Der Humboldt-Universität zu Berlin

Dissertation

Molecular approaches to direct diagnosis and characterization of *Leishmania donovani* in clinical isolates

zur Erlangung des akademischen Grades

doctor rerum naturalium

(Dr. rer. Nat.)

Mathematisch-Naturwissenschaftlichen Fakultät I

M. Sc Biologie: Frau Nahla O. A. El Tai

Prof. Dr.Bernhard Ronacher

1. Prof. Dr. Wolfgang Presber

2. Prof. Dr. Richard Liucius

3. Prof. Dr. Christian Bogdan

Datum der Promotion:26.09.2002

Iam pleased to dedicate this work to my beloved, parents,

husband, son Ahmed and daughter Reem

Contents

Abbreviations:

A	Adenine
APS	Ammonium peroxodisulfate
BM	Bone marrow
C	Cytosine
CL	Cutaneous leishmaniasis
DAT	Direct agglutination test
DCL	Diffuse cutaneous leishmaniasis
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediamine-tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
G	Guanine
Gp63	Glycolipid-anchored Zinc protease
GRNA	Guide RNA
IFAT	Indirect immunofluoresent antibody test
IPS	impulse per second
ITS	Internal transcribed spacer
Kbp	Kilo base-pair
KDNA	Kinetoplast DNA
L	*Leishmania*
LN	Lymph node
MCL	Mucocutaneous leishmaniasis

mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
NASBA	Nucleic acid sequence based amplification
NTS	Non-transcribed spacer
OFAGE	Orthogonal field alternate gel electrophoresis
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
PKDL	Post kala-azar dermal leishmaniasis
RFLP	Ristriction fragment length polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT-PCR	Reverse-transcriptase PCR
SDS	Sodium dodecyl sulphate
SSCP	Single-stranded conformation polymorphisms
SSU	Small sub-unit
T	Thymine
Taq	*Thermus aquaticus*
TBE	Tris borate EDTA
TE	Tris EDTA
TEMED	NNNN-Tetramethylene diamine
TRNA	Transfer RNA
U	Uracil
UV	Ultra violet
VL	Visceral leishmaniasis
WHO	World Health Organization

Abstract

This study was carried out in clusters of villages that represent an endemic focus of visceral leishmaniasis (VL). These villages were located in Gedaref state, eastern Sudan. For diagnostic purposes polymerase chain reaction (PCR) was performed successfully, directly from clinical samples spotted on filter papers with no prior cultivation from 100 patients suspected of having kala-azar or post kala-azar dermal leishmaniasis. Mainly the ribosomal internal transcribed spacer (ITS1 & ITS2) were targeted in PCR because this region is more variable and allows clear species identification and also strain differences could be expected by further analysis of these PCR products. PCR was found to be more sensitive compared to the gold standard microscopic method. Four PCR based approaches were used to analyse diversity within Sudanese isolates of *Leishmania donovani*. Methods compared were fingerprinting with single non-specific primers, restriction analysis of the amplified ITS locus (RFLP), single-stranded conformation polymorphism (SSCP) of the ITS region, major surface protease (gp63) gene, anonymous DNA fragments and sequencing of these targeted regions. When PCR fingerprinting and restriction analysis of ITS region were applied, highly similar fragment patterns were observed for all strains of *L. donovani* studied. The ITS1 locus gave 12 different SSCP profiles among the 86 Sudanese isolates, where as the ITS2 locus was highly conserved among the 86 samples with the exception of 1 isolate. Strains of *L. donovani* derived from other geographical areas were found to have different ITS2 patterns. The gp63 locus gave 3 polymorphic patterns among 31 Sudanese isolates. Concerning most of the anonymous

DNA fragments namely, L510, L413, LK413, L0308 and L0114 unfortunately, we succeeded to get good PCR products only from DNA extracted 8 successful cultures. Only for the fragment L0110 we were able to get good PCR products from 31 samples spotted on filter papers. When these PCR products were investigated for polymorphisms using SSCP no differences were observed with exception of L0114 region, which showed 2 patterns. SSCP analysis correlates well with results of DNA sequencing and confirmed that SSCP was able to detect genetic diversity at the level of a single nucleotide. SSCP had advantages over the other methods employed for investigating of sequence variation within the species *L. donovani*. There was no correlation between the form of clinical manifestation of the disease and the PCR fingerprinting, ITS-RFLP, or ITS-SSCP characteristics. This study is beneficial particularly in epidemiological studies based on field-work where obtaining cultures can be extremely difficult especially in developing countries.

Zusammenfassung

Die vorliegende Studie wurde in einer Gruppe von Dörfern im Ostsudan, Gedaref State, durchgeführt. Bei 100 Patienten mit der Verdachtsdiagnose Kala Azar- oder Post-Kala Azar-Leishmaniose war der Erregernachweis mit der PCR direkt in klinischen Proben, die auf Filterpapier aufgebracht worden waren, ohne vorherige Kultivierung erfolgreich. In dieser PCR wurden die ribosomalen, internal transcribed spacer (ITS1 & ITS2) amplifiziert, weil sie sehr variabel sind, eine klare Speziesidentifizierung gestatten und bei weiterführenden Analysen der PCR-Produkte auch der Nachweis stammspezifischer Unterschiede erwartet werden konnte. Für die Analyse der Diversität von *Leishmania donovani*-Isolaten aus dem Sudan wurden 4 verschiedene PCR-basierte Methoden eingesetzt: das PCR-Fingerprinting mit unspezifischen Einzelprimern, die RFLP- Analyse des amplifizierten ITS-Locus, „single strand conformation polymorphism (SSCP)- Analysen der amplifizierten ITS-Region, des Gens, welches für die Hauptoberflächenprotease (gp63) kodiert, und anonymer DNA-Fragmente sowie Sequenzanalysen der entsprechenden Zielregionen. Das PCR-Fingerprinting und die Restriktionsanalyse der ITS-Region lieferten weitgehend übereinstimmende Fragmentmuster für alle untersuchten *L.donovani*-Stämme. 12 unterschiedliche Profile wurden bei der SSCP-Analyse des ITS1-Locus für 86 Isolate aus dem Sudan erhalten, während der ITS2-Locus bei diesen Stämmen hochkonserviert war und nur ein Stamm ein unterschiedliches SSCP-Muster aufwies. *L. Donovani* -Stämme anderer geographischer Herkunft hatten unterschiedliche ITS2-Profile in der SSCP. Für den gp63 - Locus waren 3 polymorphe SSCP-Muster bei 31 untersuchten sudanesischen Isolaten nachweisbar. Für die meisten der anonymen

DNA-Fragmente, L510, L413, LK413, L0308 UND L0114, konnten leider nur von 8 kultivierten Stämmen gute PCR-Produkte erhalten werden. Lediglich das Fragment L0110 konnte erfolgreich von 31 auf Filterpapier aufgebrachten Proben direkt amplifiziert werden. Die Suche nach Polymorphismen mit der SSCP ergab keine Unterschiede in diesen anonymen DNA-Regionen, mit Ausnahme des Fragments L0114, das zwei verschiedene Muster aufwies. Die Ergebnisse der SSCP-Analysen und der DNA-Sequenzierung stimmten gut überein, wodurch bestätigt wurde, dass die SSCP genetische Unterschiede auf dem Niveau einzelner Basenaustausche nachweisen kann. Die SSCP-Technik hat Vorteile gegenüber den anderen Methoden, die für die Untersuchung von Sequenzvariationen innerhalb der Spezies *L. donovani* angewandt wurden. Es konnten keine Korrelationen zwischen der Form der klinischen Manifestation und den Ergebnissen des PCR-Fingerprinting, der ITS-RFLP- und ITS-SSCP- Analysen festgestellt warden. Diese Studie ist von besonderem Nutzen in epidemiologischen Feldstudien, bei denen die Kultivierung der Erreger besonders in Entwicklungsländern extrem schwierig sein kann.

1 Introduction

1.1 Leishmaniasis:

1.1.1 The disease:

The Leishmaniasis is a globally widespread group of diseases caused by obligatory, intracellular, haemoflagellate protozoan parasites of the genus *leishmania* (family trypanosomatidae). The disease manifests itself in a variety of clinical forms, ranging from the self-healing cutaneous lesions to the more serious, potentially fatal visceralizing form, and includes the metastasising muco-cutaneous form, and the post kala-azar dermal leishmaniasis.

Leishmaniasis forced itself upon medical attention as an increasingly significant problem over the last decade. Because of its importance, leishmaniasis is considered as one of the 6 diseases selected by WHO for its special programme for research and training in tropical diseases (WHO, 1984). It ranks only second to malaria among human protozoan diseases (Chang *et al.*, 1985). It is prevalent on 4 continents: Africa, Europe, Asia, Central and Latin America and is endemic in the tropical and subtropical regions of 88 countries (WHO, 1998). Sixteen are developed countries, 72 are developing countries, 13 of them are among the least developed (Desjeux, 1996). Overall, it has been estimated that there are 12-13 million of cases of leishmaniasis world-wide, over 90% of cases are found in 3 regions: Sudan/Ethiopia/Kenya; India/Bangladesh/Nepal and Brazil (WHO, 1991; WHO, 1996), with as many as 100 000 deaths every year (Ashford *et al*., 1992). Figures of 1-1.5 million new cases of cutaneous leishmaniasis (CL), representing 50 to 75 percent of all new cases, and 500,000 cases of visceral leishmaniasis (VL) per year are likely and 350 million people

are at risk (WHO, 1998 & 2000). This only represent the 'tip of the iceberg, since not all infected individuals develop a disease (Hommel, 1999).

1.1.2 Clinial spectrum:

1.1.2.1 Visceral leishmaniasis (VL):

Also known in Asia as ' black fever' or 'kala-azar' is the most severe form of the disease, the parasite invades internal organs (spleen, liver, bone marrow) and the consequences are usually with an almost 100% mortality rate if left untreated. It is characterized by irregular fever, loss of weight, splenomegaly, hepatomegaly and/or lymphadenopathy and anaemia. Of the 500,000 new cases of VL, which occur annually, 90% are in 5 countries Bangladesh, Brazil, India, Nepal and Sudan (Desjeux, 1996; WHO, 1996 &1998). VL is caused by *L. donovani* on the Indian subcontinent and in East Africa, by *L. infantum* in the Mediterranean region and by *L. chagasi*, which is closely related to or not distinguished from *L. infantum* (Mauricio *et al*., 2000), in the New World mainly in Brazil, Peru and Paraguay (Berman, 1997).

