position 816 of the 850 nucleotide: the three different patterns of nucleotides*

Six sequences belonging to type E, showed two different sequence patterns at position 63 (850 nucleotide) (Figure 27).

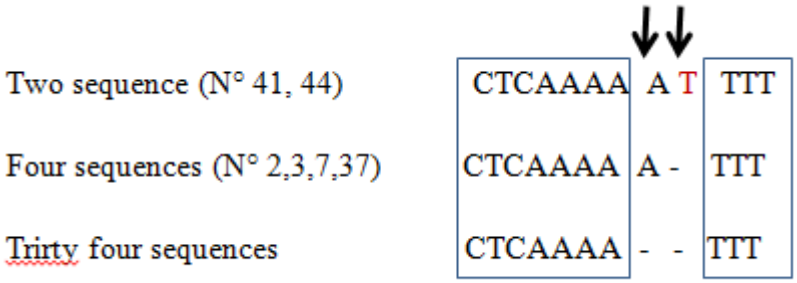


Figure 27*: 106 ML, position 63 of the 850 nucleotide: the three different patterns of nucleotides*

ITS 107 CL

Thirty four colonies of the 107CL sample were studied. Type E is the dominant with eighteen sequences. Modified type E that contain 9 G and 8 G at poly G_2 is found in ten and three sequences respectively. Type F was absent but mixed type E/F is found in three sequences (Figure 28). At poly CT only one sequence possessed 6 CT repeats while all the other possessed 5 repeats, while at "Poly GT+TA", all sequences have 5 TA repeats with the exception of two sequences with 4 repeats (Figure 28). No polymorphism was detected at "Poly AT + TA" or poly G_1

Interestingly, two sequences (both type E, but one sequence possess 9G at poly G_2 instead of 7) showed a unique pattern , their poly TA contains 7 repeats. Positions 60 and position 768 showed additional polymorphism (Figure 29)

Type	Poly TA	Poly A	Poly G_2	Poly CT	Poly GT…. Poly TA
E	TG**TATATATATATA**TGTA	GT**AAAAAAAA**GG	T**GGGGGGG**AGG	GG**CTCTCTCTCT**—GT	TGG**TATATATATAT**GT
Modified E	TG**TATATATATATA**TGTA	GT**AAAAAAAA**GG GT**AAAAAAAAA**GG	T**GGGGGGG**AGG T**GGGGGGGGG**AGG T**GGGGGGGG**AGG	GG**CTCTCTCTCTCT**GT GG**CTCTCTCTCT**—GT	TGG**TATATATATAT**GT TGG**TATATATAT**--GT
Mixed E/F	TG**TATATATATA**-- TGTA TG**TATATATATATA**TGTA	GT**AAAAAAAA**—GG GT**AAAAAAA**—GG	T**GGGGGGG**AGG	GG**CTCTCTCTCT**—GT	TGG**TATATATATAT**GT

Figure 28: *107CL polymorphic sites, among 836 pb nucleotides*

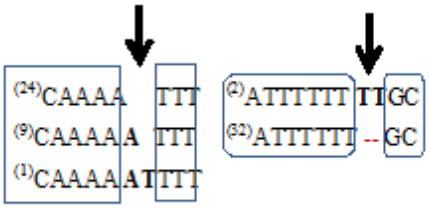


Figure 29:107 CL polymorphic sites at position 60, and 768 respectively

ITS 111 CL

Thirty colonies of the 111CL sample were studied. Type E and type F were fairly divided between colonies. Two sequences were found to be of mixed types E/F with poly TA similar to type E and poly A similar to type E. Modified type F was detected in two sequences, one with 9 A at poly A and 9 G at poly G_2, and the other with 6 A at poly A. Also modified type E exists in four sequences, two with 9 A at poly A, while the other two with 9 G at poly G_2. (Figure 30) No polymorphism was detected at site G_1, CT (all sequences with 5 CT) or poly TA (all sequences with 5 AT).

Type	Poly TA	Poly A	Poly G_2
E	TG**TATATATATATA**TGTA	GT**AAAAAAAA**GG	T**GGGGGGG**AGG
F	TG**TATATATATA**TGTA	GT**AAAAAAA**GG	T**GGGGGGG**AGG
Modified E	TG**TATATATATATA**TGTA TG**TATATATATATA**TGTA	GT**AAAAAAAAA**GG GT**AAAAAAAA**GG	T**GGGGGGG**AGG T**GGGGGGGGG**AGG
Modified F	TG**TATATATATA**TGTA	GT**AAAAAAAAA**GG GT**AAAAAA**GG	T**GGGGGGGGGG**AGG TGGGGGGGAGG
Mixed F/E	TG**TATATATATA**-- TGTA	GT**AAAAAAAA**GG	T**GGGGGGG**AGG

Figure 30: *111 CL polymorphic sites, among 848 pb nucleotides*

ITS 108 CL

Twenty two colonies were studied. Two very distinct sequence patterns were detected. In BLAST homology analysis, one pattern corresponds to *L. donovani* and the other to *L. major*. (Figure 31) demonstrates the two sequence types.

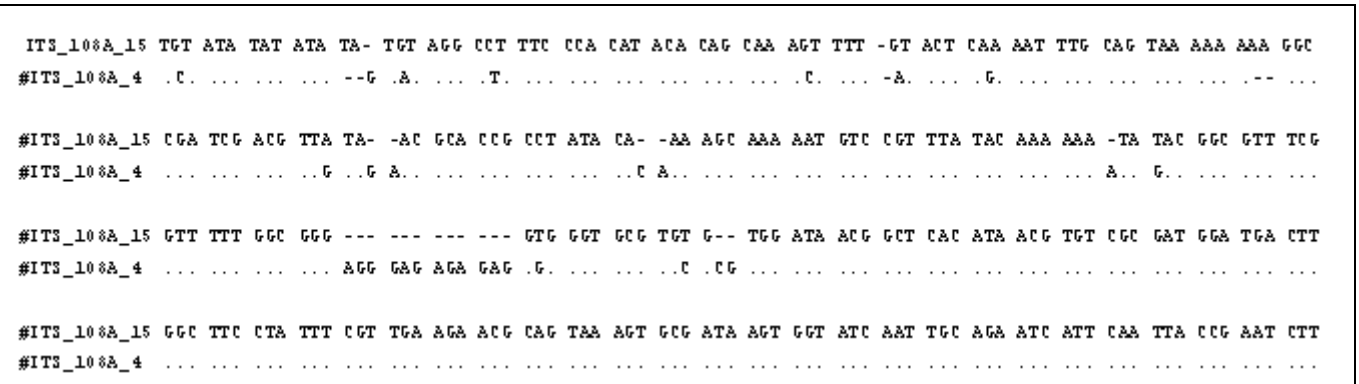


Figure 31*: showing partial seqment of the two sequence patterns optained from the cloning of 108 CL sample, the upper sequence is clony N° 15 L.donovani, the lower sequence is colony N° 4 correspond to L.major.*

Colonies of the *L. major* groups (21 colony) were alligned. Five polymorphic sites were detected. (Figure 29). One sequence related to *L. donovani* (108 15) was found to be modified type E. It has poly TA similar to type C or D (i.e. contains 3 TA repeats instead of 4).

Figure 32

First site Poly TA	Second site	Third site Poly AT*	Forth site**	Fifth site***
TCTATATATATA**TA**GT$^{(19)}$ TCTATATATATA--GT$^{(2)}$	CTTTT-AT$^{(20)}$ CTTTT**T**AT$^{(1)}$	CATATATATATATA**TATATA**TGT$^{(2)}$ CATATATATATATA**TATA**--TGT$^{(5)}$ CATATATATATATA**TA**----TGT$^{(11)}$ CATATATATATATA------TGT$^{(3)}$	AGA**AGA**CA$^{(11)}$ AGA---CA $^{(12)}$	GGTATATATATAT**AT**GT$^{(20)}$ 1.GGTATATATATAT—GT$^{(1)}$

Figure 32: *108 CL polymorphic sites of L.major related colonies at positions 8, 54, 491, 712 and 864 respectively. Polymorphic sites were shown in bold letters and underlined dots. Number between brackets indicates the number of colonies with the same sequence.* * indicates similar sites in 109 and 110 samples.

ITS 109 CL

Thirteen colonies were studied, and as in 108, *L. major* and *L. donovani* sequence patterns were detected. Ten sequences correspond to *L. major* with two polymorphic sites (Figure 33)

	Poly AT*	***
109	CATATATATATATATATA**TA**TGT$^{(4)}$	GGTATATATATA**TA****T**GT$^{(8)}$
	CATATATATATATATATA--TGT$^{(6)}$	GGTATATATATA--TGT$^{(2)}$

Figure 33*: 109 CL polymorphic sites of L.major related colonies . Polymorphic nucleotides were shown in bold letters and underlined dots. Number between brackets indicates the number of colonies with the same sequence. * indicates similar sites in 108 and 110 samples.*

Two of the three remanining sequences (109-25 and 109-28) were found to be modified type E and modified type F, respectively. The two sequences have poly TA similar to type C or D, i.e. contains 3 TA repeats instead of 4. The third sequence (109- 4) seemed to be a hybrid strain between *L. donovani* and *L. major*, it contains two genetic blocks, the first consist of 612 nucleotides identical to *L. donovani*, and the second block of 290 nucleotides identical to that of *L. major* (Figure34 and Figure 35)

Figure 34

612 pb nucleotide 100% L.donovani **CTTT** TT CT **CTTT** 290 pb nucleotides 100% L.major

Figure 34: the structure of a hybrid sequence obtained from cloning of sample 109

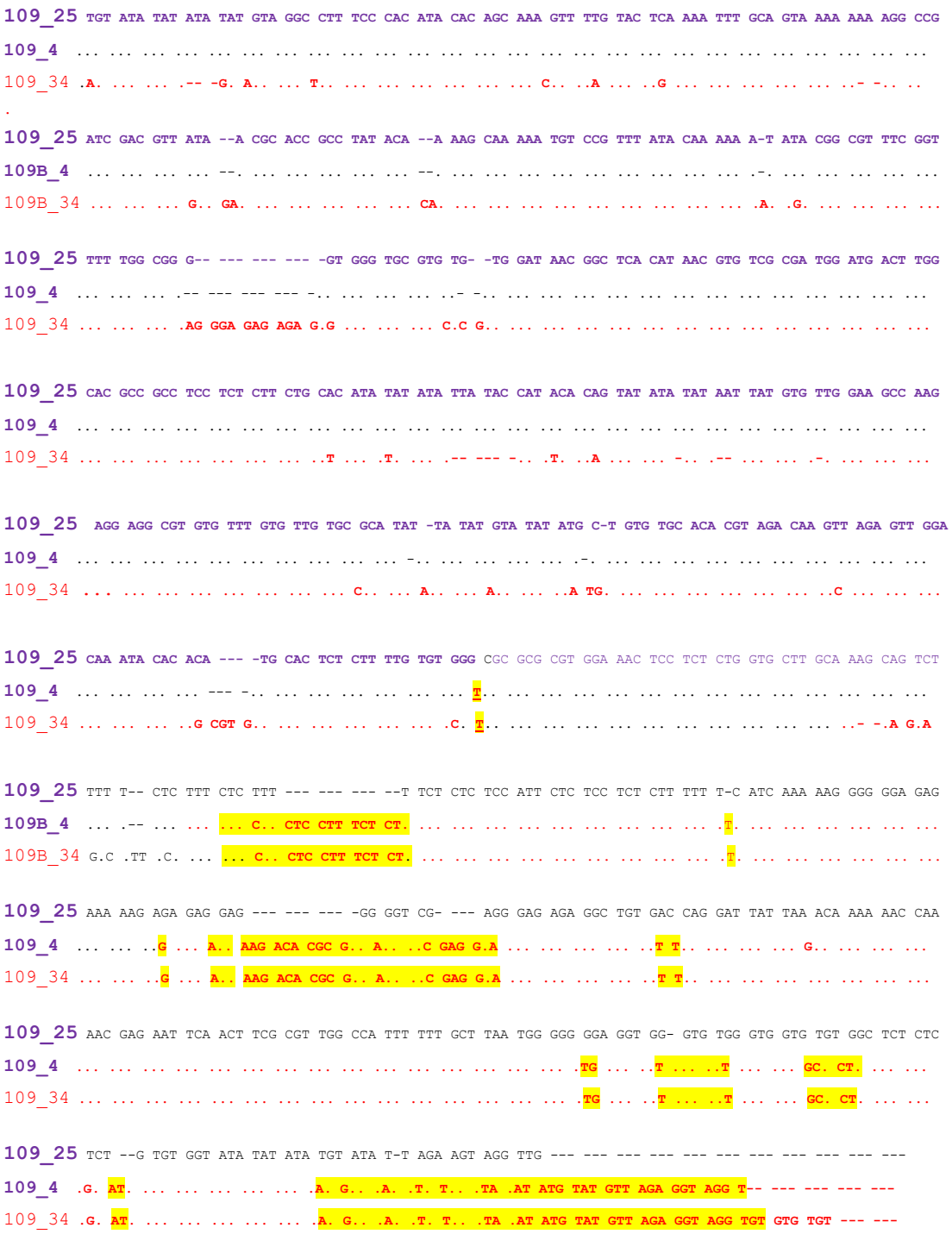


Figure 35: *hybrid sequence found in one colony of the cutaneous sample (colony N° 4) compared to two sequence patterns from the same sample attributed to L.donovani (colony N° 25) and to L.major (colony N° 34). Dots indicate similar sequence to L.donovani and the highlighted part similar sequence to L.major.*

ITS 110 CL

Fourteen colonies were studied, one sequence corresponds to *L. donovani* and thirteen to *L. major*. Three polymorphic sites of the *L. major* group were detected.

Figure 36

	Poly AT*	**	PolyA$_1$ Poly A$_2$
110	CATATATATATATATATATGT $^{(5)}$	AGAAGACA$^{(7)}$	ACAAAAAAAA**A**CCAAAAACG$^{(1)}$
	CATATATATATATATAT—GT$^{(7)}$	AGA---CA$^{(6)}$	ACAAAAAAA-CCAAAAACG$^{(1)}$
	CATATATATATATAT----GT$^{(1)}$		ACAAAAAAA-CCAAAA--CG$^{(2)}$
			ACAAAAAA--CCAAAA-CG$^{(9)}$

Figure 36*: 110CL polymorphic sitesof L.major related colonies. Polymorphic sites were shown in bold letters and underlined dots. Number between brackets indicates the number of colonies with the same sequence.* * indicates similar sites in 108 and 109 samples

The sequence related to *L. donovani* (110-2) was found to be modified type E. It has poly TA similar to type C or D (i.e., contains 3 TA repeats instead of 4).

Phylogeny of Cutaneous Samples

The phylogenetic analysis of the ITS cloning sequences (samples108, 109 and 110) revealed two different sequence patterns, corresponding to *L. donovani* with 99.5-100% genetic similarity to *L. donovani* AJ634357 GenBank™ reference sequences and 99.8-100% to *L. major* FJ753391 GenBank™ reference sequences. The genetic similarity between *L. major* and *L. donovani* used in this study was 93.5% (Figure 37).

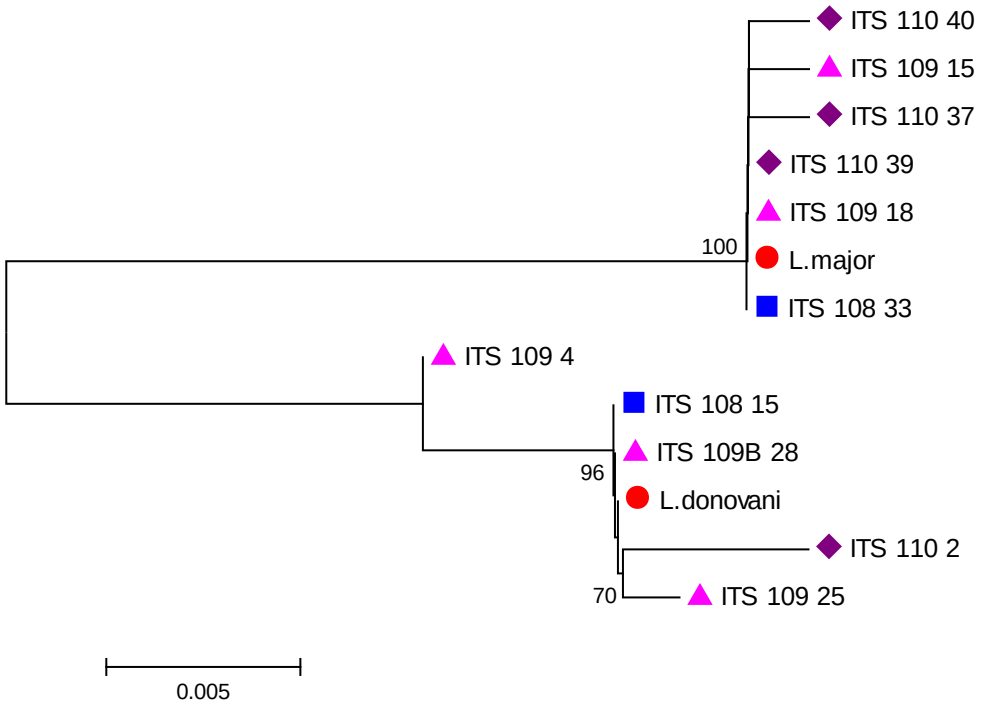


Figure 37: *Phylogenetic tree of ITS cloning sequence of cutaneous samples. Phylogenetic tree (unrouted) based on a fragment of 970 bp of ITS gene of Leishmania parasite. Sequence dataset was analyzed using MEGA 5, the neighbour-joining (NJ) method, and bootstrap analysis (1,000 replicates) based on the ClustalW algorithm. Significant bootstrapping values (>70%) are shown on the nodes. Only representative sequences from each cloned sample were used, the same structure cloured icon indicates sequences from the same patient. (●) Leishmania GenBank™ reference sequence (L.donovani GenBank™ accession number AJ634357; L.major GenBank™ accession number FJ75339*

3.6 GP63 Results

PCR Results

The PCRs targeting the Gp63 produced the expected amplificates (Figure 39) in all tested samples.

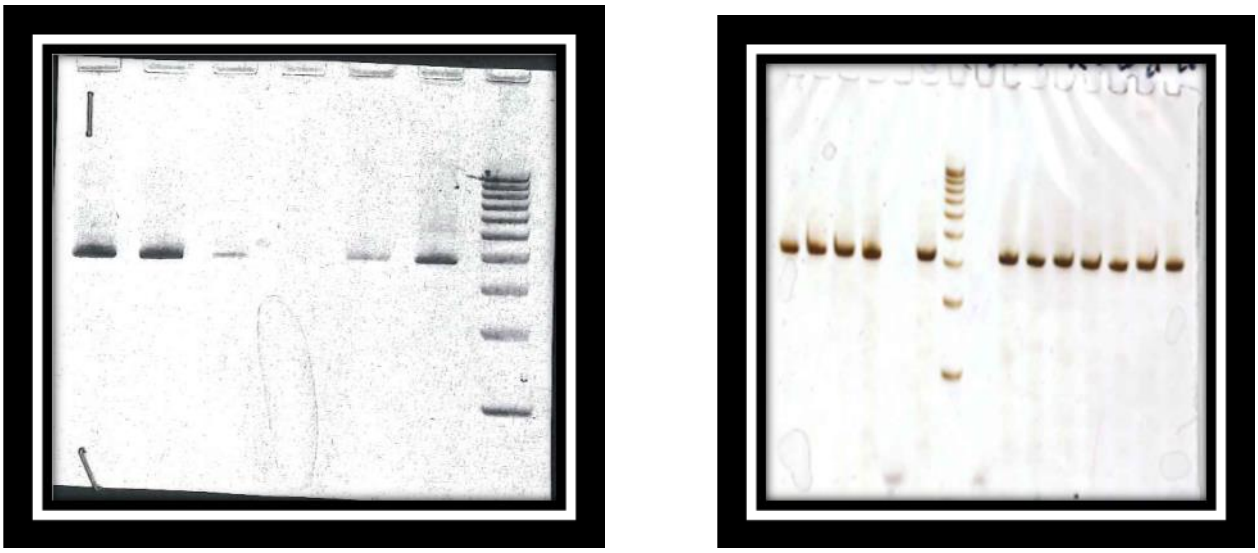


Figure 38: *showing some of the amplificates obtained by the primers gp63 1/2 to the left and primers gp63 3/4 to the right. Amplificates were loaded in acrylamide gel and stained by silver staining. Positive control in position N° 1 and the molecular ruler in position N° 7 in both gels.*

GP63 Sequence Results

Seventeen sequences were found to possess multi nucleotidic superposition (from position 810 to position 895 on the gp63 nucleotidic sequence, positions given according to the GenBank gp63 sequence accession number GQ301544) (Figure 40).

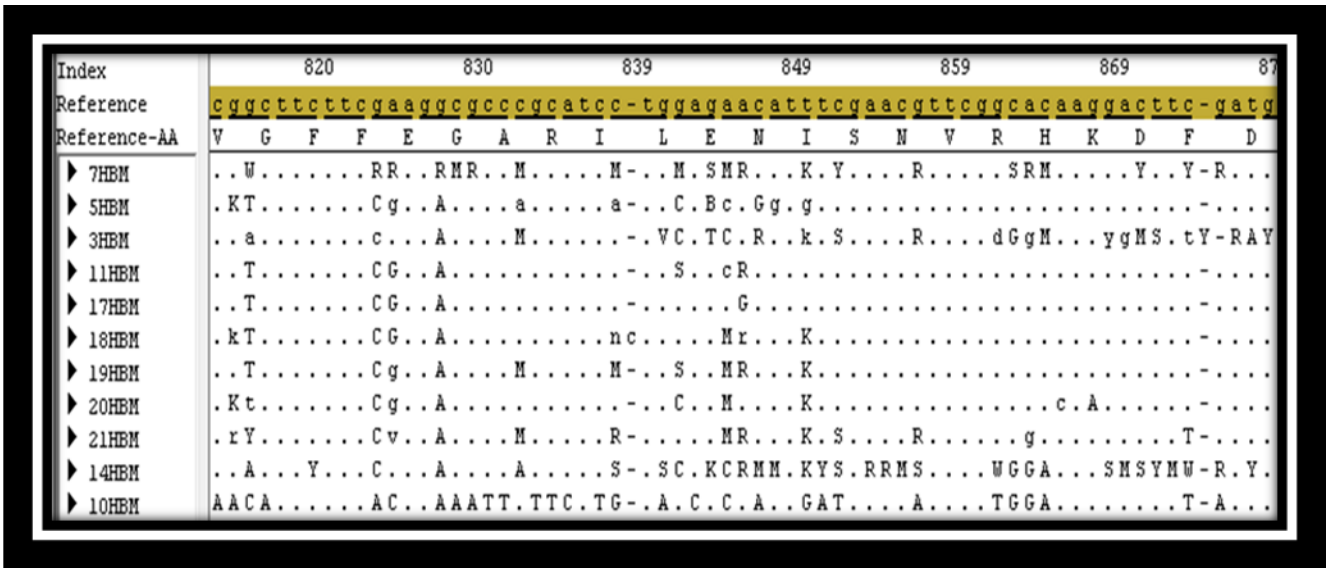


Figure 39: *schematic demonstration of gp63 3/4 sequences obtained by direct sequencing of some VL isolates showing an example of nucleotide's*
overlapping from position 810 to position 895 on the gp63 nucleotidic sequence, positions given according to the GenBankTM gp63 sequence
accession number GQ301544).

GP63 cloning Results

A total of 149 sequences were successfully obtained from colonies of the DNA from the six patients. The number of the sequence patterns obtained from each patient is shown in Table 7

	patient code	N° of sequences from cloning
Patient 1	3HBM	28
Patient 2	7HBM	19
Patient 3	10HBM	12
Patient 4	11HBM	29
Patient 5	14 HBM	26
Patient 6	17 HBM	35

Table 7: *number of the sequence patterns obtained from each patient*

GP63 Analysis

As, we aligned our sequences with those of the gp63 ^{SHORT} (Mauricio et al, 2007). Mauricio et al (2007) have determined the two groups of the GP63, i.e the gp63 ^{SHORT} and the gp63 ^{EXT} of the Old World Leishmania, using thirty-three strains of the *L. donovani* complex of diverse origins and zymodemes, thirty seven reference strains of other OW Leishmania and published sequences for gp63^{EXT} of *L. major* and *L. mexicana*). Our GP63 sequences belonged to six different clusters, one cluster associated with *L. infantum* genetic group while three clusters associated with *L. donovani* genetic groups. The remaining two clusters (group 1 and group 2) remains within the *L. donovani complex*, but differ from both *L.donovani* and *L.infantum* (Figure 40). The GP63 between group mean distance is shown in Table 8