The association of VL and HIV infection clearly confirms the fact that VL is an opportunistic infection. HIV/*Leishmania* co-infections are considered to be a real threat, especially in southern Europe, where approximately 700 cases of co-infection have been reported to WHO. Now in southern Europe, VL is the most common opportunistic parasitic infection among HIV positive persons. Most co-infections in the Americas are reported from Brazil. In eastern Africa, cases of *leishmania/*HIV co-infection have been reported in Ethiopia (27), Kenya (13), Malawi (1) and Sudan (3). The risk of overlap is increasing due to a number of factors: mass migration, civil unrest, resettlement programmes and promiscuity and prostitution in refugee camps. In

North Africa, a few cases have been reported in Algeria, Morocco, and Tunisia and in western Africa 1 case in Cameroon and 1 case in Guinea Bissau (WHO, 1996). AIDS and VL are locked in a vicious circle of mutual reinforcement. VL accelerates the onset of full-blown AIDS, and shortens the life expectancy of HIV-infected people, while HIV spurs the spread of VL. The gridlock produce cumulative deficiency of the immunoresponse, as *Leishmania* parasites and HIV destroy the same cells (WHO, 1998).

Canine VL is regarded as both an important veterinary problem and a problem concerning human health, as the dog is the main reservoir of *L. infantum* and *L. chagasi.* An increase in both VL and canine VL cases has been reported in most Mediterranean countries in recent years (WHO, 1990).

1.1.2.2 Post kala-azar dermal leishmaniasis (PKDL):

It is a dermatropic form of leishmaniasis developed by part of the ex- VL patients (WHO, 1990), but there are cases without any previous known history of VL (El-Hassan *et al*., 1992). The disease is characterized by the development of macules, papules and nodules, which first appear around the mouth; those which do not heal spontaneously become more dense and spread over the entire body (Berman, 1997). The interval between the end of treatment of VL and the onset of PKDL is variable: PKDL may appear during or directly after treatment (Zijlstra *et al*., 1995) to up to 2 years post treatment (Zijlstra *et al*., 1991). PKDL patients may be important sources of infection in VL transmission (Addy & Nandy, 1992; WHO, 1990).

1.1.2.3 Cutaneous leishmaniasis (CL):

It is known, as 'little sister' in some countries that the disease is so common that is part of the family. In the Old World is known as oriental sore. It produces skin lesions, sometimes as many as 200 on the face, arms and legs, causing serious disability and permanent scars (WHO, 1998). In the Old World is caused by *Leishmania major*, *Leishmania tropica* and *Leishmania aethiopica.* In the New World CL is caused by *L. mexicana* and *L. braziliensis* complexes. Some *L. infantum* and *L. donovani* strains can also cause lesions. Ninety percent of all cases of CL occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria, with 1-1.5 million new cases reported annually world-wide (WHO, 1996*). L. major* usually produces self-healing lesions, on the other hand, *L. tropica* is usually more chronic, and its most severe form, recidivans leishmaniasis, is very difficult to treat. In the New World, *L. mexicana* usually produces relatively benign lesions but some locations such as the ear's pinna are very difficult to treat in general (Desjeux, 1996).

1.1.2.4 Diffuse cutaneous leishmaniasis (DCL):

It is less common, chronic in evolution and especially difficult to treat. It produces lesions resembling leprosy, which do not heal spontaneously, due to deficiency of the immune response (Desjeux, 1996; WHO, 1998). DCL is due to *L. aethiopica* and *L. amazonensis* (Desjeux, 1996).

1.1.2.5 Mucocutaneous leishmaniasis (MCL):

Also called 'espundia', it produces disfiguring lesions to the face, destroying the mucous membranes of the nose, mouth and throat (Desjeux, 1996; WHO, 1998). It is mostly related to *Leishmania* species of the New World such as *L. braziliensis*, *L.*

panamensis and *L. guyanensis*, but mucosal lesions have been reported in the Old World due to *L. donovani, L. major* and *L. infantum* in immunosupresed patients (Desjeux, 1996). Ninety percent of all cases of MCL occur in Bolivia, Brazil and Peru (WHO, 1996).

1.1.3 Treatment:

Accurate parasitological diagnosis is essential in leishmaniasis to determine the correct treatment. Some infections, especially simple cutaneous lesions due to *L. major*, are often self-healing and induce immunity to reinfection and treatment of these is generally not recommended, unless the lesions do not heal within 6-9 months. In case of chronic lesions due to *L. tropica* the treatment is based on pentavalent antimonials intramuscularly or intravenously at 10-20 mg/kg/day until cure.

Treatment of other forms, such as VL and MCL infections, mainly relies on the pentavalent antimonials sodium stibogluconate (Pentostam) or meglumine antimoniate (Glucantime), the first -line drugs except when resistance exists, and the usual dose is 20 mg/kg/day for 30 days. They are expensive and need to be given by injection. The second-line drugs in case of resistance -amphotericin B and pentamidine, used in cases unresponsive to antimonials, need careful management to avoid serious side effects. They are used intravenously over several hours on alternate days from 0.1 mg/kg/day up to 1 mg/kg/day with a maximum total dose of 3g. For VL, aminosidine, alone or in association with pentavalent antimonials, has shown good efficacy but it is still under evaluation. Amphotericin B, included in liposomes, has proven to be very efficient but its use is still limited and expensive (Baily & Nandy, 1994; Desjeux, 1996; WHO, 1998).

PKDL cases should be given high priority as they are considered to be residual reservoir, able to disseminate the disease. Patients should be treated by pentavalent antimonials at 20 mg/kg/day for 3 to 4 months (Desjeux, 1996).

1.1.4 Parasite and life cycle:

The life cycle of *Leishmania* species is commonly viewed as consisting simply of two different morphological stages: the intracellular amastigote in the vertebrate host and the extracellular promastigote in the invertebrate host (Molyneux & Killick-Kendrick, 1987; Hommel, 1999). Briefly, the female sandfly picks up infected cells from the skin with their blood meal. The amastigotes are released in the midgut of the insect, transform to the procyclic stage and start multiplying actively without penetrating the hemocoele. After few days, numerous procyclic conquer the gut of the insect. Then the elongated procyclic promastigote attach to the midgut epithelium by inserting their long flagella between the microvilli that line the midgut. They migrate to the cardiac valve, where they transform into short, spherical, non-dividing promastigotes. Then the parasites are released from the midgut and penetrate the pharynx (proboscis) as metacyclic promastigotes, also termed paramastigote. From proboscis the metacyclic promastigotes are ousted to the new mammalian host.

Metacyclic promastigotes enter the skin of the vertebrate host when the infected sandfly takes its blood meal. It may inoculate 10-200 promastigotes into the dermis. Within the macrophages and related cell types, they rapidly transform into amastigotes, remain within the phagocytic vacuole, where they develops and multiply, at some stage this infected cell, which may harbour up to 20 or more amastigotes, bursts and

released free amastigotes, which infect other cells. Infected macrophages move from the skin to other tissues, infecting the spleen, liver and bone marrow, while certain parasites exhibit a specific tropism for each given host e.g. viscerotropism or dermatotropism. The features that control this tropism has not yet been elucidated but are thought to include, both host and parasite genetics as well as the status of immunity (Molyneux & Killick-Kendrick, 1987; Hommel, 1999).

Most leishmaniasis are zoonotic (transmitted to humans from animal, reservoir hosts), and humans become infected only when accidentally exposed to the transmitting sandflies. However, in the anthroponotic form (those transmitted from human to human through the sandfly vector), humans are probably the sole reservoir host (WHO, 1996). The reservoir of *L. infantum* and *L. chagasi* is usually dog, but in several Old World and New World foxes, rats, opossum or racoon dogs may also act as reservoirs in some areas. The reservoir of *L. donovani* is mainly humans (Hommel, 1999), although other species are incriminated in Africa e.g. *Arvicanthis niloticus* in Sudan (El-Hassan *et al.,* 1995). Domestic dog is incriminated as a reservoir host of American cutaneous leishmaniasis caused by *L. braziliensis*, *L. panamensis* and *L. peruviana* (Reithinger & Davies, 1999). Gerbils (*Psammomys obesus*) have been implicated as a reservoirs host of *L. major* in different Asian countries (Elbihari & El-Hassan, 1987; Rioux *et al.,* 1990; El-Sibae *et al*., 1993). Also *Psammomys obesus is* suspected as a reservoir host of *L. major* in Egypt (Morsy *et al.*, 1996).

1.1.5 The vector:

Sandflies that are the vectors of *Leishmania* parasites are insects of the order Diptera, family Psychodidae, subfamily Phlebotominae of the genus *Phlebotomus* (Old World),

Lutzomyia (New World). There are more sandfly species in the New World than the Old World and this is often quoted as the reason that there are more species of *Leishmania* in the New World (León *et al*., 1996).

Vectors of *L. donovani* in which infection in the fly gut was confirmed are: *P. argentipes* in India, *P. chinensis* in China, *P. pernicrosis* in North Africa, Italy, France and Portugal, *P. perfiliewi* in Greece, *P. orientalis* in Sudan and Ethiopia, *P. martini* in Kenya (Le Blancq & Peters, 1986). *L. infantum* is transmitted by *P. perniciosus*, *P. ariasi*, *P. perfiliewi* and *P. neglectus*. *L. chagasi* is transmitted by the *L. longipalpis* (WHO, 1990). Sandflies usually repose during the day in burrows, tree hollows, caves or buildings. After sundown, they leave the shelters to remain active throughout the night.

1.1.6 The *Leishmania* genome:

1.1.6.1 Nuclear DNA:

The study of trypanosomatid chromosomes has been done by pulse field gel electrophoresis (PFGE) and variations of this technique like the orthogonal field alternate gel electrophoresis (OFAGE), since there is no evidence available for typical chromosome condensation during mitosis (Bishop & Miles, 1987; Lighthall & Giannini, 1992; León *et al*., 1996).