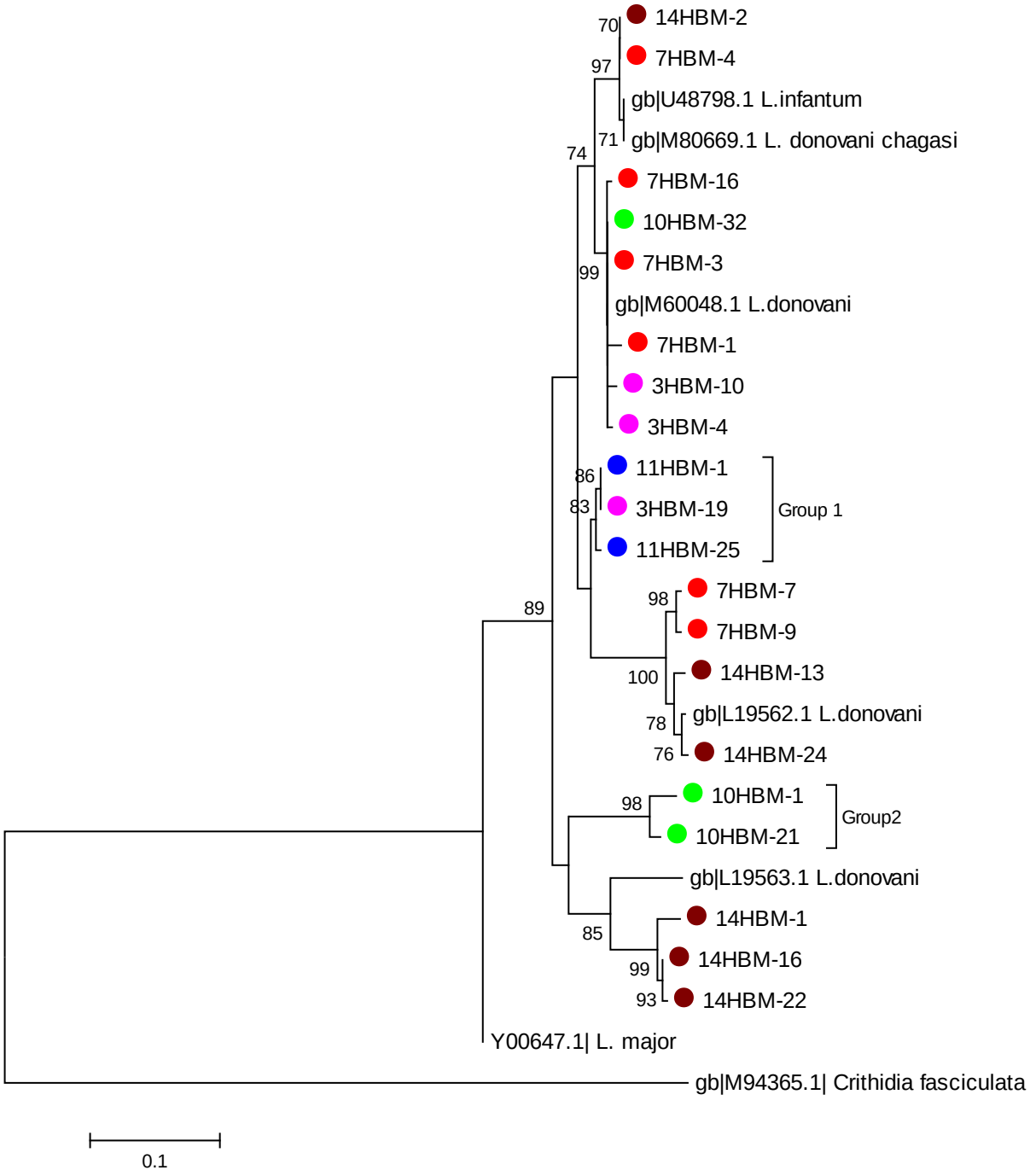


Figure 40: *Phylogenetic analysis of the sequences obtained from cloning of five samples from five VL patients and the gp63* ^{SHORT} *(Mauricio et al 2007) published sequences. The same colour indicates sequences obtained from the same patient. Phylogenetic tree based on a fragment of 370 bp of* gp63 ^{SHORT} *gene of Leishmania parasite. Sequence dataset was analyzed using MEGA 5, the neighbour-joining (NJ) method, and bootstrap analysis (1,000 replicates) based on the ClustalW algorithm. Significant bootstrapping values (>70%) are shown on the nodes.*

gp63 ^{SHORT} groups	1	2	3	4	5	6
1.L.infantum cluster						
2.L.donovani M60048 cluster	3.6					
3.L.donovani 19562.1 cluster	12.7	11.1				
4.L.donovani 19563.1 cluster	14.0	13.4	14.5			
5.Group1	7.2	4.3	7.7	13.2		
6.Group2	14.1	14.3	18.5	19.1	14.2	

Table 8: gp63 between groups mean distance

3.7 MSPC Results

Twenty nine and nineteen sequences were obtained from the colonies of the 14 HBM and 11HBM samples, respectively. Sequences were aligned with the the GP63 ^{EXT} published sequences (Mauricio et al, 2007) (Figure 41 and Figure 42).

All 14 HBM sequences were clustered into one group, GP63 ^{EXT} MON 31and MON 83 (Mauricio et al, 2007). The within group mean distance is 0.9 for both, and all 11 HBM sequences 18 30

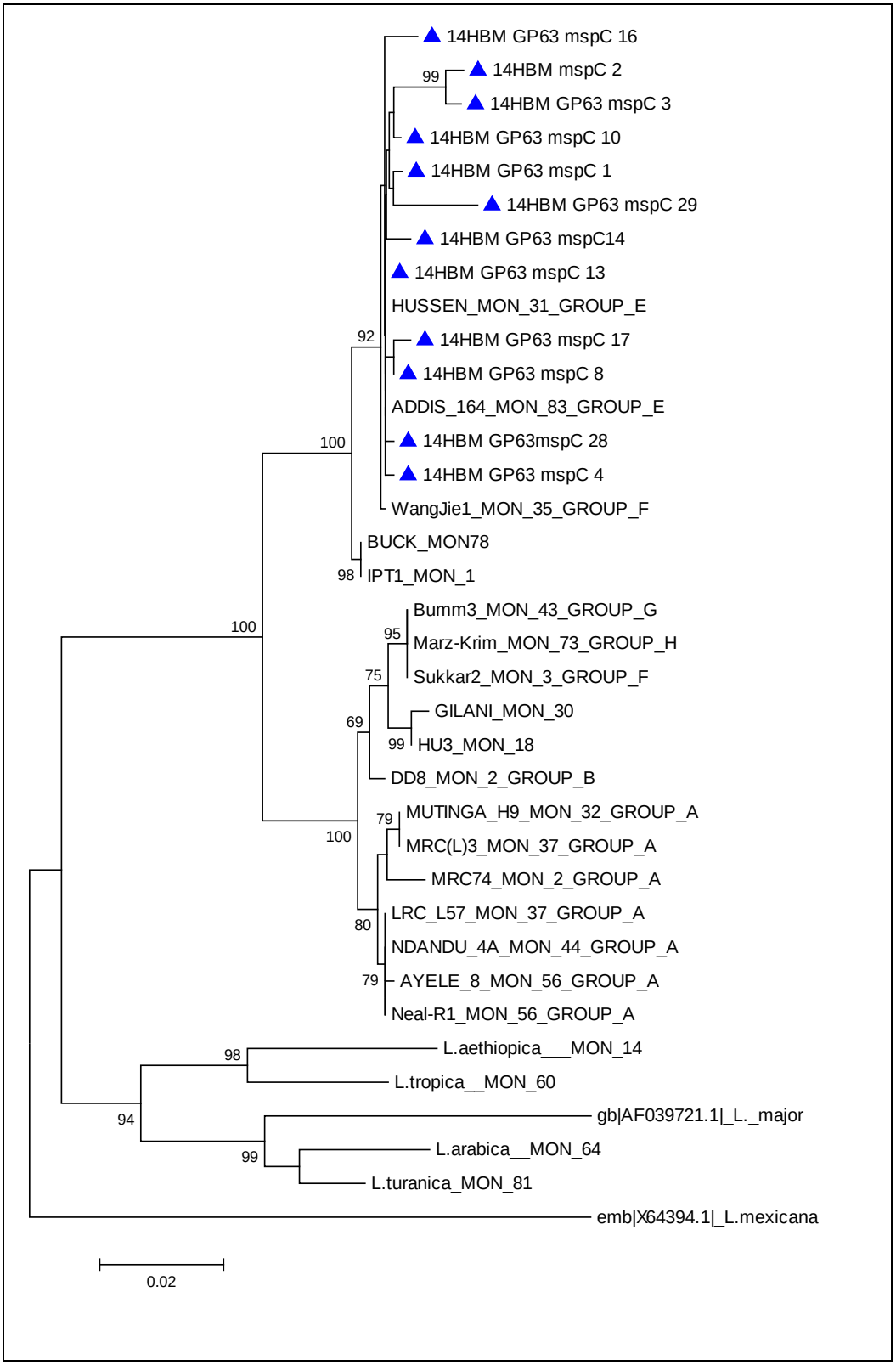


Figure 41: *Phylogenetic analysis of the sequences obtained from cloning of sample 14 HBM and the gp63* EXT published sequences *(Mauricio et al 2007). MON Zymodemes are shown., gp63 ext groups are shown in letters*

from A-H. Phylogenetic tree based on a fragment of 1044 bp of gp63 gene of Leishmania parasite. Sequence dataset was analyzed using MEGA 5, the neighbour-joining (NJ) method, and bootstrap analysis (1,000 replicates) based on the ClustalW algorithm. Significant bootstrapping values (>70%) are shown on the nodes.

Figure 42

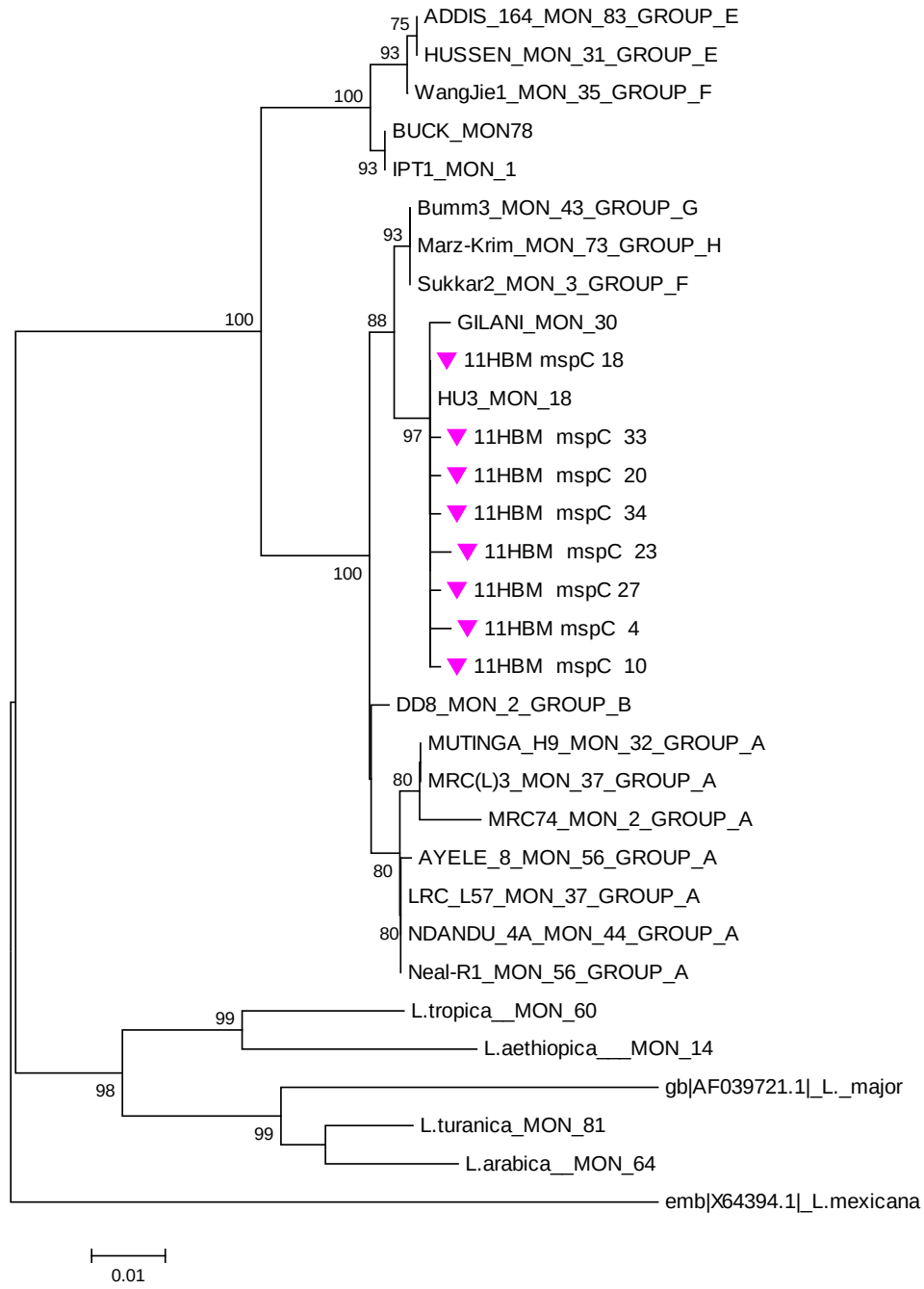


Figure 42: *Phylogenetic analysis of the sequences obtained from cloning of sample 11 HBM and the gp63* [EXT] published sequences *(Mauricio et al 2007). MON Zymodemes are shown., gp63 ext groups are shown in letters from A-H. Phylogenetic tree based on a fragment of 1044 bp of gp63 gene of Leishmania parasite. Sequence dataset was analyzed using MEGA 5, the neighbour-joining (NJ) method, and bootstrap analysis (1,000replicates) based on the ClustalW algorithm. Significant bootstrapping values (>70%) are shown on the nodes.*

CHAPTER FOUR

DISCUSSION

DISCUSSION

This chapter will summarize findings of this study and will discuss issues raised as the presence of mixed infection, the frequency and the transmission route in nature of such infections, the role of the culture media and other selection pressures in Leishmaniasis and the robustness of the present taxonomy.

In the present study, five CL samples from five patients have been examined, three of them have been cloned for COII and ITS genes. The five patients were presented with typical localized cutaneous ulcers with neither evident signs of systemic illness or immunosuppression nor VL. All patients originated from a VL non-endemic areas and had no history of travelling to a known VL endemic area. However, even if visceral involvement or immunosuppression were not clinically suspected, no other clinical or laboratory examinations were carried out.

COII and ITS analysis of two CL samples (107CL and 111CL) confirmed *L. donovani* as the merely etiological agent, findings concordant with other's results (El Amin *et al.,* 2004). moreover, the ITS analysis showed that at least two different strains / hybrids of *L. donovani* co-exist within the same patient..

ITS sequence data of these samples (107CL and 111CL) showed different ITS results

The 107 sample which has been extracted after being mass cultured, showed dominant *L. donovani* type E and absence of type F (type E is characteristic of *L. infantum*, MON 30, MON81 and *L. archibaldi* MON 82, while type F is characteristic of *L. infantum* MON 30 and MON 267 and *L. donovani* MON 18 according to Kuhls et al 2005 characetrization). GG and GGG insertions were intensively present at poly G_2 and some sequences showed a unique poly TA pattern.

Regarding Sample 111CL that has been extracted at the primary phase of culture, both type E and type F were fairly presented among colonies. Also GGG insertion at poly G_2 was detected. Polymorphisms at poly A witnessed both insertions and deletions of A in some uncharacterized sequences. We can conclude that the ITS sequence data support the presence of more than one strain of *L. donovani* in some CL patients.

We also assessed the presence of mixed infection with two *Leishmania s*pecies from a single cutaneous aspirate in the other three CL patients (samples 108, 109 and 110). The COII and the ITS genes analysis gave concordant results and confirmed -for the first time- naturally mixed infection

with *L. major* and L. *donovani*. (Figure 17 and Figure 38). The ITS sequence data analysis of *L. major* related colonies in the three CL samples revealed the presence of more than one strain /hybrid of *L. major* as different polymorphic sites were detected within and between patients (five polymorphic sites in 108, two in 109 and three in 110 samples) (Figures 32, 33 and 36). Only few *L. donovani* related colonies were detected possessing polymorphic sites belonging in some sites to type E, while in other sites to type D or C (type D is characteristic of *L. archibaldi MON 82, MON 258, MON 257, L. donovani MON 274* while type C is characteristic of *L. donovani MON 35*).

Most interestingly, one hybrid strain containing *L. donovani* sequence at the initial part and *L. major* at the last part was detected in one CL patient (sample 109 Figure 34).

L. infantum similar COII sequence has been found in one VL sample (14 HBM), regarding the same sample, the GP63 sequence data analysis confirmed some colonies as *L. infantum* (Figure 40). At the same time, two out of the six identified polymorphic sites (poly G_2 and poly TA) in the ITS were similar to those of types A, B, (types A and B were characteristic for *L. infantum* different zymodems) (Figure 22). On the other hand, the MSPC gene put this sample (14HBM) within one group belonging to *L. donovani MON 31 and MON 83.* As the 14HBM, another VL sample, namely 7HBM, shared some colonies with *L. infantum* genetic group in the GP63 (Figure 40).

Although COII gene analysis identified ***L. donovani*** as the merely causative agent of the PKDL (sample 105) and ML (sample 106) the number of samples from these forms was not sufficient to draw a conclusion., However, ITS data analysis pointed type E as the dominant type in both samples, but considerable different sequence patterns were detected between colonies from the two samples, poly G2 in sample 105 witnessed insertion of G,GG and deletion of G while positions 63 and 850 witnessed insertions of AT, A in the first sample and TCT, T and C at the second sample (Figure 26)

For other two samples (3HBM and 17HBM) attributed to VL identified by the COII gene as *L. donovani*, ITS data pointed in the first sample types E and D as the dominant types, while in the second sample type F. Insertion of G at poly G_2 was present in both samples (Figures 21, 23).

Two genetic groups, both within the *L. donovani complex* but different from *L. infantum* and *L. donovani* in the Gp63 sequence data analysis of some colonies attributed to VL samples (namely 10 HBM, 3HBM and 11HBM) have been identified (Figure 40). In the MSPC gene, sample 11 HBM colonies clustered within *L. donovani* MON 30 and MON 18 (Figure 42)

It has been generally accepted that Leishmaniasis in Sudan is caused by *L. major* and by the members of *L. donovani complex*, even if the presence of *L. infantum* has been denied by some authors (Jamjoom et al., 2004). No other species were reported. *L. major* and *L. donovani* have different transmission vectors and distinct biologic properties and epidemiologic features, both have been reported as etiologic agents of CL in Sudan. CL due to *L. major* LON1 is transmitted by *P. papatasi* in arid and semi-arid areas and circulates among rodents (el-Hassan& Zijlstra, 2001), while the vector that transmit CL due to *L. donovani* has not been established yet. On the other hand, Sudanese *L. donovani complex* are considered to be genetically homogenous group regardless the wide differences in clinical manifestations.

Co-infection with different *Leishmania* has been reported in closely related *Leishmania* species such as *L. braziliensis* and *L. panamensis* (Belli *et al*., 1994) and *L. major* and *L. arabica* (Shirian *et al*., 2012). The only two reports on concomitant natural infection with *L. donovani* and *L. major* were reported in Iraq where the two species were isolated from two different sites, the bone marrow and cutaneous lesions of a Kala azar suffering patients (Al-Diwany J. *et al*; 1995) and in Kenya where isolation succeeded from spleen aspirate culture from a clinically relapsed Kala azar suffering patient (Mebrahtu *et al.,* 1991). Concomitant infection with divergent Leishmania species has also been reported. The two species L. infantum and L. major have been isolated from the spleen of VL/HIV immune-compromised patient (Ravel *et al*., 2006).

Most laboratories in practice rely on culture for *Leishmania* isolation, diagnosis and DNA extraction for genetic typing. However, it has been demonstrated that in culture some strains overgrow and tend to eliminate other strains (Ibrahim *et al*., 1994). Previous studies carried out on the gp63 gene in Sudanese patients from the same area resulted on genetic homogeneity of the *Leishmania* isolates (PhD thesis, Khartoum University, unpublished data). Differently, in our study DNA was extracted directly from biological material, likely maintaining intact the different strains composition of the samples. The selection of a single strain in culture, if corroborated by other evidences, could also make necessary to reconsider the association of *Leishmania* species with clinical manifestations, such as *L. donovani* causing CL (Elamin *et al*, 2008) or *L.infantum* and PKDL (Dereure *et al*, 2003)

In the present study, the two species were likely maintained because the cultured parasites were harvested and extracted during early growth at the third day of culture. However, after cloning, the number of colonies related to *L. major* genetic group was higher compared to that related to *L. donovani* genetic group in both COII and ITS genes. This may suggest the dominance

of *L. major* species in the co-culture; however, it is also possible that the PCR conditions were more efficient towards *L. major* genome.

The occurrence and frequency of natural infection with more than one *Leishmania*, especially where foci of two species overlap, is believed to be more prevalent than reported and it has been demonstrated that in co-cultivation of more than one *Leishmania*, the dominant species tend to inhibit the growth of the other; as a consequence, the degree of laboratory detection of such phenomenon remains unclear and likely underestimated (Pacheco *et al*., 1987; Ibrahim *et al*; 1994).

The transmission route of naturally mixed infection remains debated. However, the presence of genetically different populations of *P. papatasi* in Sudan has been recently reported (Khalid NM *et al.*, 2012). Moreover, high polymorphism has been attributed to *L. major* causing cutaneous diseases in Iran (Oryan A *et al*., 2013).

P. papatasi is not a vector of visceral leishmaniasis and is refractory to the infection by *L. infantum* and *L. donovani* (Pimenta *et al*., 1994; Myskova *et al*; 2007). However, an experimental evidence of *P. papatasi* infection with hybrid strains of *L. infantum/L. major* causing visceral disease in *HIV*-positive patients (Volf *et al*., 2007) has been documented. Based on these results *P. papatasi* has been suggested as vector of this hybrid in nature (Volf *et al*., 2007).

In our study several different strains of *L. donovani* and *of L.major* were simultaneously found within the same patient and for most of them it was not possible the identification at the species level. This in one hand confirms that some genes can provide the basis for genotyping of strains within the *L. donovani* complex, but also highlights the internationally recognized need for a revision of the taxonomic status of *Leishmania* species (Schonian *et al*., 2010; Van der Auwera *et al.*, 2011; Boitè *et al*., 2012; Almeida and Araujo, 2012).

High percentage of VL Sudanese patients develop PKDL shortly after treatment. However, the responsibility of some strains to the subsequent onset of PKDL or other clinical manifestations remains an open question and need further insights.

GP63 ^{SHORT} and MSPC (also known as Gp63^{EXT}) are the two classes of the GP63 gene family in the subgenus L. (Leishmania) (Medina-Acosta *et al*., 1993). In the *L. infantum* genome, four identical copies of the GP63^{SHORT} were identified while the MSPC seems to be single copy gene (Ramamoorthy *et al.*, 1992). In the present study, the GP63 ^{SHORT} phylogenetic analysis of the 14 HBM samples gave different sequence patterns grouped into three main groups, while the MSPC analysis gave only one group. This could be explained by either that the 14HBM sample contains

more than one genome with much similar MSPC sequence or that the same genome contained non identical GP63^{SHORT} copies, even though finding of other genes supported the hypothesis that this sample could contain more than one genome, at least one of them contain non identical copies.

Biomolecular Discussion

The modified real time PCR used in this study revealed to be a useful tool for the screening of a large number of samples. The Cytochrome Oxidase gene used in this study for differentiating the positive isolates at species level and it was not able to reveal the intraspecific polymorphism. While the GP63 ^{SHORT} and the ITS genes are suitable genes for intraspecific polymorphism and strains detection. The MSPC gene showed gene conservation in the two tesed VL samples. However, the data obtained from the kDNA gene that has been abondonated seemed to supports finding of this study, even if no cloning technique was done

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CHAPTER FIVE

CONCLUSION

CONCLUSION

This study attempted to understand the correlation between different clinical manifestations and the genetic profile of *Leishmania* positive isolates from Sudanese patients diagnosed with different types of leishmaniasis using the COII, the ITS and the GP63 (gp63 3-4 and MSPC) genes as targets for characterization and identification of intraspecific polymorphisms using direct sequencing and cloning techniques. From the results we can in summary conclude:

- The study affirmed the presence of mixed infection with *L. donovani* and *L. major* from a single cutaneous aspirate in CL patients. To the best of our knowledge this is the first report of *L. donovani* and *L. major* mixed-infection in Sudan. Additionally no other cases of such mixed-infection from the same cutaneous sample were reported elsewhere.
- The study assessed the circulation of more than one strain/hybrid of *L. major* and *L. donovani* between Sudanese patients.
- The presence of *L. infantum* as an etiological agent of VL in Sudan was confirmed by CO II and ITS genes.
- *Leishmania* isolates with mixed species or strains might be subjected to selective pressure upon parasite culture, so direct extraction from biological materials is important for characterization.

These findings have important implications regarding the diagnosis, the choice of the most appropriate therapy and the possibility of developing drug resistance. The presence of *L. donovani* in all the five cutaneous samples examined suggests caution in the follow up of CL patients who could later develop VL symptoms.

Many aspects of leishmaniasis in Sudan still need to be explored. However, these findings have also set the stage for future studies such as the prevalence of different species and vectors and the competence of Phlebotomus spp. in transmitting different *Leishmania*.

References

Abdalla Hassan Sharief, El-Tahir Awad Gasim Khalil, Douglas Cameron Barker, Samia Ali Omer, Hamid Suliman Abdalla **and** Muntasir Eltayeb Ibrahim**. (2011).** Simple and Direct Characterization of Leishmania donovani Isolates Based on Cytochrome Oxidase II Gene Sequences. The Open Tropical Medicine Journal, 4:1-5

Ajdary S, Alimohammadian MH, Eslami MB, Kemp K, and Kharazmi A. 2000. Comparison of the immune profile of nonhealing cutaneous Leishmaniasis patients with those with active lesions and those who have recovered from infection. Infection and immunity 68: 1760-1764.

Alborzi A, Pouladfar GR, Ghadimi Moghadam A, Attar A, Drakhshan N, Khosravi Maharlooei M, and Kalantari M. 2013. First molecular-based detection of mucocutaneous leishmaniasis caused by Leishmania major in Iran. Journal of infection in developing countries 7: 413-416.