Several studies suggest that *Leishmania* chromosomes are mainly diploid (Lovannisci *et al.,* 1984; Beverley *et al*, 1988), with some chromosomes being aneuploid (Bard, 1989). Now diploidy of Leishmania is widely accepted (Wong, 1995). The most refined studies identified 22 to 33 chromosomes in different *Leishmania* species, a

figure that is very likely an underestimate of the actual number of chromosomes (Bard, 1989). León *et al.* (1996) reported that in both New and Old World different species exhibit number of chromosomes ranging between 20-25 pairs. However, Wincker *et al*. (1996) managed to characterize 36 chromosomes demonstrated in *L. infantum* and *L. donovani*. The chromosomes are linear between 200-4000kb in length and they possess telomeres but centromeres have not been identified (Lighthall & Giannini, 1992). Chromosome size variability is characteristic of some *Leishmania* species, even between homologus, chromosomes (Blaineau *et al.,* 1991) which complicates the use of karyotype in taxonomic studies. *Leishmania* genes are often found to be organized in tandem arrays or at least to have 2 or more copies spread through the genome (Bard, 1989).

1.1.6.2 Kinetoplast DNA (kDNA):

kDNA represents the mitochondrial DNA (mtDNA) of the kinetoplastida and corresponds to 10-20% of the total DNA (Simpson, 1987). It is a network of concatenated circular DNA, divided into 2 classes: the homogenous maxicircles (~25-50 molecules of 20 kb) and the heterogeneous minicircles (~0.8kb), which has many copies (~10^4). The maxicircle is the functional counterpart of the mitochondrial DNA, nevertheless its role in the editing of Uracil residues into mRNA nucleotides has been demonstrated (León, 1996). The minicircles encode guide RNAs (gRNA) for editing of cytoplasm oxidase subunit III mRNA (Sturm & Simpson, 1990). Also Singh & Rastogi (1999) describe an unprecedented finding in the variable region in one of the minicircles sequence classes of *L. donovani,* which is transcribed and translated to a protein product.

1.1.7 Classification of *Leishmania* species:

The genus *Leishmania* is divided in 2 subgenera based in the distribution of the parasite in the midgut of the sandfly vectors. In the subgenus *Leishmania*, the promastigotes develop in the midgut and foregut of the insect - section Suprapylaria-, whereas in the subgenus *Viannia* parasites are restricted to the hindgut -section Peripylaria- (Lainson & Shaw, 1987). Species of the subgenus *Leishmania* belong to the *L. donovani* complex (*L. donovani, L. infantum, L. chagasi* [American *L. infantum*], *L. archibaldi*); *L. tropica* complex (*L. tropica, L. aethiopica, L. major*) and *L.* mexicana complex (*L. mexicana*, *L. amazonensis*, *L. pifanoi, L. garhami, L. venezuelensis*). On the other hand, parasites of the subgenus *Viannia* only occur in the New World (Shaw, 1994). These are the species of *L. braziliensis* complex (*L. guyanensis*, *L. naiffi*, *L. peruviana, L. panamensis* and *L. shawi*). The 2 subgenera *Leishmania* and *Viannia* belong to the Kingdom Protista Haeckel, 1866; Sub-kingdom Protozoa Goldfuss, 1817; Phylum Sarcomastigophora Honigberg & Balamuth, 1963; Sub-phylum Mastigophora Deising, 1866; Class Zoomastigophorea Calkins, 1909; Order Kinetoplastida Honigberg, 1963, emend. Vickerman, 1976; Sub-order Trypanosomatina Kent, 1880; Family Trypanosomatidae Dolfein, 1901, emend. Grobben, 1905; Genus *Leishmania* Ross 1903 (Lainson & Shaw, 1987).

1.1.8 Diagnosis of visceral leishmaniasis:

Routine diagnosis of VL relies on a combination of the following methods (i) the clinical suspicion i.e. long-term unexplained fever, cachexia and hepatosplenomegaly (ii) microscopic detection of leishmanial amastigotes in stained smears of lymphnode, bone marrow or splenic aspirates (iii) the culture of the parasite (iv) immunological

tests for the detection of anti-leishmanial antibodies and leishmanial antigenes and (v) molecular techniques including PCR.

Microscopic finding of the parasite is considered the golden standard for diagnosis, although the method is relatively simple and cheap, it suffers from low sensitivity (Weiss, 1995; Osman *et al*., 1997), and there is no possibility to distinguish between *leishmania* amastigotes belonging to the different species (Neva & Sacks, 1990; Weiss, 1995). It has been estimated that the sensitivity of microscopy may only be 50-85% when, a single specimen is examined by a competent microscopist (Hommel, 1999). Also microscopy suffers from low parasitaemia in the target organs (Wilson, 1991). Better results were obtained when using invitro culture, but the major drawbacks of culturing, especially under field conditions, is its vulnerability to contamination (Wilson, 1991; Oskam, *et al*., 1998).

Serological tests reveal the presence of antibody and are useful in both individual diagnosis and epidemiological surveys. A number of methods have been described (extensively reviewed by Kar, 1995), including indirect immunofluoresent antibody test, IFAT, (Badaro *et al*., 1983; Pappas *et al*., 1985); enzyme- linked immunosorbent assay, ELISA, (Hommel *et al*., 1978; Pappas *et al*., 1985); direct agglutination tests, DAT, (Harith *et al*., 1986 & 88; Meredith, *et al.,* 1995; Hommel *et al*., 1997), and a variety of immunoblotting methods (Evans & Pearson, 1988; Rolland *et al*., 1994). There are various problems with serological assays, including the fact that persistence of antibodies may be a problem in endemic area; certain individuals may have a high level of reactive antibodies in the absence of the organism (false positive), conversely anti-parasite antibodies when induced may not be present until some time after the

initiation of infection (false negative). The possible cross-reactivity with other pathogens, e.g. malaria, trypanosomiasis, schistosomiasis and leprosy (Abdallah, 1980) and the fact that most serological tests cannot readily distinguish between current, subclinical or past infections (Hommel *et al*., 1997). The performance of serological tests is particularly poor in patients co- infected with HIV (Hommel, 1999).

Recently, several molecular biological techniques have been developed for the sensitive detection and identification of pathogens. The main approaches to nucleic-acid-based detection are (i) hybridization using DNA probes (ii) amplification techniques including the polymerase chain reaction (PCR) for the detection of DNA, nucleic acid sequence based amplification (NASBA) and reverse-transcriptase PCR (RT-PCR) for the detection of RNA.

PCR, which is used in this study, is a technique, which allows the sensitive, specific and fast detection of minute amounts of pathogen DNA. PCR is based on the amplification of a known, specific sequence using oligonucleotide primers (typically 20-mers), which specifically bind to the DNA flanking the region of interest. Then the target sequence is amplified using a heat-stable DNA polymerase isolated from *Thermus aquaticus* (Saiki *et al*., 1988). The amplification procedure consists of denaturation of a double stranded DNA, annealing and extension that results in thousands of copies of DNA. This technique is useful in detecting infections with low parasites level.

1.1.9 Identification and characterization of *Leishmania:*

1.1.9.1 Phenotypic and immunological methods:

Leishmania organisms have been classified as different species primarily on the basis of clinical, biological, geographical and epidemiological criteria. During the last decade a number of methods have become available for the differentiation of *Leishmania* isolates. For epidemiological purposes, the most useful taxonomic techniques has long been isoenzyme analysis (Miles *et al*., 1980; Evans *et al*., 1984; WHO, 1990; Andresen *et al*., 1996). The technique still remains the 'gold standard' for *Leishmania* taxonomy and its principal advantage is to provide a stable marker for clusters of geographical isolates within each given species (Hommel, 1999). However, it is restricted in the way that, it assays the genotype indirectly, so that nucleotide substitutions that do not change the amino acid composition remain undetected, changes in the amino acid composition that do not change the electrophoretic mobility may also be not observed (Lewin, 2000). Further more isoenzyme analysis is slow, laborious, and expensive, requiring cultivation of the isolates and the estimation of the profiles of 10-20 different enzymes (Andresen *et al.*, 1996; Noyes *et al.*, 1996). Other techniques include monoclonal antibodies, which are currently being assessed to identify New World species but suffer from their relative lack of specificity for isolates from widely spread foci (McMahon-Pratt & David, 1981; Noyes *et al.*, 1996).

1.1.9.2 Molecular biological methods:

Different DNA-based methods have been used for characterization of isolates of *Leishmania* at

(a) Genus level: by performing PCR using *Leishmania* common primers depending on direct identification of the parasite in clinical materials (Uliana *et al*., 1991; Hassan *et al*., 1993; Piarroux *et al.,* 1993 & 1994; Andresen *et al*., 1997; Osman *et al*., 1997 & 1998).

(b) Species level: different methods could be applied, such as PCR which depends on species specific primers (Ibrahim *et al*., 1994), analysis of restriction fragment length polymorphism (RFLP), where the substrate can be whole genomic DNA, kDNA or PCR amplification products, analysis of kinetoplast and nuclear DNA including Southern blot hybridisation with specific DNA probes (Jackson *et al*., 1984; Beverley *et al*., 1987; Barker, 1989; Van Eys *et al*., 1989&1991), DNA fingerprinting using DNA probes complementary to repetitive DNA sequences (Macedo *et al*., 1992), PCR fingerprinting approaches using single arbitrary or non-specific primers (Williams *et al*., 1990; Tibayrenc *et al*.,1993; Pogue *et al*., 1995a&b; Schönian *et al*, 1996), molecular karyotyping (Lighthall and Giannini,1992) and sequencing of species specific genes e.g. sequence of the first part of ITS (located between the small subunit rRNA and the 5.8S rRNA genes), obtained at Microbiology laboratory, Institüt fur Microbiologie und Hygiene, Berlin, Germany, are found to be almost species specific.

(c) Strain level: these methods are capable to detect variation among isolates of *Leishmania* belonging to the same species such as sequencing, PCR-fingerprinting approach which uses single non specific primers and the restriction analysis of the amplified ITS region (Schönian *et al*., 2000; Schönian *et al.,* 2001), single-stranded conformation polymorphisms, SSCP (Van Eyes *et al*., 1992; Lewin, 2000).

High levels of intra- and inter-species variation were observed in New World *Leishmania* species of the *Viannia* subgenus by amplifying and restricting the rapidly evolving internal transcribed spacers (ITS) in the ribosomal operons. Using this aproach it has been made possible to distinguish species and strains of New World *Leishmania* isolates based on their characteristic restriction patterns (Cupolillo *et al.*, 1995). However, the disadvantage of this technique is that it detects either point mutations that affect the restriction site or deletions or insertions which change the size of the fragment (Orita *et al*., 1989). To obtain information about the part of the sequence not covered by this analysis, other techniques must be employed.

PCR fingerprinting methods do not depend upon previous knowledge or availability of the DNA sequence of the target (Welsh & McCllelland, 1990; Williams *et al*., 1990; Tibayrenc *et al.*, 1993). Using these PCR techniques, detection of genetic polymorphisms among species and strains of *Leishmania* has been described (Pogue *et al*., 1995a&b; Noyes *et al.*, 1996; Schönian *et al.*, 1996). However, these methods can only be used on cultured parasites. Contaminating host DNA would mask the signal from the parasite DNA (Noyes *et al.,* 1996).

Analysis of kinetoplast sequences as well as of electrophoretic karyotyping have proven less reliable for identifying *Leishmania* isolates because extensive polymorphism has been observed by these methods among strains of the same species (Rogers & Wirth, 1987; Bishop & Akinsehinwa, 1989; Bastien *et al.,* 1990; Henriksson *et al.*, 1996; Wincker *et al.*, 1997).

Direct DNA sequencing of PCR products is another approach commonly used for development of typing methods, population genetic studies, designing specific primer and detection of sequence variation in a particular gene (Bevan *et al*., 1992; Reddy, 1995). Although it is possible to detect polymorphisms in a sequence, it is not possible to separate and hence define the number of sequence variants, if significant levels of size and sequence heterogeneity exist in a particular DNA e.g. (hypervariable region of DNA) then it may be difficult or even impossible to read a sequence (Gasser & Chilton, 1995). To overcome this problem PCR products are usually cloned into a plasmid vector. Once PCR product is cloned, it may be a tedious and time consuming (Gasser, 1997). Although nucleic acid sequencing is the most informative technique available for genotypic studies, it is labour intensive, difficult and expensive. Sequencing can be faster and easier if an automated sequencer is used.

PCR- based mutation scanning techniques provide powerful alternatives for studying genetic variation in populations (Cotton, 1993; Lessa & Applebaum, 1993). These methods, which rely on physical properties or the modification of DNA molecules of the same or very similar size, that differ by one or more nucleotides, their excellent capacity and potentiality to resolve sequence variability has not been exploited for the study of genetic variation in parasites (Gasser, 1997).

Single-stranded conformation polymorphisms (SSCP) analysis has been developed to scan genes for single base differences which could be useful as genetic markers (Orita *et al*., 1989; Gasser, 1997; Gasser & Zhu, 1999). This method relies mainly on the principle that the electrophoretic mobility of a single-stranded DNA molecule in a non-denaturing gel is dependant on its structure and sizes (length). The single stranded molecules have secondary and tertiary structures (conformations) as a result of base pairing between complementary nucleotides within each strand. SSCP showed species-specific patterns as well as intra-species variation in an entomological study (Hiss *et al*., 1994). So far, only a single report describes the use of isotopic SSCP as a tool for the identification of *Leishmania* at the complex level based on the observation of point mutations in the central part of the SSU rRNA genes (Van Eys *et al*., 1992).

1.2 Leishmaniasis in the Sudan:

Sudan is considered as one of the most important areas of leishmaniasis in the world where sharp epidemics involving thousands of people with many deaths were recorded. All forms of leishmaniasis i.e. CL, MCL, VL and PKDL occur in Sudan. This group of diseases causes serious economic loss in the country, both in terms of the disability of affected individuals and in the cost of treatment, especially as most of those with leishmaniasis are on low incomes and live in rural areas.

Visceral leishmaniasis (VL) is the one of the most important endemic diseases in the country and is known to occur in the Sudan since 1904 when Neave (1904) described the first patient in the country. The main endemic area is in the eastern part of the

country, from the banks of the White Nile in the West to the Ethiopian border in the East, and from Kassala in the North towards Malakal in the South see Fig.(1). Other smaller foci have also been described in Kapoeta in Equatoria and parts of Kordofan and Darfur provinces. Occasional severe outbreaks occur, like the one in the southern Fung in Blue Nile province in 1956-1960, which caused thousands of death (Sati, 1958). An outbreak of kala-azar was reported in Khartoum among displaced people (de Beer *et al*, 1990). Also, epidemics have occurred in recent years in war zones of southern Sudan where about 100,000 people died of leishmaniasis since 1984 (Seaman *et al*, 1996). Recently Roberts *et al.* (2000) reported that more than 10% of the population in southern Sudan died from visceral leishmaniasis over the past 5 years. Zeese and Frank (1987) estimated that of the total number of 1300 patients reported annually in the Sudan, more than 75% were treated in the hospitals of Gedaref and Hawata, a small rural town ~100km south to Gedaref, situated along the Rahad River.

L. donovani senso lato is incriminated as the aetiological agent of VL (Hoogstraal & heyneman, 1969; El- Hassan *et al*., 1995). The incubation period of kala-azar appears to be between 2 months to 2 years (Zijlstra *et al*., 1991). *P. orientalis* is the only known proven vector of kala-azar in the Sudan (Hoogstraal & Heyneman, 1969; Elnaiem *et al.,* 1997). However, in the Kapoeta area in South Sudan, where *P. orientalis* is not known to be present *P. martini* may be the main vector of VL (Miniter *et al*, 1962). The Nile Rat (*Arvicanthis niloticus*) is being incriminated as the reservoir host for VL in Sudan (Hoogstraal & Heyneman, 1969; El- Hassan *et al*., 1995).

Figure 1: Endemic areas of visceral and cutaneous leishmaniasis in Sudan.

: Study area

\\\\\ : Cutaneous leishmaniasis

: Visceral leishmaniasis

Also Hoogstraal & Heyneman, (1969) reported the rodent *Acomys aligena*, The Spiny Mouse and two species of carnivores: *Genetta g. senegalensis*, The Senegal Genet and *Felis serval phillipsi*, The Sudanese Serval Cat to be infected.

The diagnosis of kala-azar is classically made on clinical grounds as well as parasitologically by examination of smears of lymph node, bone marrow or splenic aspirates. Lymph node aspirate has been recommended as a safe procedure with sensitivity of 78% in Sudanese kala-azar (Siddig *et al*., 1989). However, other serological tests were applied such as the enzyme- linked immunosorbent assay (ELISA) and the direct agglutination test -DAT- (Harith *et al*., 1986; El-Safi & Evans, 1989). Recently the PCR has been introduced for the diagnosis of leishmaniasis (Osman *et al*., 1997).

Kirk & Sati (1947) introduced the treatment of kala-azar with sodium stibogluconate in the Sudan and antimony is well established as the drug of choice (Zijlstra *et al*., 1993). The only drugs affordable in poor countries are the pentavalent antimonials. Drug costs vary between about $20 and $200 for treating an average patient.

Post kala-azar dermal leishmaniasis (PKDL) is a known complication of VL in Sudan, although cases without previous history of kala-azar were reported in Sudan (El-Hassan *et al*., 1992).

Cutaneous leishmaniasis (CL) is common in the North and is characterized by ulcers in the skin that usually self heal, leaving a scar. In recent years a major epidemic occurred in large parts of northern Sudan, causing thousands of cases (El-Safi & Peters, 1991). CL is caused by *L. major* (Abdallah *et al*., 1973). *P. papatasi* was incriminated as a vector for CL (Abdallah & Sherif, 1978). *Arvicanthis niloticus* is believed to be the reservoir of cutaneous leishmaniasis in the Khartoum area (El-Safi & Peters, 1991).

Mucocutaneous leishmaniasis (MCL) in the Sudan is a rare condition, less than 100 cases have been described. The condition may be caused by *L. major* or it may follow kala-azar, which is caused by *L. donovani* (Ghalib *et al*., 1992).

1.3 Selection of genomic targets for the differentiation of species and strains of *Leishmania:*

Studies involving PCR, sequencing and SSCP require the selection of appropriate areas of genome that must be chosen with respect to variability. Sequences coding for vital proteins or RNAs are useful for comparing between distantly related taxa, because they are highly conserved. On the contrary, genes for proteins with antigenic properties can be expected to vary considerably, thus making their use difficult, but they can give valuable information on adaptation and speciation. Likewise, intergenic regions that are expected to vary randomly since they are not influenced by natural selection are the most useful for determination of temporal distance. The following genomic regions were targeted in this study.

1.3.1 Ribosomal internal transcribed spacer (ITS):

Leishmania rRNA genes are found as a tandem repeat units separated by a non-transcribed spacer (NTS) regions. The units are composed of the small sub-unit (SSU) rRNA, 5.8S rRNA and of the large sub-unit (LSU) rRNA genes which are highly conserved in *Leishmania*. These coding regions are separated by an internal transcribed spacers (ITS). Both NTS and the ITS regions show extensive variability (Cupolillo *et al*., 1995). ITS is particularly interesting for its relatively small size (~ 1 kb in *Leishmania*). It is flanked by highly conserved segments to which PCR primers can be designed (Cupolillo *et al*., 1995).

1.3.2 gp63 genes:

Gp63 is a surface glycolipid-anchored zinc protease of approximately, 63 kDa present in all *Leishmania* species (Roberts *et al*., 1993). It has been demonstrated that Gp63 is present in both the promastigote and amastigote life stages of *Leishmania* (Frommel *et al*., 1990). Also it is known as a major surface protease (msp). This molecule is important for both, the entry of the parasite to the mammalian macrophages and its survival within the macrophage phagolysomes (Wilson *et al*., 1989; Bordier, 1987).

Although the gp63 gene organization has a different degree of complexity among various *Leishmania* species and even within the same species it forms, in general, a family whose members are distributed in tandem arrays along the genome with varying copy numbers (Morales *et al*., 1997). In *L. chagasi* the genes are described to be arranged in 3 distinct classes (Ramamoorthy *et al*., 1992).