Al-Diwany LJ, Al-Awkati NA, Atia M, and Rassam MB. 1995. Concomitant natural infection with L. donovani and L. major: a case report from Iraq. Sozial-und Präventivmedizin 40: 234-238.

Allahverdiyev AM, Uzun S, Bagirova M, Durdu M, and Memisoglu HR. 2004. A sensitive new microculture method for diagnosis of cutaneous leishmaniasis. The American Journal of Tropical Medicine and Hygiene 70: 294-297.

Almedia LA, Araujor R. (2012). Highlights on molecular identification of closely related species. Infect Genet Evol.,

Alvar J, Aparicio P, Aseffa A, Den Boer M, Canavate C, Dedet JP, Gradoni L, Ter Horst R, Lopez-Velez R, and Moreno J. 2008. The relationship between leishmaniasis and AIDS: the second 10 years. Clinical microbiology reviews 21: 334-59, table of contents.

Alvar J, Canavate C, Molina R, Moreno J, and Nieto J. 2004. Canine leishmaniasis. Advances in Parasitology 57: 1-88.

Amato VS, Tuon FF, Bacha HA, Neto VA, and Nicodemo AC. 2008. Mucosal leishmaniasis: current scenario and prospects for treatment. Acta Tropica 105: 1-9.

Andresen K, Gasim S, Elhassan AM, Khalil EA, Barker DC, Theander TG, and Kharazmi A. 1997. Diagnosis of visceral leishmaniasis by the polymerase chain reaction using blood, bone marrow and lymph node samples from patients from the Sudan. Tropical medicine & international health : TM & IH 2: 440-444.

Antoine J, Prina E, Courret N, andLang T.2004. Leishmania spp.: on the Interactions They Establish with Antigen-Presenting Cells of their Mammalian Hosts. In: Anonymous Advances in Parasitology: Academic Press, 1-68.

Antoniou M, Doulgerakis C, Pratlong F, Dedet J, and Tselentis Y. 2004. Short report: treatment failure due to mixed infection by different strains of the parasite Leishmania infantum. The American Journal of Tropical Medicine and Hygiene 71: 71-72.

Aquino DM, Caldas AJ, Miranda JC, Silva AA, Barral-Netto M, and Barral A. 2010. Epidemiological study of the association between anti-Lutzomyia longipalpis saliva antibodies and development of delayed-type hypersensitivity to Leishmania antigen. The American Journal of Tropical Medicine and Hygiene 83: 825-827.

Arias JR, Naif RD, Miles MA, and de Souza AA. 1981. The opossum, Didelphis marsupialis (Marsupialia: Didelphidae), as a reservoir host of Leishmania braziliensis guyanensis in the Amazon Basin of Brazil. Transactions of the Royal Society of Tropical Medicine and Hygiene 75: 537-541.

Arnot DE, Barker DC, Arnot DE, and Barker DC. 1981. Biochemical identification of cutaneous leishmanias by analysis of kinetoplast DNA. II. Sequence homologies in Leishmania kDNA. Molecular and biochemical parasitology 3: 47-56.

Ashford RW, Schnur LF, Chance ML, Samaan SA, and Ahmed HN. 1977. Cutaneous leishmaniasis in the Libyan Arab Republic: preliminary ecological findings. Annals of Tropical Medicine and Parasitology 71: 265-271.

Ashford RW, Seaman J, Schorscher J, and Pratlong F. 1992. Epidemic visceral leishmaniasis in southern Sudan: identity and systematic position of the parasites from patients and vectors. Transactions of the Royal Society of Tropical Medicine and Hygiene 86: 379-380.

Ashford RW. 1996. Leishmaniasis reservoirs and their significance in control. Clinics in dermatology 14: 523-532.

Attar ZJ, Chance ML, el-Safi S, Carney J, Azazy A, El-Hadi M, Dourado C, and Hommel M. 2001. Latex agglutination test for the detection of urinary antigens in visceral leishmaniasis. Acta Tropica 78: 11-16.

Bacellar O, Lessa H, Schriefer A, Machado P, Ribeiro de Jesus A, Dutra WO, Gollob KJ, and Carvalho EM. 2002. Up-regulation of Th1-type responses in mucosal leishmaniasis patients. Infection and immunity 70: 6734-6740.

Badaro R, Jones TC, Carvalho EM, Sampaio D, Reed SG, Barral A, Teixeira R, and Johnson WD,Jr. 1986. New perspectives on a subclinical form of visceral leishmaniasis. The Journal of infectious diseases 154: 1003-1011.

Bañuls A, Hide M, and Prugnolle F.2007. Leishmania and the Leishmaniases: A Parasite Genetic Update and Advances in Taxonomy, Epidemiology and Pathogenicity in Humans. In: Anonymous Advances in Parasitology: Academic Press, 1-458.

Barral A, Honda E, Caldas A, Costa J, Vinhas V, Rowton ED, Valenzuela JG, Charlab R, Barral-Netto M, and Ribeiro JM. 2000. Human immune response to sand fly salivary gland antigens: a useful epidemiological marker? The American Journal of Tropical Medicine and Hygiene 62: 740-745.

Basu MK, and Ray M. 2005. Macrophage and Leishmania: an unacceptable coexistence. Critical reviews in microbiology 31: 145-154.

Belli AA, Miles MA, Kelly JM, 1994. A putative Leishmania panamensis/Leishmania braziliensis hybrid is a causative agent of human cutaneous Leishmaniasis in Nicaragua. Parasitology 109 (Pt 4): 435-442.

Berman J. 1997. Human leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last 10 years. Clinical infectious diseases 24: 684-703.

Berman JD, Dwyer D, and Wyler D. 1979. Multiplication of Leishmania in human macrophages in vitro. Infection and immunity 26: 375-379.

Blackwell JM, Fakiola M, Ibrahim ME, Jamieson SE, Jeronimo SB, Miller EN, Mishra A, Mohamed HS, Peacock CS, Raju M, Sundar S, and Wilson ME. 2009. Genetics and visceral leishmaniasis: of mice and

man. Parasite immunology 31: 254-266.

Blackwell JM, Mohamed HS, and Ibrahim ME. 2004. Genetics and visceral leishmaniasis in the Sudan: seeking a link. Trends in parasitology 20: 268-274.

Blaineau C, Bastien P, Rioux JA, Roizes G, and Pages M. 1991. Long-range restriction maps of size-variable homologous chromosomes in Leishmania infantum. Molecular and biochemical parasitology 46: 292-302.

Boitè MC, Mauricio IL, Miles MA, Cupollilo E. (2012). New insights on taxonomy, phylogeny and population genetics of leishmania (viannia) parasites based on multilocus sequence analysis. PLoS Negl Trop 6(11): e1888

Bray RS, Ashford RW, Mukherjee AM, and Sen Gupta PC. 1973. Studies on the immunology and serology of leishmaniasis. Serological investigation of the parasites of Indian kala-azar and Indian post-kala-azar dermal leishmaniasis. Transactions of the Royal Society of Tropical Medicine and Hygiene 67: 125-129.

Bray RS. 1974. Leishmania. Annual Review of Microbiology 28: 189-217.

Bucheton B, Abel L, El-Safi S, Kheir MM, Pavek S, Lemainque A, and Dessein AJ. 2003. A major susceptibility locus on chromosome 22q12 plays a critical role in the control of kala-azar. American Journal of Human Genetics 73: 1052-1060.

Bucheton B, Argiro L, Chevillard C, Marquet S, Kheir MM, Mergani A, El-Safi SH, and Dessein AJ. 2007. Identification of a novel G245R polymorphism in the IL-2 receptor beta membrane proximal domain associated with human visceral leishmaniasis. Genes and immunity 8: 79-83.

Cabello PH, Lima AM, Azevedo ES, and Krieger H. 1995. Familial aggregation of Leishmania chagasi infection in northeastern Brazil. The American Journal of Tropical Medicine and Hygiene 52: 364-365.

Carvalho EM, Johnson WD, Barreto E, Marsden PD, Costa JL, Reed S, and Rocha H. 1985. Cell mediated immunity in American cutaneous and mucosal leishmaniasis. Journal of immunology (Baltimore, Md.: 1950) 135: 4144-4148.

Cerf BJ, Jones TC, Badaro R, Sampaio D, Teixeira R, and Johnson WD,Jr. 1987. Malnutrition as a risk factor for severe visceral leishmaniasis. The Journal of infectious diseases 156: 1030-1033.

Chang K, and Dwyer DM. 1976. Multiplication of a human parasite (Leishmania donovani) in phagolysosomes of hamster macrophages in vitro. Science 193: 678-680.

Chang KP, and McGwire BS. 2002. Molecular determinants and regulation of Leishmania virulence. Kinetoplastid biology and disease 1: 1.

Costa DJ, Favali C, Clarencio J, Afonso L, Conceicao V, Miranda JC, Titus RG, Valenzuela J, Barral-Netto M, Barral A, and Brodskyn CI. 2004. Lutzomyia longipalpis salivary gland homogenate impairs cytokine production and costimulatory molecule expression on human monocytes and dendritic cells. Infection and immunity 72: 1298-1305.

Cunningham AC. 2002. Parasitic adaptive mechanisms in infection by leishmania. Experimental and molecular pathology 72: 132-141.

Cupolillo E, Momen H, and Grimaldi Jr G. 1998. Genetic diversity in natural populations of New World Leishmania. Memórias do Instituto Oswaldo Cruz 93: 663-668.

Dantas-Torres F, Lorusso V, Testini G, de Paiva-Cavalcanti M, Figueredo LA, Stanneck D, Mencke N, Brandao-Filho SP, Alves LC, and Otranto D. 2010. Detection of Leishmania infantum in Rhipicephalus

sanguineus ticks from Brazil and Italy. Parasitology research 106: 857-860.

Dantas-Torres F. 2011. Ticks as vectors of Leishmania parasites. Trends in parasitology 27: 155-159.

David CV, and Craft N. 2009. Cutaneous and mucocutaneous leishmaniasis. Dermatologic therapy 22: 491-502.

Davies CR, and Mazloumi Gavgani AS. 1999. Age, acquired immunity and the risk of visceral leishmaniasis: a prospective study in Iran. Parasitology 119 (Pt 3): 247-257.

De Colmenares M, Portus M, Riera C, Gallego M, Aisa MJ, 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. The American Journal of Tropical Medicine and Hygiene 52: 427-428.

De Ibarra AA, Howard JG, and Snary D. 1982. Monoclonal antibodies to Leishmania tropica major: specificities and antigen location. Parasitology 85 (Pt 3): 523-531.

Dereure J, Boni M, Pratlong F, el Hadi Osman M, Bucheton B, el-Safi S, Feugier E, Musa MK, Davoust B, Dessein A, and Dedet JP. 2000. Visceral leishmaniasis in Sudan: first identifications of Leishmania from dogs. Transactions of the Royal Society of Tropical Medicine and Hygiene 94: 154-155.

Dereure J, El-Safi SH, Bucheton B, Boni M, Kheir MM, Davoust B, Pratlong F, Feugier E, Lambert M, Dessein A, and Dedet JP. 2003. Visceral leishmaniasis in eastern Sudan: parasite identification in humans and dogs; host-parasite relationships. Microbes and infection / Institut Pasteur 5: 1103-1108.

Desjeux P. 1996. Leishmaniasis. Public health aspects and control. Clinics in dermatology 14: 417-423.

Desjeux P. 2001. The increase in risk factors for leishmaniasis worldwide. Transactions of the Royal Society of Tropical Medicine and Hygiene 95: 239-243.

Desjeux P. 2004. Leishmaniasis. Nature reviews.Microbiology 2: 692.

Desjeux P. 2004. Leishmaniasis: current situation and new perspectives. Comparative immunology, microbiology and infectious diseases 27: 305-318.

Desjeux, P. (1991). Information on the epidemiology and control of Leishmaniasis by country or territory.

WHO/Leish/91.30.

Dougall AM, Alexander B, Holt DC, Harris T, Sultan AH, Bates PA, Rose K, and Walton SF. 2011. Evidence incriminating midges (Diptera: Ceratopogonidae) as potential vectors of Leishmania in Australia. International journal for parasitology 41: 571-579.

Dowlati Y. 1996. Cutaneous leishmaniasis: clinical aspect. Clinics in dermatology 14: 425-431.

Ehlert N, Seilmaier M, Guggemos W, Loscher T, Meurer A, and Wendtner CM. 2013. Severe oral mucositis in a patient with HIV infection. Deutsche medizinische Wochenschrift (1946) 138: 1601-1605.

el Harith A, Kolk AH, Leeuwenburg J, Muigai R, Huigen E, Jelsma T, and Kager PA. 1988. Improvement of a direct agglutination test for field studies of visceral leishmaniasis. Journal of clinical microbiology 26: 1321-1325.