Low virulent promastigotes in early logarithmic phase of growth, express gp63 RNA of 2.7 kb (mspL); where as the highly virulent stationary phase promastigotes contain gp63 RNA of 3.0 kb (mspS). Cells in intermediate stages of growth have varying amounts of both the RNA classes. Constitutively expressed RNAs of 2.6 and 3.1 kb which form the third class mspC are also present at constant low levels throughout the promastigote growth cycle (Ramamoorthy *et al*., 1992). The organizations of the gp63 genes in several other *Leishmania* species have been investigated. In *L. donovani* at least 7 tandem genes as 3 kb repeats are separated from a single gene down stream and at least 2 other genes occur elsewhere (Webb *et al*., 1991). The finding that multiple

gp63 genes occur within a species indicates that different forms of the protein might be expressed (Roberts *et al*., 1993).

1.3.3 Anonymous DNA regions:

All anonymous DNA regions targeted in this study are nuclear genes, specific for *Leishmania* and represent single copy genes with exception of one marker (LK413) which is found in 2 chromosomes. These codominant anonymous markers namely L0110, L510, L413, LK413, L0308 and L0114 are characterized by small sizes ranging between 120 and 580 base pairs and are located at chromosomes number 29, 33, 14, 13 &26, 28 and 34, respectively. These genes do not show any homology to known genes of *Leishmania* found in data bank. Primers for these randomly amplified targets are developed at the Institüt für Mikrobiologie und Hygiene, Berlin, Germany as described by Lewin (2000) for population genetic studies.

1.4 Objectives of this study:

Correct diagnosis and identification of the parasite are crucial for choosing the effective treatment against different forms of leishmaniasis, for epidemiological and population genetic studies and for development of genetic markers. Therefore diagnosis of causative agent of visceral leishmaniasis must now contemplate typing of the organism. These typing methods should be quick, easy to perform, produce results from low parasites number and contaminated samples to be performed particularly in endemic regions of developing countries, especially like the situation in this study where the samples were collected under field conditions in remote rural areas. This

study tried mainly to contribute towards development and evaluation of sensitive methods based on amplification of parasite DNA directly from clinical samples without prior culturing. For the above mentioned reasons this work aimed towards

1. To evaluate the performance of the nested PCR based on the detection of the *Leishmania* ITS1 rRNA gene, directly from biological materials spotted on filter papers as a diagnostic tool.
2. To examine genetic heterogeneity among the causative agents of visceral leishmaniasis in some endemic Sudanese leishmaniasis foci directly from biological materials spotted on filter papers. Mainly the internal transcribed spacer (ITS), has been utilized. Also some other gene loci have been targeted such as the gene coding for the antigenic proteins (gp63) and other 6 anonymous DNA regions. Then to evaluate the use of these mentioned PCR products as potential genetic markers for strain differentiation using SSCP technique.
3. To compare 3 PCR based methods (in order to screen for genetic variability within the causative agents of VL and PKDL in eastern Sudan) namely PCR-fingerprinting as well as PCR-RFLP and PCR- SSCP utilizing ITS as a target and to correlate the data obtained by the 3 methods and to validate the results by sequencing.

2 Materials and Methods

2.1 Study area:

This study was carried out in clusters of villages that represent an endemic focus of visceral leishmaniasis (VL). These villages were located in Gedaref State, eastern Sudan (Figure 1). The disease is well known among people in these villages as kala-azar. The climate of the study area is tropical and could be divided through the year into a hot dry summer (March-June), a warm wet autumn (July-October) and a moderately warm winter (November-February). The average minimum/maximum temperature of the area is 28/44^{o}C in summer and 18/34^{o}C in autumn and winter, with annual rainfall of about 600mm.

This study area is generally characterised by reduced vegetation and grass with scattered *Acacia* trees. The area is flat and composed of cracked alluvial clay soil. People usually live in huts constructed of wood, bamboo and grass (Figure 2). The villages are surrounded by large scale sorghum and sesame fields. Inhabitants mainly work as farmers, woodcutters, and shepherds. Many nomadic tribes roam the area.

2.2 Samples collection:

Clinical samples were collected from 100 patients suspected of having kala-azar (patients in whom parasites were not demonstrated microscopically, but who had clinical symptoms and signs suggestive of (VL) or post kala-azar dermal leishmaniasis

(PKDL). These patients had at least one of the following features: fever for more than two months, left upper quadrant abdominal pain lymphadenopathy, splenomegaly, wasting or nodules and papules on the skin in case of PKDL. When kala-azar was suspected, inguinal lymph node aspiration was performed and Giemsa-stained thin films were examined for *Leishmania* parasites; if negative or when no palpable lymph nodes were present, a bone marrow aspiration was performed and the marrow was also microscopically examined. In case of PKDL, skin smear was examined.

Figure 2: A picture of the endemic villages showing the construction of living places and the cracked clay soil.

The clinical samples were collected from Gedaref hospital (the main referral hospital in the area), Gedaref clinic (Gedaref State, eastern Sudan, 411 km from Khartoum) as outpatients and from kala-azar suspected patients in their villages between April 1997 and November 1998. All patients were originating from an endemic area in eastern Sudan. In total 71 cases were found to be microscopically positive. Only the microscopically confirmed kala-azar cases (patients in whom *Leishmania* amastigotes were demonstrated by microscopy on either lymph node or bone marrow aspirates) were included in this study for molecular characterization purposes. The remaining 29 were included for diagnostic purposes to compare the microscopic method with the polymerase chain reaction (PCR) method for detection of *Leishmania* parasite.

About 30µl from inguinal lymph node, bone marrow aspirates or skin scraping were collected and spotted on Whatman filter paper #3. Each filter paper sample was stored in a separate polyethylene bag at room temperature for further molecular biological analysis. Five samples were collected from lymph node aspirate from Sudanese patients who had diseases other than VL and were used as negative control.

Forty from the 71 microscopically positive aspirates were simultaneously attempted for culturing. In this case part of the aspirate was also aseptically inoculated into 2 tubes containing NNN (1.4% agar, 0.6% NaCl) and Difco 4N (4% difco 4N blood agar) media, respectively. These media were prepared as described by Evans (1989). 10 ml of defibrinated rabbit blood containing 100 µl gentamicin solution were added to 100 ml of the previous melted medium. 2 ml of either NNN or Difco 4N media were distributed into small sterile tubes, then kept in sloped position until the agar solidified. The tubes were stored in 4^{o}C until use. Just before inoculation 200 µl of 0.9% NaCl

were added to each tube. The cultures were kept at ambient temperature 25^{o}C and transported to Institüt für Mikrobiologie und Hygiene, Humboldt-Universität zu Berlin, Germany for further sub-culturing in RPMI 1640 medium supplemented with 15% fetal bovine serum and 100 µg/ml penicillin and 100 µg/ml streptomycin. These cultures were sub-cultured weekly (1 ml from the previous culture plus 10 ml from the RPMI medium).

Structural questionnaire filled in by; direct interview for each patient was used for collection of demographic and clinical information. This questionnaire covered the following topics: name of the patient, patient code, sex, age, tribe, residence (usual place of residence and present residence), date of isolation of the parasite, clinical symptoms, microscopy test and stage of treatment.

DNA isolated from further 18 *Leishmania donovani* strains used in this study were obtained from the collection of the Royal Tropical Institute, Amsterdam. Twelve of them were isolated from the same above-mentioned endemic study area in eastern Sudan (Gedaref State). Six were WHO references namely, MHOM/KE/85/NLB323, MHOM/IN/71/LRC-L51a, MHOM/CN/??/Wangjie1, MHOM/SD/75/LV139, MHOM/SD/68/1S and MHOM/SD/62/LRC-L61.

2.3 DNA extraction:

2.3.1 DNA extraction from clinical samples spotted on filter papers:

DNA was isolated as described previously by Meredith *et al.* (1993). Briefly, filter papers with spotted biological material (Lymph node, bone marrow aspirates and skin smear) were punched out with a paper puncher. After each sample was obtained a clean sheet of paper sprayed with 90% alcohol was punched 10 times in order to prevent DNA contamination from one sample to the next. Two punched out discs (approximately 15 µl of aspirate) were placed in 250 µl lysis buffer (50 mM NaCl, 50 mM Tris- HCl; 10 mM EDTA pH 7.4; 1% {vol/vol} Triton X-100; 200 µg of Proteinase K per ml) and incubated in water bath for 3 hours or overnight at 60^{o}C. Thereafter the mixture was subjected to phenol -chloroform extraction. An equal volume of bufferd phenol was added, shaked gently for 10 minutes and centrifuged at 13000 rpm. The upper water phase was then transferred to a new 1.5 ml eppendorf tube containing an equal volume of chloroform-isoamyl alcohol (24:1), shaked and centrifuged as mentioned above. Again the upper water phase (±250 µl) was extracted and for precipitation 1/10 volume of 3M NaAc and 2 volumes of absolute ethanol were added. The tubes were left overnight at - 20^{o}C. Thereafter, samples were centrifuged for 15 minutes at 12000 rpm. DNA pellets were washed with 70% ethanol then dried using speed vacuum dryer (Speed Vac, Savant, Hicksville, NY, USA) for 5 minutes. The dry pellets were then resuspended in 50 µl TE buffer (10mM Tris; 1mM EDTA pH 7.5) and stored at 4^{o}C until use.

2.3.2 DNA extraction from cultured *Leishmania*:

DNA was isolated from 8 successful cultures as described previously by Schönian *et al.* (1996) with slight modifications, 2 ml cultured cells were suspended in 5 ml Net buffer (50 mM NaCl; 10 mM EDTA; 50 mM Tris- HCl pH 7.4). Sodium dodecyl sulphate (SDS) was added to a final concentration of 0.5% and the mixture was shaked until the solution was viscous. RNase was added to a concentration of 100 µg/ml and the mixture was incubated in a water bath for 30 min at 60^{o}C. Proteinase K was added to a final concentration of 100µg/ml and the samples were incubated again at 60^{o}C in a water bath for 3 hours or overnight. Then samples were subjected to phenol/chloroform extraction, precipitation with NaAc and ethanol and redissolution in 200 µl TE buffer as described above for extraction of DNA from filter papers. The DNA concentration was measured spectrophotometrically using UV/ Visible Spectrophotometer (Pharmacia LKB. Ultrospec 111).

2.4 PCR amplification:

2.4.1 Internal transcribed spacer (ITS):

The entire ITS region was amplified with the following *leishmania* specific primers: LITSR (5$^{'}$ -CTGGATCATTTTCCGATG-3$^{'}$) and LITSV (5$^{'}$-ACACTCAGGTCTGTAAAC-3$^{'}$). No amplification products were observed when human, *Trypanosoma cruzi*, *Escherichia coli*, *Candida albicans, Trychophyton terrestre* and *Microsporum audouinii* DNA was used as template in this PCR approach. The ITS1 and ITS2 regions (see Figure 3) were separately amplified with the

primerpairs L5.8S (5'- TGATACCACTTATCGCACTT-3')/LITSR and L5.8SR/ (5'-AAGTGCGATAAGTGGTA-3')/LITSV; respectively. Primers were synthesized commercially (TIB-MOLBIOL, Berlin, Germany).

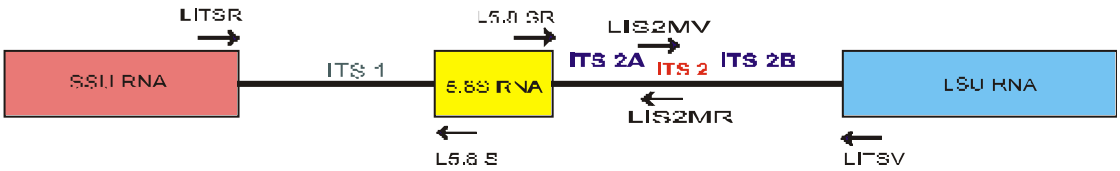


Figure 3: The position of the internal transcribed spacer (ITS) in the ribosomal operon amplified with *leishmani*a specific primers. Primer sequences are given in the text.

For SSCP and sequence analysis the two parts of the ITS2, A and B (Figure 3) were amplified separately using the primer combinations LIS2MR (5'-AGAGTGCATGTGTGTAT-3')/ L5.8SR and LIS2MV (5'-ATACACACATGCACTCTC-3')/ LITSV, respectively.

Amplification reactions were performed in volumes of 50 µl. 3 µl DNA or 1 µl DNA (for nested PCR) were added to a PCR mix containing 200 µM of each dNTP (Pharmacia Biotech, USA); 1x PCR buffer (10 mM Tris-HCl, pH 8.0; 50 mM KCl; 1.5 mM $MgCl_2$); 2 U *Taq* polymerase (Perkin Elmer, Roche Molecualr System, USA) and 25 pmol of each primer. Samples were overlaid with sterile, light mineral oil (Sigma, Deisenhofen, Germany) and amplified as follows: initial denaturation at 95°C for 2 min followed by 34 cycles consisting of denaturation at 95°C for 20 sec, annealing at 53°C for primer pairs LITSR/LITSV; LISTR/L5.8S and LITSV/L5.8SR or at 57°C for primer pairs LITSV/LIS2MV and L5.8SR/LIS2MR for 30 sec and extension at 72°C for 1min. This was followed by a final extension cycle at 72°C for 6 min.

PCR was run in Robocycler Gradient 40, Stratagene. Amplification products were subjected to electrophoresis in 1% agarose NA (Pharmacia Biotech AB, Uppsala, Sweden) at 100 Volts in 0.5x TBE buffer (0.023 M Tris-borate, 0.5 mM EDTA) and visualized under UV light after staining for 15 min in ethidium bromide (0.5 µg/ml). One kilo base pairs (1kbp) DNA ladder (Life Tech, USA), was used as a molecular size marker. Amplified PCR products were documented by photography (on Polaroid films, USA) or by a camera (Gene Eagle eye 11, Stratagene, Heidelberg).

Nested PCR was performed to obtain sufficient PCR products for subsequent single-stranded conformation polymorphisms (SSCP) analysis or sequencing. ITS1 and ITS2 were amplified by nested PCR from previous ITS PCR products and, ITS2 A and B from ITS2 products.

2.4.2 Major surface protease msp (gp63) gene:

To design primers for the amplification of gp63 sequences, sequences of *L. donovani, L. infantum, L. chagasi* and *L. major* as well as of different classes of gp63 genes (S, L & C) that were submitted to Gene Bank were aligned. Then primer sequences were designed from the highly conserved regions (with close annealing temperature, according to the equation {4(G+C) +2(A+T)}-3 to 5^{o}C across the above mentioned species. Part of the coding region of the gp63 gene (from position 445-461 to position 1802-1817) was amplified with the following primers: gp63-1 (5'-TCCACCGAGGACCTCACCGA-3') and gp63-6 (5'-CTGGCACACCTCCACGTACG-3'). For SSCP and sequence analysis (Figure 4), this part was divided into 3 parts X (from position 445-461 to position 869-887), Y (from

position 850-868 to position 1209-1224) and Z (from position 1189-1204 to position 1802-1817), which were amplified separately using the primer combinations gp63-1/gp63-2 (5'-GTCGTACCGCGACGCAATGT-3'); gp63-3(5-ACATTGCGTCGCGGTACGAC-3')/gp63-4 (5'-GTAGAAGCCGAGGTCCTGGA-3') and gp63-5 (5'-TCCAGGACCTCGGCTTCTAC-3')/GP63-6, respectively. These primers were synthesised commercially (TIB-MOLBOL, Berlin, Germany).

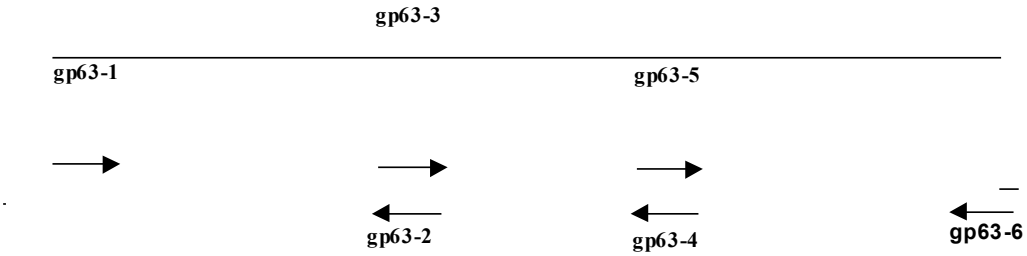


Figure 4: Schematic presentation showing the division of gp63 gene into 3 parts. Primer sequences are given in the text.

Amplification reactions were performed in volumes of 50 µl. 3 µl DNA were added to a PCR mix containing 200 µM of each dNTP (Pharmacia Biotech, USA); 1x PCR buffer (10 mM Tris-HCl, pH 8.0; 50 mM KCl; 1.5 mM $MgCl_2$); 2.5 U *Taq* polymerase (Perkin Elmer, Roche Molecular System, USA) and 10 pmol of each primer. Samples were overlaid with sterile, light mineral oil (Sigma, Deisenhofen, Germany) and amplified as follows; initial denaturation at 94°C for 3 min followed by 30 cycles consisting of denaturation at 94°C for 30 sec, annealing at 63°C for primer pairs gp63-1/gp63-6 and gp63-1/gp63-2; at 64°C for primer pairs gp63-3/gp63-4 and at 65°C for the primer pairs gp63-5/gp63-6 for 30 sec and extension at 72°C for 1 min. This was

followed by a final extension cycle at 72^{o}C for 6 min. PCR was run in Robocycler Gradient 40, Stratagene. Visualization and documentation of PCR products were performed as mentioned above for ITS region.

To obtain enough PCR products for subsequent SSCP analysis or sequencing, X, Y and Z regions were amplified by nested PCR from previous XYZ region.

2.4.3 Anonymous DNA markers:

Six anonymous DNA regions were amplified, using primers developed at the Institüt für Mikrobiologie und Hygiene, Humboldt Universität zu Berlin as described by (Lewin, 2000) for population genetic studies in *Leishmania*. These primers were synthesized commercially (TIB-MOLBIOL, Berlin, Germany). The primer sequences and the PCR conditions were shown in table (1).

Table 1: Specific primer pairs and PCR conditions (per 50 µl) for the amplification of anonymous DNA markers. One U Taq polymerase and 1x PCR buffer (10 mM Tris-HCl, pH 8.0; 50 mM KCl; 1.5 mM MgCl2) were added into all reactions.

Prime pair	Sequence	Primer each pmol	DNTPs µM	DNA ng	Annealing temp.°C.	cycles
L413H	5′-CTCACGCTTTGTGCTTGTGT-3′	15	50	6	54	30
L413R	5′-CAACAAGGCGTATTTCCACG-3′					
L0114H	5′-CTACCAAGAAGGGTGGCAAG-3′	60	200	8	51	34
L0114R	5′-GGTGCAGTACTCGTACCTAC-3′					
LK413H	5′-ACAGACGTACACACGGGC-3′	15	50	6	50	31
LK413R	5′-TCGTCTTCTTGTTGCTTGCC-3′					
L510H	5′-ATAGGTTAACGGCAACGCAC-3′	30	100	10	52	34
L510R	5′-TGACAGAGACACACAACGAC-3′					
L0308H	5′-ACACCATTCACGGCAGGCAA-3′	60	200	8	52	34
L0308R	5′-CCCTCAATCTCTACCTCCAC-3′					
L0110H	5′-GGCAAAGAAAAAGAGCAGCG-3′	15	100	6	50	34
L0110R	5′-CTTGTCGTGCGTTGAATATC-3′					

All amplification reactions were performed in volumes of 50 µl and amplified as follows; initial denaturation at 95°C for 3 min followed by number of cycles depending on the primer (Table 1). These cycles consist of denaturation at 94°C for 1 min, annealing temperature (Table 1) for 30 sec; extension at 72°C for 1 min and a final extension at 72°C for 5 min. Amplification reactions were performed in Perkin Elmer Thermocycler 9600. The PCR products were visualized and documented as described previously for ITS region.

2.5 Optimization of PCR protocols:

PCR protocols, applied with different primers were optimized mainly with regard to $MgCl_2$ and primer concentration, amount of template DNA and cycling program for both direct and nested PCR. To detect DNA contamination from other samples a negative control (PCR mix plus water instead of DNA) were done. For diagnostic purposes further additional negative control (PCR mix plus DNA from patients who had diseases other than VL) was performed for diagnostic purposes. These two negative controls were performed with each set of PCR amplification reaction.