El Hassan AM, and Khalil EA. 2001. Post-kala-azar dermal leishmaniasis: does it play a role in the transmission of Leishmania donovani in the Sudan? Tropical medicine & international health : TM & IH 6: 743-744.

el Hassan AM, Ghalib HW, Zijlstra EE, Eltoum IA, Satti M, Ali MS, and Ali HM. 1992. Post kala-azar dermal leishmaniasis in the Sudan: clinical features, pathology and treatment. Transactions of the Royal Society of Tropical Medicine and Hygiene 86: 245-248.

El Tai NO, El Fari M, Mauricio I, Miles MA, Oskam L, El Safi SH, Presber WH, Schonian G, 2001. Leishmania donovani: Intraspecific polymorphisms of Sudanese isolates revealed by PCR-based analyses and DNA sequencing. Exp Parasitol 97: 35-44.

El Tai NO, Osman OF, el Fari M, Presber W, Schönian G. 2000. Genetic heterogeneity of ribosomal internal transcribed spacer in clinical samples of Leishmania donovani spotted on filter paper as revealed by single-strand conformation polymorphisms and sequencing. Trans R Soc Trop Med Hyg. 2000 Sep-Oct;94(5):575-9.

Elamin EM, Guerbouj S, Musa AM, Guizani I, Khalil EA, Mukhtar MM, Elkadaro AM, Mohamed HS, Ibrahim ME, Abdel Hamid MM, El Azhari M, and El Hassan AM. 2005. Uncommon clinical presentations of cutaneous leishmaniasis in Sudan. Transactions of the Royal Society of Tropical Medicine and Hygiene 99: 803-808.

Elamin, E. M., Guizani, I., Guerbouj, S., Gramiccia, M., El Hassan, A. M., Di Muccio, T., et al. (2008). Identification of leishmania donovani as a cause of cutaneous leishmaniasis in sudan. Transactions of the Royal Society of Tropical Medicine and Hygiene, 102(1), 54-57.

el-Hassan AM, and Zijlstra EE. 2001. Leishmaniasis in Sudan. Cutaneous leishmaniasis. Transactions of the Royal Society of Tropical Medicine and Hygiene 95 Suppl 1: S1-17.

el-Hassan AM, Meredith SE, Yagi HI, Khalil EA, Ghalib HW, Abbas K, Zijlstra EE, Kroon CC, Schoone GJ, and Ismail A. 1995. Sudanese mucosal leishmaniasis: epidemiology, clinical features, diagnosis, immune responses and treatment. Transactions of the Royal Society of Tropical Medicine and Hygiene 89: 647-652.

el-Hassan AM, Yagi HI, Zijlstra EE, and el-Kadarow AI. 1994. Leishmaniasis of the nose caused by different species of Leishmania in the Sudan. Distinguishing features of the different clinical forms. Tropical and geographical medicine 46: 33-36.

el-Hassan AM, Zijlstra EE, Ismael A, and Ghalib HW. 1995. Recent observations on the epidemiology of kala-azar in the eastern and central states of the Sudan. Tropical and geographical medicine 47: 151-156.

Elnaiem D, Hassan M, Maingon R, Nureldin G, Mekawi A, Miles M, and Ward R. 2001. The Egyptian mongoose, Herpestes ichneumon, is a possible reservoir host of visceral leishmaniasis in eastern Sudan. Parasitology 122: 531-536.

Elnaiem DA, Ward RD, Hassan HK, Miles MA, and Frame IA. 1998a. Infection rates of Leishmania donovani in Phlebotomus orientalis from a focus of visceral leishmaniasis in eastern Sudan. Annals of Tropical Medicine and Parasitology 92: 229-232.

Elnaiem DA, Connor SJ, Thomson MC, Hassan MM, Hassan HK, Aboud MA, and Ashford RW. 1998b. Environmental determinants of the distribution of Phlebotomus orientalis in Sudan. Annals of Tropical Medicine and Parasitology 92: 877-887.

Elnaiem DA, Hassan HK, and Ward RD. 1997. Phlebotomine sandflies in a focus of visceral leishmaniasis in a border area of eastern Sudan. Annals of Tropical Medicine and Parasitology 91: 307-318.

Elnaiem DE, Schorscher J, Bendall A, Obsomer V, Osman ME, Mekkawi AM, Connor SJ, Ashford RW, and Thomson MC. 2003. Risk mapping of visceral leishmaniasis: the role of local variation in rainfall and altitude on the presence and incidence of kala-azar in eastern Sudan. The American Journal of Tropical

Medicine and Hygiene 68: 10-17.

el-Safi SH, and Peters W. 1991. Studies on the leishmaniases in the Sudan. Epidemic of cutaneous leishmaniasis in Khartoum. Transactions of the Royal Society of Tropical Medicine and Hygiene 85: 44-47.

El-Sayed, S. M. (1990).Ecological studies on phlebotomine sandflies (Diptera; Psychodidae) of Surogia village (Khartoum Province) and Dinder National Park (Blue Nile Province), Sudan.Ph D thesis, University of Khartoum.

Enright AJ, Van Dongen S, and Ouzounis CA. 2002. An efficient algorithm for large-scale detection of protein families. Nucleic acids research 30: 1575-1584.

Evans D (1989). Handbook on isolation characterization and cryopreservation of Leishmania. UNDP/ World Bank/ WHO Special Program for Research and Training in Tropical Diseases (TDR).Genova http://apps.who.int/iris/bitstream/10665/60795/1/a33651.pdf

Fichet-Calvet E, Jomaa I, Ben Ismail R, and Ashford R. 2003. Leishmania major infection in the fat sand rat Psammomys obesus in Tunisia: interaction of host and parasite populations. Annals of Tropical Medicine and Parasitology 97: 593-603.

Follador I, Araujo C, Bacellar O, Araujo CB, Carvalho LP, Almeida RP, and Carvalho EM. 2002. Epidemiologic and immunologic findings for the subclinical form of Leishmania braziliensis infection. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 34: E54-8.

Gama ME, Costa JM, Gomes CM, and Corbett CE. 2004. Subclinical form of the American visceral leishmaniasis. Memorias do Instituto Oswaldo Cruz 99: 889-893.

Gasim S, Elhassan AM, Kharazmi A, Khalil EA, Ismail A, and Theander TG. 2000. The development of post-kala-azar dermal leishmaniasis (PKDL) is associated with acquisition of Leishmania reactivity by peripheral blood mononuclear cells (PBMC). Clinical and experimental immunology 119: 523-529.

Ghalib HW, Piuvezam MR, Skeiky YA, Siddig M, Hashim FA, el-Hassan AM, Russo DM, and Reed SG. 1993. Interleukin 10 production correlates with pathology in human Leishmania donovani infections. The Journal of clinical investigation 92: 324-329.

Ghosh MK, Nandy A, Addy M, Maitra TK, and Ghose AC. 1995. Subpopulations of T lymphocytes in the peripheral blood, dermal lesions and lymph nodes of post kala-azar dermal leishmaniasis patients. Scandinavian Journal of Immunology 41: 11-17.

Gomes ,Regis, and Oliveira ,Fabiano. The immune response to sand fly salivary proteins and its influence on Leishmania immunity..

Gomes R, Teixeira C, Teixeira MJ, Oliveira F, Menezes MJ, Silva C, de Oliveira CI, Miranda JC, Elnaiem DE, Kamhawi S, Valenzuela JG, and Brodskyn CI. 2008. Immunity to a salivary protein of a sand fly vector protects against the fatal outcome of visceral leishmaniasis in a hamster model. Proceedings of the National Academy of Sciences of the United States of America 105: 7845-7850.

Grevelink SA, and Lerner EA. 1996. Leishmaniasis. Journal of the American Academy of Dermatology 34: 257-272.

Guerin PJ, Olliaro P, Sundar S, Boelaert M, Croft SL, Desjeux P, Wasunna MK, and Bryceson AD. 2002. Visceral leishmaniasis: current status of control, diagnosis, and treatment, and a proposed research and development agenda. The Lancet Infectious Diseases 2: 494-501.

Guerra JA, Prestes SR, Silveira H, Coelho LI, Gama P, Moura A, Amato V, Barbosa M, and Ferreira LC. 2011. Mucosal Leishmaniasis caused by Leishmania (Viannia) braziliensis and Leishmania (Viannia)

guyanensis in the Brazilian Amazon. PLoS neglected tropical diseases 5: e980.

Haldar JP, Ghose S, Saha KC, and Ghose AC. 1983. Cell-mediated immune response in Indian kala-azar and post-kala-azar dermal leishmaniasis. Infection and immunity 42: 702-707.

Haldar JP, Saha KC, and Ghose AC. 1981. Serological profiles in Indian post kala-azar dermal leishmaniasis. Transactions of the Royal Society of Tropical Medicine and Hygiene 75: 514-517.

Handman E, Elso C, andFoote S.. Genes and Susceptibility to Leishmaniasis. In: Anonymous Advances in Parasitology: Academic Press, 1-75.

Hassan MM, Elraba'a FM, Ward RD, Maingon RD, and Elnaiem DA. 2004. Detection of high rates of in-village transmission of Leishmania donovani in eastern Sudan. Acta Tropica 92: 77-82.

Hassan MM, Osman OF, El-Raba'a FM, Schallig HD, and Elnaiem DE. 2009. Role of the domestic dog as a reservoir host of Leishmania donovani in eastern Sudan. Parasites & vectors 2: 26-3305-2-26.

Hassan MM, Widaa SO, Ibrahim MA, Abu Shara R, Osman OM, Numairy MS, and El Khider ET. 2007. Studies on the ecology of sandflies (Diptera: Psychodidae) in Sudan: the first records of Phlebotomus orientalis and P. rodhaini in northern Sudan. Annals of Tropical Medicine and Parasitology 101: 653-655.

Herwaldt BL. 1999. Leishmaniasis. Lancet 354: 1191-1199.

Herwaldt BL. 2001. Laboratory-acquired parasitic infections from accidental exposures. Clinical microbiology reviews 14: 659-688.

Hockmeyer WT, Wellde BT, Sabwa CL, Smith DH, Rees PH, and Kager PA. 1984. A complement fixation test for visceral leishmaniasis using homologous parasite antigen I. Annals of Tropical Medicine and Parasitology 78: 489-493.

Hoogsraal H, and Dietlein DR. 1964. Leishmaniasis in the Sudan Republic: Recent Results. Bulletin of the World Health Organization 31: 137-143.

Hoogsraal H, Dietlein DR, and Heyneman D. 1962. Leishmaniasis in the Sudan Republic. 4. Preliminary observations on man-biting sandflies (Psychodiae: Phlebotomus) in certain Upper Nile endemic Nile endemic areas. Transactions of the Royal Society of Tropical Medicine and Hygiene 56: 411-422.

Ibrahim M, Smyth A, Ali M, Barker D, Kharazmi A, 1994. The polymerase chain reaction can reveal the occurrence of naturally mixed infections with Leishmania parasites. Acta Trop 57: 327-332.

Iovannisci DM, Goebel D, Allen K, Kaur K, and Ullman B. 1984. Genetic analysis of adenine metabolism in Leishmania donovani promastigotes. Evidence for diploidy at the adenine phosphoribosyltransferase locus. Journal of Biological Chemistry 259: 14617-14623.

Ivens AC, Peacock CS, Worthey EA, Murphy L, Aggarwal G, Berriman M, Sisk E, Rajandream MA, Adlem E, Aert R, Anupama A, Apostolou Z, Attipoe P, Bason N, Bauser C, Beck A, Beverley SM, Bianchettin G, Borzym K, Bothe G, Bruschi CV, Collins M, Cadag E, Ciarloni L, Clayton C, Coulson RM, Cronin A, Cruz AK, Davies RM, De Gaudenzi J, Dobson DE, Duesterhoeft A, Fazelina G, Fosker N, Frasch AC, Fraser A, Fuchs M, Gabel C, Goble A, Goffeau A, Harris D, Hertz-Fowler C, Hilbert H, Horn D, Huang Y, Klages S, Knights A, Kube M, Larke N, Litvin L, Lord A, Louie T, Marra M, Masuy D, Matthews K, Michaeli S, Mottram JC, Muller-Auer S, Munden H, Nelson S, Norbertczak H, Oliver K, O'neil S, Pentony M, Pohl TM, Price C, Purnelle B, Quail MA, Rabbinowitsch E, Reinhardt R, Rieger M, Rinta J, Robben J, Robertson L, Ruiz JC, Rutter S, Saunders D, Schafer M, Schein J, Schwartz DC, Seeger K, Seyler A, Sharp S, Shin H, Sivam D, Squares R, Squares S, Tosato V, Vogt C, Volckaert G, Wambutt R, Warren T, Wedler H, Woodward J, Zhou S, Zimmermann W, Smith DF, Blackwell JM, Stuart KD, Barrell B, and Myler PJ. 2005.

The genome of the kinetoplastid parasite, Leishmania major. Science (New York, N.Y.) 309: 436-442.