2.6 Single stranded conformation polymorphism (SSCP):

SSCP is performed by denaturing the double -stranded DNA products as follows: 10 µl of each PCR product were added to 3 µl denaturing buffer (0.01 M EDTA, 1% SDS) and 2 µl loading buffer (80% Glycerine, 0.1 M EDTA pH 8, 10 mM Tris-HCl pH 8, 0.1% Bromophenol blue). The mixture was heated to 98^{o}C for 15 min and immediately chilled on ice and kept at 4^{o}C for 15 min. These samples were loaded on acrylamide gel 350x450x0.8 mm [37.5 ml MDE-gels solution (FMC Bioproducts, Rockland, USA), 9 ml 10x TBE (10.8%Tris, 5.5% Boric, 0.02 M EDTA pH 8), 103.5 ml distilled water, 60 µl NNNN-Tetramethylene diamine (TEMED), 600 µl 10% APS (Ammonium peroxide sulphate)]. The samples were subjected to electrophoresis in 0.5xTBE (0.023 M Tris-borate, 0.5 mM EDTA) which was run in a cold room (7-8^{o}C) at 3 kV, 300 mV and amount of Watt and running time depending on the region under investigation (Table 2). Following electrophoresis, the gel was fixed in 1% Nitric acid for 15 min, subsequently the gel was washed in distilled water for 20 sec, then stained in 2% $AgNo_3$ for 25 min. After washing for 10 min in distilled water, the gel was

placed in freshly prepared developing solution (3% Sodium carbonate with addition of formaldehyde up to 0.05%).

Table 2: Conditions for SSCP analysis of different PCR products

DNA region	Watt	Running time (hr)
ITS1	12	17
ITS2	27	17
ITS2 A	10	15
ITS2 B	15	20
Gp63-X	18	18
Gp63-y	14	17
Gp63-Z	25	17
413	10	10
114	10	10
K413	10	10
510	10	12
308	10	10
110	20	18

2.7 PCR- fingerprinting:

The PCR- fingerprinting technique was performed as described by Schönian *et al.* (1996). The following oligonucleotides which anneal to mini- and microsatellite DNA sequences were used as single primers in the PCR experiments: The core sequence of phage M13 (Huey and Hall, 1989) 5'- GAGGGTGGCGGTTCT-3'; the simple repeat

sequence $(GTG)_5$ 5'-GTGGTGGTGGTGGTG-3'; and $(GACA)_4$ 5'-GACAGACAGACAGACA-3' (Ali *et al*., 1986) as well as the T3B oligonucleotide 5'-AGGTCGCGGGTTCGAATCC-3' which was derived from an intergenic spacer of tRNa genes (McClelland *et al*., 1992).

Table 3: PCR program for PCR-fingerprinting using the primers T3B, (GTG)5, M13 core and (GACA)4. *ID: Initial denaturation. ** F: Final

PCR-program	Primer			
	T3B	$(GTG)_5$	M13 core	$(GACA)_4$
*ID	95°C 2min	95°C 2min	95°C 2min	95°C 2min
Denaturation	95°C 20sec	95°C 20sec	95°C 20sec	95°C 20sec
Annealing	52 °C 30sec	50°C 30sec	50°C 60sec	50°C 60sec
Extension	72°C 80sec	72°C 80sec	72°C 20sec	72°C 20sec
No. of cycles	32	32	27	35
**F. extension	72°C 6min	72°C 6min	72°C 6min	72°C 6min

Amplification reactions were performed in volumes of 50 µl containing 10 ng template DNA for the primers $(GACA)_4$ and T3B, 50 ng for the primer $(GTG)_5$ and 25 ng for the M13 core primer; 1x PCR buffer (10 mM Tris-HCl, pH 8.0; 50 mM KCl; 1.5 mM $MgCl_2$); 3 mM Mg-acetate; 200 µM each of dNTPs (Pharmacia Biotech, USA) and 2.5 U *Taq* polymerase (Perkin Elmer, Roche Molecular System, USA). The primers $(GTG)_5$ and $(GACA)_4$ were added to a final concentration of 5 pmol per 50 µl assay where as T3B and M13 core were added at final concentrations of 10 pmol and 25 pmol, respectively. Samples were overlaid with sterile, light mineral oil (Stigma, Deisenhofen, Germany) and amplified in a Perkin-Elmer Thermocycler 9600 as shown in table (3).

Samples were concentrated to a volume of approximately 20 µl using speed vacuum dryer (Speed Vac. Savant-Hicksville, NY, USA) and subjected to electrophoresis in 1.2% agarose NA gel (Pharmacia Biotech AB, Uppsala, Sweden) for 5 hours at 100 Volts in 0.5x TBE buffer (0.023 M Tris-borate, 0.5 mM EDTA). Amplification products were detected under UV light after staining the gel with Ethidium bromide (0.5 µg/ml) for 15 minutes. The gel was run once again for band sharpening at 125 Volts for 15 min. Finger printing profiles were documented by photography (on Polaroid films, USA) or by a camera (Eagle Eye 11, Stratagene, Heidelberg).

2.8 Restriction fragment length analysis (RFLA):

The entire amplified ITS region, was digested using 10 different enzymes (*Alu*1*, Bst* U1, *Cfo*1, *Dde*1, *Eco*R1, *Fsp*1, *Mse*1, *Msp*1, *Nde*1 and *Taq*1) according to the conditions recommended by the supplier. Briefly, 17 µl of the DNA were restricted by addition of 10 units of each enzyme and 2 µl of the corresponding buffer and incubated at 37^{o}C (with exception of *Taq*1 which is incubated at 65^{o}C) for 2 hours. Restriction products plus 3 µl loading buffer (80% glycerine, 0.1 M EDTA pH 8.0, 10 mM Tris - HCl pH 8.0) were subjected to electrophoresis in 1.3 % metaphore agarose (for fine analytical separation of small nucleic acids and PCR products, FMC Bio Products Rockland, ME, USA) for 2 hours at 80 Volts in 0.5 TBE buffer (0.023 M. Tris-borate, 0.5 mM EDTA). DNA fragments were visualized under UV light after staining for 15 min in ethidium bromide (0.5µg/ml). The gel was run again for 15 min at 100 Volts for increased resolution. Restriction products were documented by photography (on Polaroid films, USA) or by a camera (Gene Eagle Eye 11,Stratagene, Heidelberg).

2.9 Radioactive cycle sequencing:

2.9.1 Template purification:

The quality of the sequence data depends directly on the purity of DNA products. Hence prior to sequencing, 100 µl of the PCR products were purified by loading them on 1% agarose NA gel (Pharmacia Biotech AB, Uppsala, Sweden). Electrophoresis was performed in 0.5x TBE buffer (0.023 M Tris-borate, o.5 mM EDTA). Agarose blocks containing amplified DNA fragments were cut out from the gel under UV light using sterile scalpel and purified using QIA quick gel extraction kit (QIAGEN, Hilden, Germany) according to the instructions given by the manufacturer.

2.9.2 Sequencing cycles:

Sequencing was performed using ThermoSequenase radiolabeled terminator cycle sequencing kit (Amersham Life Science, Inc.26111 Miles Rd., Cleveland, OH44128 USA) according to the instructions given by the supplier. Briefly, purified PCR products were amplified twice using the forward primer on the first amplification and the reverse primer on the second amplification. 7 µl purified DNA, 8 µl distilled water, 2 µl Thermo Sequenase^{TM} reaction buffer, 1 µl of 3 pmol solution single primer and 2 µl Thermo Sequenase^{TM} DNA polymerase were mixed together. The mixture was divided into four 0.5 ml microcentrifuge tubes, labelled A, C, G and T each one containing 4.5 µl reaction mix. To each tube the corresponding 2.5 µl radiolabeled termination reaction mix (0.5 µl of [α-^{33}P] ddNTP, 2 µl dGTP) was added. Then each tube was overlaid with sterile light mineral oil (Sigma, Deisenhofen, Germany). Thereafter amplification reactions were performed in Biometra- Trio-Thermoblock as follows: initial denaturation at 95^{o}C for 2 min, followed by 32 cycles consisting of

denaturation at 95°C for 30 sec, annealing according to the primers (the same annealing temperature used for PCR amplification of the specific region), and extension at 72°C for 80 sec. After PCR cycle sequencing is completed 4µl from the stop buffer were added to each tube. The tubes were stored at -4°C until use for electrophoresis.

2.9.3 Preparation of the sequencing plates and electrophoresis:

Long run ranging sequencing gel glass plates (450x350mm) were cleaned twice with distilled water, and once with 70% ethanol. One of the glasses (earless glass) was wiped with acrylease (Stratagene, Heidelberg). Spacers (0.4 mm) were placed between the plates of both sides and the bottom side. The two glasses were fixed together using clips.

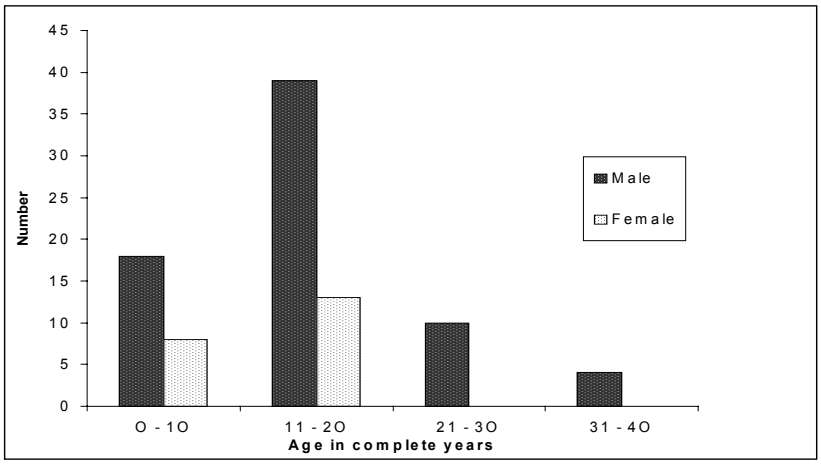
Amplification products were subjected to electrophoresis. Polyacrylamide gel 450x350x0.4mm (48% urea, 6% rotiphorese gel 40 [Fa. Roth, Karlsruhe], 15ml 20x Glycerol tolerant buffer, added to this 20 µl TEMED and 1 ml 10% APS) was poured into the prepared plates. Then a comb was inserted and the gel was allowed to polymerize at room temperature for 2 hours. Samples were preheated at 70°C for 10 min before loading onto the gel to prevent formation of secondary structure. Three µl of each sample (labelled A, C, G and T respectively) were loaded on the gel. The samples were run in 1x Glycerol- tolerant gel buffer (21.6% Tris-hydroxymethyl-aminomethane GR, 7.2% Taurine, 0.4% EDTA) at 1500 Volts for 3 hours (short run), for 6 hours (middle run) and at 1000 Volts for 13 hours (long run). Then the gel was

fixed in 10% glacial acetic acid for 20 min, transferred to GB002 Gel blotting paper 460x570mm, wrapped with Saran Wrap and dried in a Slab gel dryer (Savant-Hicksville, N.Y., USA) at 80^{o}C for 1.5 hour. After complete drying Saran Wrap was peeled off and the radiation was measured in impulses per second using a LB122 Berthold β- δ detector. The dried gel was attached to X-ray hyperfilm^{TM} MP (Amersham Buchler GmbH, Braunschweig, Germany) and kept in the dark room on carton file. After a time of exposure dependant on the amount of measured radioactivity (if less than 200 IPS, 10 days; greater than 200 IPS, 3 days; more than 800 IPS one day), the autoradiograph was developed using (Hyperprocessor, Amersham). The sequence was read and interpreted in both directions using the computer program for sequencing (Esee, the eye ball sequence editor, version 1.09e).