Jamjoom MB, Ashford RW, Bates PA, Chance ML, Kemp SJ, Watts PC, and Noyes HA. 2004. Leishmania donovani is the only cause of visceral leishmaniasis in East Africa; previous descriptions of L. infantum and "L. archibaldi" from this region are a consequence of convergent evolution in the isoenzyme data. Parasitology 129: 399-409.

Kamali-Sarvestani E, Rasouli M, Mortazavi H, and Gharesi-Fard B. 2006. Cytokine gene polymorphisms and susceptibility to cutaneous leishmaniasis in Iranian patients. Cytokine 35: 159-165.

Kemp M, Hey AS, Kurtzhals JA, Christensen CB, Gaafar A, Mustafa MD, Kordofani AA, Ismail A, Kharazmi A, and Theander TG. 1994. Dichotomy of the human T cell response to Leishmania antigens. I. Th1-like response to Leishmania major promastigote antigens in individuals recovered from cutaneous leishmaniasis. Clinical and experimental immunology 96: 410-415.

Kemp M, Kurtzhals JA, Bendtzen K, Poulsen LK, Hansen MB, Koech DK, Kharazmi A, and Theander TG. 1993. Leishmania donovani-reactive Th1- and Th2-like T-cell clones from individuals who have recovered from visceral leishmaniasis. Infection and immunity 61: 1069-1073.

Khalid NM, Aboud MA, Alrabba FM, Elnaiem DE, Tripet F, 2012. Evidence for genetic differentiation at the microgeographic scale in Phlebotomus papatasi populations from Sudan. Parasit Vectors 5: 249-3305-5-249

Khalil EA, Musa AM, Elgawi SH, Meshasha A, Gamar Eldawla I, Elhassan MO, Eljaleel KA, Younis BM, Elfaki ME, and El-Hassan AM. 2008. Revival of a focus of visceral leishmaniasis in central Sudan. Annals of Tropical Medicine and Parasitology 102: 79-80.

Khalil EA, Zijlstra EE, Kager PA, and El Hassan AM. 2002. Epidemiology and clinical manifestations of Leishmania donovani infection in two villages in an endemic area in eastern Sudan. Tropical medicine & international health : TM & IH 7: 35-44.

Killick-Kendrick R, and Killick-Kendrick M. 1987. Honeydew of aphids as a source of sugar for Phlebotomus ariasi. Medical and veterinary entomology 1: 297-302.

Killick-Kendrick R. 1990. Phlebotomine vectors of the leishmaniases: a review. Medical and veterinary entomology 4: 1-24.

Killick-Kendrick R. 1999. The biology and control of phlebotomine sand flies. Clinics in dermatology 17: 279-289.

Kubba R, Al-Gindan Y, el-Hassan AM, and Omer AH. 1987. Clinical diagnosis of cutaneous leishmaniasis (oriental sore). Journal of the American Academy of Dermatology 16: 1183-1189.

Lachaud L, Chabbert E, Dubessay P, Reynes J, Lamothe J, and Bastien P. 2001. Comparison of various sample preparation methods for PCR diagnosis of visceral leishmaniasis using peripheral
Lainson 1984. Leishmaniases in the New World. The Lancet 324: 497-499.

Lainson R, Ishikawa EAY, and Silveira FT. 2002. American visceral leishmaniasis: wild animal hosts. Transactions of the Royal Society of Tropical Medicine and Hygiene 96: 630-631.

Lainson R, Shaw JJ, Peters W, and Killick-Kendrick R. 1987. Evolution, classification and geographical distribution.: Academic Press.

Lambert M, Dereure J, El-Safi SH, Bucheton B, Dessein A, Boni M, Feugier E, and Dedet JP. 2002. The sandfly fauna in the visceral-leishmaniasis focus of Gedaref, in the Atbara-River area of eastern Sudan.

Annals of Tropical Medicine and Parasitology 96: 631-636.

Lambson, B., and Barker, D.C., (2002). Basic molecular tools, Homologous minicircles in Leishmania donovani. Transactions of the Royal Society of Tropical Medicine and Hygiene. Supplement 1 S1/75-S1/80.

Lane RP, and Crosskey RW. 1993. Medical insects and arachnids.: Chapman & Hall Ltd.

Lightner LK, Chulay JD, and Bryceson AD. 1983. Comparison of microscopy and culture in the detection of Leishmania donovani from splenic aspirates. The American Journal of Tropical Medicine and Hygiene 32: 296-299.

Magill AJ. 1995. Epidemiology of the leishmaniases. Dermatologic clinics 13: 505-523.

Maniatis, T., Fritsch, E.F., Sambrook, K.J. (1986). Purification of nucleic acids: Molecular Cloning, thirteenth ed. Cold Spring Harbor Laboratory, New York. 458-459

Mannaert A, Downing T, Imamura H, and Dujardin JC. 2012. Adaptive mechanisms in pathogens: universal aneuploidy in Leishmania. Trends in parasitology 28: 370-376.

Maroli M, Feliciangeli MD, Bichaud L, Charrel RN, and Gradoni L. 2013. Phlebotomine sandflies and the spreading of leishmaniases and other diseases of public health concern. Medical and veterinary entomology 27: 123-147.

Martinez P, de la Vega E, Laguna F, Soriano V, Puente S, Moreno V, Sentchordi MJ, Garcia-Aguado C, and Gonzalez-Lahoz J. 1993. Diagnosis of visceral leishmaniasis in HIV-infected individuals using peripheral blood smears. AIDS (London, England) 7: 227-230.

Martinez-Calvillo S, Sunkin SM, Yan S, Fox M, Stuart K, and Myler PJ. 2001. Genomic organization and functional characterization of the Leishmania major Friedlin ribosomal RNA gene locus. Molecular and biochemical parasitology 116: 147-157.

Mbow ML, Bleyenberg JA, Hall LR, and Titus RG. 1998. Phlebotomus papatasi sand fly salivary gland lysate down-regulates a Th1, but up-regulates a Th2, response in mice infected with Leishmania major. Journal of immunology (Baltimore, Md.: 1950) 161: 5571-5577.

Mebrahtu YB, Lawyer PG, Hendricks LD, Muigai R, Oster CN, Perkins PV, Koech DK, Pamba H, and Roberts CR. 1991. Concurrent infection with Leishmania donovani and Leishmania major in a Kenyan patient: clinical description and parasite characterization. The American Journal of Tropical Medicine and Hygiene 45: 290-296.

Miles MA, Lainson R, Shaw JJ, Povoa M, and de Souza AA. 1981. Leishmaniasis in Brazil: XV. Biochemical distinction of Leishmania mexicana amazonensis, L. braziliensis braziliensis and L. braziliensis guyanensis--aetiological agents of cutaneous leishmaniasis in the Amazon Basin of Brazil. Transactions of the Royal Society of Tropical Medicine and Hygiene 75: 524-529.

Mohamed HS, Ibrahim ME, Miller EN, Peacock CS, Khalil EA, Cordell HJ, Howson JM, El Hassan AM, Bereir RE, and Blackwell JM. 2003. Genetic susceptibility to visceral leishmaniasis in The Sudan: linkage and association with IL4 and IFNGR1. Genes and immunity 4: 351-355.

Molyneux DH, Moore J, and Maroli M. 1991. Sugars in sandflies. Parassitologia 33 Suppl: 431-436.

Monge-Maillo B, and Lopez-Velez R. 2013a. Therapeutic options for old world cutaneous leishmaniasis and new world cutaneous and mucocutaneous leishmaniasis. Drugs 73: 1889-1920.

Monge-Maillo B, and Lopez-Velez R. 2013b. Therapeutic options for visceral leishmaniasis. Drugs 73: 1863-1888.

Morsy TA, el Kady GA, Salama MM, Habib KM, and Ghareb MA. 1993. Leishmania major in Meriones sacramenti Thomas 1922 in Nakhel, North Sinai, Egypt. Journal of the Egyptian Society of Parasitology 23: 373-379.

Murray HW, Berman JD, Davies CR, and Saravia NG. 2005. Advances in leishmaniasis. Lancet 366: 1561-1577.

Myler PJ, and Stuart KD. 2000. Recent developments from the Leishmania genome project. Current opinion in microbiology 3: 412-416.

MylerPJ, Nicolas Fasel, 2008, Leishmania: After the Genome book

Myskova J, Svobodova M, Beverley SM, Volf P, 2007. A lipophosphoglycan-independent development of Leishmania in permissive sand flies. Microbes Infect 9: 317-324.
Nadler SA. 1995. Microevolution and the genetic structure of parasite populations. The Journal of parasitology: 395-403.

Oryan A, Shirian S, Tabandeh MR, Hatam GR, Randau G, Daneshbod Y, 2013. Genetic diversity of Leishmania major strains isolated from different clinical forms of cutaneous Leishmaniasis in southern Iran based on minicircle kDNA. Infect Genet Evol 19: 226-231

Osman OF, Kager PA, and Oskam L. 2000. Leishmaniasis in the Sudan: a literature review with emphasis on clinical aspects. Tropical medicine & international health : TM & IH 5: 553-562.

Osman OF, Oskam L, Zijlstra EE, Kroon NC, Schoone GJ, Khalil ET, El-Hassan AM, and Kager PA. 1997. Evaluation of PCR for diagnosis of visceral leishmaniasis. Journal of clinical microbiology 35: 2454-2457.

Pacheco RS, Grimaldi Junior G, Morel CM, 1987. Inhibition of growth of Leishmania mexicana mexicana by Leishmania mexicana amazonensis during "in vitro" co-cultivation. Mem Inst Oswaldo Cruz 82: 537-542.

Pattabhi S, Whittle J, Mohamath R, El-Safi S, Moulton GG, Guderian JA, Colombara D, Abdoon AO, Mukhtar MM, Mondal D, Esfandiari J, Kumar S, Chun P, Reed SG, and Bhatia A. 2010. Design, development and evaluation of rK28-based point-of-care tests for improving rapid diagnosis of visceral leishmaniasis. PLoS neglected tropical diseases 4: 10.1371/journal.pntd.0000822.

Peacock CS, Seeger K, Harris D, Murphy L, Ruiz JC, Quail MA, Peters N, Adlem E, Tivey A, Aslett M, Kerhornou A, Ivens A, Fraser A, Rajandream MA, Carver T, Norbertczak H, Chillingworth T, Hance Z, Jagels K, Moule S, Ormond D, Rutter S, Squares R, Whitehead S, Rabbinowitsch E, Arrowsmith C, White B, Thurston S, Bringaud F, Baldauf SL, Faulconbridge A, Jeffares D, Depledge DP, Oyola SO, Hilley JD, Brito LO, Tosi LR, Barrell B, Cruz AK, Mottram JC, Smith DF, and Berriman M. 2007. Comparative genomic analysis of three Leishmania species that cause diverse human disease. Nature genetics 39: 839-847.

Pimenta PF, Saraiva EM, Rowton E, Modi GB, Garraway LA, Beverley SM, Turco SJ, Sacks DL, 1994. Evidence that the vectorial competence of phlebotomine sand flies for different species of Leishmania is controlled by structural polymorphisms in the surface lipophosphoglycan. Proc Natl Acad Sci U S A 91: 9155-9159.

Pintado V, Martin-Rabadan P, Rivera ML, Moreno S, and Bouza E. 2001. Visceral leishmaniasis in human immunodeficiency virus (HIV)-infected and non-HIV-infected patients. A comparative study. Medicine 80: 54-73.

Poli A, Abramo F, Barsotti P, Leva S, Gramiccia M, Ludovisi A, and Mancianti F. 2002. Feline leishmaniosis due to Leishmania infantum in Italy. Veterinary parasitology 106: 181-191.

Pulimood SA, Rupali P, Ajjampur SS, Thomas M, Mehrotra S, and Sundar S. 2012. Atypical mucocutaneous involvement with Leishmania donovani. The National medical journal of India 25: 148-150.

Qureshi AA, Asahina A, Ohnuma M, Tajima M, Granstein RD, and Lerner EA. 1996. Immunomodulatory properties of maxadilan, the vasodilator peptide from sand fly salivary gland extracts. The American Journal of Tropical Medicine and Hygiene 54: 665-671.

Ramesh V, and Mukherjee A. 1995. Post-kala-azar dermal leishmaniasis. International journal of dermatology 34: 85-91.

Ravel C, Cortes S, Pratlong F, Morio F, Dedet JP, Campino L, 2006. First report of genetic hybrids between two very divergent Leishmania species: Leishmania infantum and Leishmania major. Int J Parasitol 36: 1383-1388

Ready PD. 2013. Biology of phlebotomine sand flies as vectors of disease agents. Annual Review of Entomology 58: 227-250.

Reed SG, Carvalho EM, Sherbert CH, Sampaio DP, Russo DM, Bacelar O, Pihl DL, Scott JM, Barral A, and Grabstein KH. 1990. In vitro responses to Leishmania antigens by lymphocytes from patients with leishmaniasis or Chagas' disease. The Journal of clinical investigation 85: 690-696.

Reithinger R, Dujardin JC, Louzir H, Pirmez C, Alexander B, and Brooker S. 2007. Cutaneous leishmaniasis. The Lancet infectious diseases 7: 581-596.

Riera C, Fisa R, Udina M, Gallego M, and Portus M. 2004. Detection of Leishmania infantum cryptic infection in asymptomatic blood donors living in an endemic area (Eivissa, Balearic Islands, Spain) by different diagnostic methods. Transactions of the Royal Society of Tropical Medicine and Hygiene 98: 102-110.

Rioux JA, Lanotte G, Serres E, Pratlong F, Bastien P, and Perieres J. 1990. Taxonomy of Leishmania. Use of isoenzymes. Suggestions for a new classification. Annales de Parasitologie Humaine et Comparee 65: 111-125.

Ritmeijer K, Melaku Y, Mueller M, Kipngetich S, O'keeffe C, and Davidson RN. 2006. Evaluation of a new recombinant K39 rapid diagnostic test for Sudanese visceral leishmaniasis. The American Journal of Tropical Medicine and Hygiene 74: 76-80.

Rogers KA, and Titus RG. 2003. Immunomodulatory effects of Maxadilan and Phlebotomus papatasi sand fly salivary gland lysates on human primary in vitro immune responses. Parasite immunology 25: 127-134.

Rogers MB, Hilley JD, Dickens NJ, Wilkes J, Bates PA, Depledge DP, Harris D, Her Y, Herzyk P, and Imamura H. 2011. Chromosome and gene copy number variation allow major structural change between species and strains of Leishmania. Genome research 21: 2129-2142.

Rohousova I, and Volf P. 2006. Sand fly saliva: effects on host immune response and Leishmania transmission. Folia parasitologica 53: 161-171.

Rohousova I, Ozensoy S, Ozbel Y, and Volf P. 2005. Detection of species-specific antibody response of humans and mice bitten by sand flies. Parasitology 130: 493-499.

Sacks D, and Kamhawi S. 2001. Molecular Aspects of Parasite-Vector and Vector-Host Interactions in Leishmaniasis 1. Annual Reviews in Microbiology 55: 453-483.

Sacks D, Phillip Lawyer and shadenkamhawi, The Biology of Leishmania - Sandfly Interaction- book. Page 20

Sacks D. 1997. A novel role for the peritrophic matrix in protecting Leishmania from the hydrolytic activities of the sand fly midgut. Parasitology 115: 359-369.

Sacks DL, and Perkins PV. 1985. Development of infective stage Leishmania promastigotes within phlebotomine sand flies. The American Journal of Tropical Medicine and Hygiene 34: 456-459.

Sacks DL, Kenney RT, Kreutzer RD, Jaffe CL, Gupta AK, Sharma MC, Sinha SP, Neva FA, and Saran R. 1995. Indian kala-azar caused by Leishmania tropica. Lancet 345: 959-961.

Sacks DL, Modi G, Rowton E, Späth G, Epstein L, Turco SJ, and Beverley SM. 2000. The role of phosphoglycans in Leishmania–sand fly interactions. Proceedings of the National Academy of Sciences 97: 406-411.

Sacks DL. 2001. Leishmania–sand fly interactions controlling species-specific vector competence. Cellular microbiology 3: 189-196.

Sakthianandeswaren A, Foote SJ, and Handman E. 2009. The role of host genetics in leishmaniasis. Trends in parasitology 25: 383-391.

Salah Eldin, H. (1997). Cutaneous leishmaniasis in Tuti Island, Khartoum, Sudan, a cross-sectional study of schoolchildren and a sample of population.M. Sc. Thesis, University of Khartoum.

Salhi A, Rodrigues V,Jr, Santoro F, Dessein H, Romano A, Castellano LR, Sertorio M, Rafati S, Chevillard C, Prata A, Alcais A, Argiro L, and Dessein A. 2008. Immunological and genetic evidence for a crucial role of IL-10 in cutaneous lesions in humans infected with Leishmania braziliensis. Journal of immunology (Baltimore, Md.: 1950) 180: 6139-6148.

Saliba EK, and Oumeish OY. 1999. Reservoir hosts of cutaneous leishmaniasis. Clinics in dermatology 17: 275-277.

Saliba EK, Disi AM, Ayed RE, Saleh N, al-Younes H, Oumeish O, and al-Ouran R. 1994. Rodents as reservoir hosts of cutaneous leishmaniasis in Jordan. Annals of Tropical Medicine and Parasitology 88: 617-622.

Salman SM, Rubeiz NG, and Kibbi AG. 1999. Cutaneous leishmaniasis: clinical features and diagnosis. Clinics in dermatology 17: 291-296.

Saravia NG, Weigle K, Segura I, Giannini SH, Pacheco R, Labrada LA, and Goncalves A. 1990. Recurrent lesions in human Leishmania braziliensis infection--reactivation or reinfection? Lancet 336: 398-402.

Savani ES, de Oliveira Camargo MC, de Carvalho MR, Zampieri RA, dos Santos MG, D'Auria SR, Shaw JJ, and Floeter-Winter LM. 2004. The first record in the Americas of an autochthonous case of Leishmania (Leishmania) infantum chagasi in a domestic cat (Felix catus) from Cotia County, Sao Paulo State, Brazil. Veterinary parasitology 120: 229-233.

Schlein Y, and Jacobson RL. 1996. Why Is Man an Unsuitable Reservoir for the Transmission of< i> Leishmania major?</i>. Experimental parasitology 82: 298-305.

Schlein Y, Warburg A, Schnur L, Le Blancq S, and Gunders A. 1984. Leishmaniasis in Israel: reservoir hosts, sandfly vectors and leishmanial strains in the Negev, Central Arava and along the Dead Sea. Transactions of the Royal Society of Tropical Medicine and Hygiene 78: 480-484.

Schonian G, Mauricio I, and Cupolillo E. 2010. Is it time to revise the nomenclature of Leishmania? Trends in parasitology 26: 466-469.

Sharma M, Gupta A, Das V, Verma N, Kumar N, Saran R, and Kar S. 2000. < i> Leishmania donovani</i> in blood smears of asymptomatic persons. Acta Tropica 76: 195-196.

Shirian S, Oryan A, Hatam GR, Daneshbod Y, 2012. Mixed mucosal Leishmaniasis infection caused by Leishmania tropica and Leishmania major. J Clin Microbiol 50: 3805-3808.

Siddig M, Ghalib H, Shillington DC, and Petersen EA. 1988. Visceral leishmaniasis in the Sudan: comparative parasitological methods of diagnosis. Transactions of the Royal Society of Tropical Medicine and Hygiene 82: 66-68.

Simpson L. 1987. The mitochondrial genome of kinetoplastid protozoa: genomic organization, transcription, replication, and evolution. Annual Review of Microbiology 41: 363-382.

Singh S, Sharma U, and Mishra J. 2011. Post-kala-azar dermal leishmaniasis: recent developments. International journal of dermatology 50: 1099-1108.

Sinha R, and Sehgal S. 1994. Comparative evaluation of serological tests in Indian kala-azar. The Journal of tropical medicine and hygiene 97: 333-340.

Sinha R, Datta U, and Sehgal S. 1993. Importance of bone marrow culture for diagnosis of Kala azar. Scandinavian Journal of Infectious Diseases 25: 787-789.

Srivastava P, Dayama A, Mehrotra S, and Sundar S. 2011. Diagnosis of visceral leishmaniasis. Transactions of the Royal Society of Tropical Medicine and Hygiene 105: 1-6.

Sterkers Y, Lachaud L, Bourgeois N, Crobu L, Bastien P, and Pages M. 2012. Novel insights into genome plasticity in Eukaryotes: mosaic aneuploidy in Leishmania. Molecular microbiology 86: 15-23.

Strazzulla A, Cocuzza S, Pinzone MR, Postorino MC, Cosentino S, Serra A, Cacopardo B, and Nunnari G. 2013. Mucosal Leishmaniasis: An Underestimated Presentation of a Neglected Disease. BioMed Research International 2013.

Sturm NR, and Simpson L. 1990. Kinetoplast DNA minicircles encode guide RNAs for editing of cytochrome oxidase subunit III mRNA. Cell 61: 879-884.

Sundar S, and Rai M. 2002. Laboratory diagnosis of visceral leishmaniasis. Clinical and diagnostic laboratory immunology 9: 951-958.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, and Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution.

Tremblay M, Olivier M, and Bernier R. 1996a. Leishmania and the pathogenesis of HIV infection. Parasitology today (Personal ed.) 12: 257-261.

Van der Auwera G, Fraga J, Montalvo AM, and Dujardin JC. 2011. Leishmania taxonomy up for promotion? Trends in parasitology 27: 49-50.

van Griensven J, and Diro E. 2012. Visceral leishmaniasis. Infectious disease clinics of North America 26: 309-322.

Vinayak VK, Mahajan D, Sobti RC, Singla N, and Sundar S. 1994. Anti-66 kDa antileishmanial antibodies as specific immunodiagnostic probe for visceral leishmaniasis. The Indian journal of medical research 99: 109-114.

Vlkova M, Rohousova I, Drahota J, Stanneck D, Kruedewagen EM, Mencke N, Otranto D, and Volf P. 2011. Canine antibody response to Phlebotomus perniciosus bites negatively correlates with the risk of Leishmania infantum transmission. PLoS neglected tropical diseases 5: e1344.

Volf P, and Volfova V. 2011. Establishment and maintenance of sand fly colonies. Journal of vector ecology : journal of the Society for Vector Ecology 36 Suppl 1: S1-9.

Volf P, Benkova I, Myskova J, Sadlova J, Campino L, and Ravel C. 2007. Increased transmission potential of Leishmania major/Leishmania infantum hybrids. International journal for parasitology 37: 589-593.

Weigle K, and Saravia NG. 1996. Natural history, clinical evolution, and the host-parasite interaction in New World cutaneous Leishmaniasis. Clinics in dermatology 14: 433-450.

WHO Technical Report Series 494 : Report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, 22–26 March 2010: Control of the leishmaniases available at: http://whqlibdoc.who.int/trs/WHO_TRS_949_eng.pdf

Wirth DF, and Pratt DM. 1982. Rapid identification of Leishmania species by specific hybridization of kinetoplast DNA in cutaneous lesions. Proceedings of the National Academy of Sciences of the United States of America 79: 6999-7003.

World Health Organization .2014 . disease information accessible at:

World Health Organization .2014 disease fact sheet accessible at http://www.who.int/ith/diseases/leishmaniasis/en/

World Health Organization. 1995. Bridging the gap. The World Health Report, Geneva. 2002. p. 192–

Wortmann, G., Hochberg, L., Houng, H. H., Sweeney, C., Zapor, M., Aronson, N., et al. (2005). Rapid identification of leishmania complexes by a real-time PCR assay. The American Journal of Tropical Medicine and Hygiene, 73(6), 999-1004.

Yaghoobi-Ershadi M, and Javadian E. 1996. Epidemiological study of reservoir hosts in an endemic area of zoonotic cutaneous leishmaniasis in Iran. Bulletin of the World Health Organization 74: 587.

Yan S, Lodes MJ, Fox M, Myler PJ, and Stuart K. 1999. Characterization of the Leishmania donovani ribosomal RNA promoter. Molecular and biochemical parasitology 103: 197-210.

Zer R, Yaroslavski I, Rosen L, and Warburg A. 2001. Effect of sand fly saliva on< i> Leishmania</i> uptake by murine macrophages. International journal for parasitology 31: 810-814.

Zijlstra EE, Ali MS, el-Hassan AM, el-Toum IA, Satti M, Ghalib HW, and Kager PA. 1992. Kala-azar: a comparative study of parasitological methods and the direct agglutination test in diagnosis. Transactions of the Royal Society of Tropical Medicine and Hygiene 86: 505-507.

Zijlstra EE, and el-Hassan AM. 2001a. Leishmaniasis in Sudan. Visceral leishmaniasis. Transactions of the Royal Society of Tropical Medicine and Hygiene 95 Suppl 1: S27-58.

Zijlstra EE, and el-Hassan AM. 2001b. Leishmaniasis in Sudan. Post kala-azar dermal leishmaniasis. Transactions of the Royal Society of Tropical Medicine and Hygiene 95 Suppl 1: S59-76.

Zijlstra EE, el-Hassan AM, and Ismael A. 1995. Endemic kala-azar in eastern Sudan: post-kala-azar dermal leishmaniasis. The American Journal of Tropical Medicine and Hygiene 52: 299-305.

Zijlstra EE, el-Hassan AM, Ismael A, and Ghalib HW. 1994. Endemic kala-azar in eastern Sudan: a longitudinal study on the incidence of clinical and subclinical infection and post-kala-azar dermal leishmaniasis. The American Journal of Tropical Medicine and Hygiene 51: 826-836.

Zijlstra EE, Musa AM, Khalil EA, el-Hassan IM, and el-Hassan AM. 2003. Post-kala-azar dermal leishmaniasis. The Lancet infectious diseases 3: 87-98

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