3 Results

3.1 Clinical manifestation:

The disease in this study area was found to affect mainly children below 11 years of age and young adults between age 11 and 30 (Figure 5). There was male predominance (79%). Most of the patients (86%) were migrants of different tribes from western Sudan or Falata from Chad and Nigeria. There were also 5 patients from Eretria. The disease was found to affect mainly those of low socio-economic class (for details see appendix 1).



Figur. 5: Age and sex distribution of kala-azar patient from eastern Sudan around El Gedaref between April 1997 and November 1998

3.2 Direct detection of *Leishmania* parasites in clinical samples:

3.2.1 Microscopy:

Overall from the 100 Giemsa-stained thin films, 71 samples were found to be microscopically positive. Twenty two of these were found to be negative when microscopic examination was performed on samples collected from lymph node aspirates. These samples were found to be positive when examination was carried out on samples collected from bone marrow aspirates and one sample from skin smear.

3.2.2 PCR results:

All the 71 selected microscopically positive clinical samples, which were collected from the field work were also positive in nested PCR when ITS1 and ITS2 regions were used as targets for amplification. In the first round of ITS-PCR these samples were also positive, but some showed weak PCR products. Fourteen of the 29 microscopically negative clinical samples were found to be PCR positive, whereas the remaining 15 clinical samples were also PCR negative. All negative controls were negative; i.e. no contamination or inhibition was detected.

3.2.3 Lymph node aspirates: microscopy versus PCR:

Lymph node aspirates were subjected to microscopy and PCR (ITS1 and ITS2 regions). A comparison of the results is given in table (4). PCR detected significantly more Leishmania parasites than did microscopy ($P<0.001$), in the clinical samples collected from lymph node aspirates. Of the 36 lymph node samples with a positive PCR results but a negative microscopy result, 21 were from patients with confirmed visceral leishmaniasis (VL) on the basis of microscopy of bone marrow aspirates, 15 were from

VL suspects (microscopically negative on basis of bone marrow and lymph node aspirates

Table 4: Comparison of the results obtained by PCR and microscopy with clinical samples collected from lymph node aspirates

		Microscopy		Total
		Positive	Negative	
PCR	Positive	**49**	**36**	**85**
	Negative	**0**	**15**	*15*
	Total	**49**	**51**	*100*

P **< 0.001**

X^2 =16.9555

but patients had clinical symptoms of leishmaniasis). Of the 15 samples, which were found to be negative by microscopy and PCR, one sample was collected from a lymph node of a post kala-azar dermal leishmaniasis patient (PKDL).

3.2.4 Bone marrow aspirates: microscopy versus PCR:

Bone marrow aspirates were subjected to microscopy and PCR (ITS1 and ITS2 regions). A comparison of the results is given in table (5). PCR detected significantly more Leishmania parasites than did microscopy (P<0.001), in the clinical samples collected from bone marrow aspirates. Of the 15 samples, which were found to be negative by microscopy and PCR one sample was collected from a bone marrow of a PKDL patient.

Table. 5: Comparison of the results obtained by PCR and microscopy with clinical samples collected from bone marrow aspirates

		Microscopy		Total
		Positive	Negative	
PCR	Positive	**21**	**15**	**36**
	Negative	**0**	**15**	15
	Total	**21**	**30**	51

P **< 0.001**

X^2 **= 14.875**

3.3 Detection of DNA polymorphisms in the ITS sequences:

The ITS sequences were amplified from genomic DNA extracted from the clinical samples collected in Sudan but also from 18 cultured strains of L. donovani.

Samples were scored as positive for the presence of Leishmania if a PCR product of approximately 1020 bp was obtained for the entire ITS region. When ITS1 and ITS2 were amplified separately, amplification products of about 320 and 700 bp, respectively, were obtained. When ITS2A and B were amplified separately, PCR products of about 300 and 450bp, respectively, were observed (Figure 6).

To test for sequence heterogeneity which could be detected by RFLP, the entire ITS region was cut with 10 different enzymes, the profiles of all 86 (71 microscopically confirmed cases plus the 15 Sudanese cultured samples) studied strains were, however

indistinguishable. Figure (7) showed the restriction pattern obtained by Cfo1 as an example.

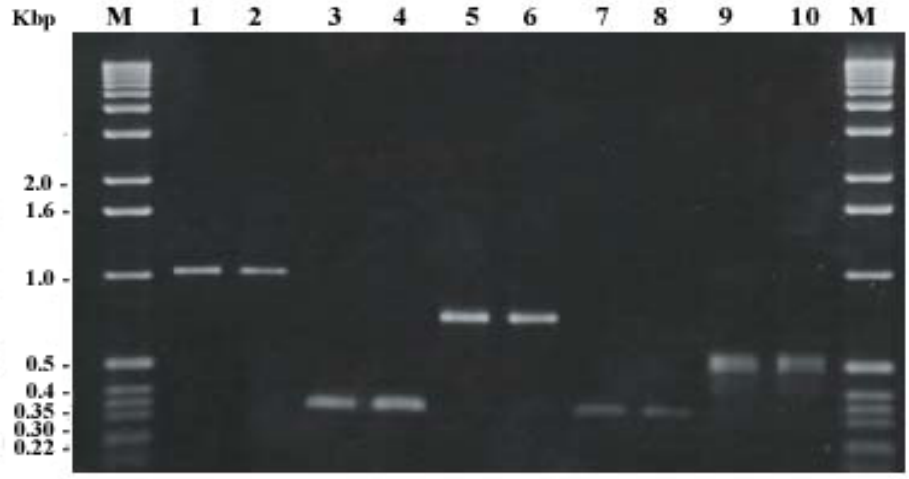


Figure. 6: PCR products amplified from 2 different clinical isolates of Leishmania. Lanes 1&2: ITS PCR products (1020bp); Lanes 3&4: ITS1 PCR products (320bp); Lanes 5&6: ITS2 PCR products (700bp); Lanes 7&8: ITS2A PCR products (300bp); Lanes 9&10: ITS2B PCR products (450bp); M: Molecular size (weight) standard marker; Kbp: Kilobase-pairs

When ITS1 PCR products were subjected to SSCP, in total 14 ITS1 polymorphic profiles (Figures 8a & 8b; Appendix 2) could be distinguished among all 89 (71 microscopically confirmed cases plus the 18 {15 from Sudan and 3 from other regions} cultured samples) tested isolates. Two of these patterns namely K and N were demonstrated by the samples of Kenya, India and China.

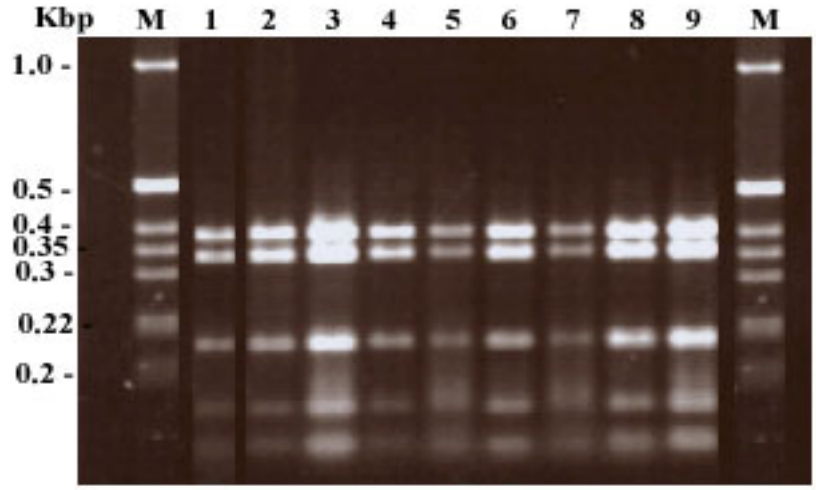


Figure 7: RFLP banding patterns obtained by digesting the amplified ITS regions of different clinical samples of L. donovani with Cfo1 enzyme. M: molecular size (weight) standard marker; Kbp: Kilobase-pairs**.**

The Kenyan and Indian samples had the same pattern K, whereas the Chinese isolate exhibited pattern N. The other 12 patterns were demonstrated by the samples from Sudan. The dominant pattern, A, was found in 66 of the 86 VL cases (76.7%) from Sudan, followed by pattern D, C and H, which were observed in 5, 4 and 3 cases, respectively i.e. (5.8%, 4.6% and 3.5%). Each of the other 8 patterns, namely J, E, L, B, F, G, I and M, were obtained from only one VL case each.

In total 5 ITS2 polymorphic SSCP profiles could be distinguished among all 89 (71 microscopically confirmed cases plus 18 cultured samples) tested isolates (Figure 9; appendix 2). Concerning the 86 Sudanese isolates, all of them had the same pattern A with exception of one sample, which had the pattern J. This sample had the pattern J in

ITS1 SSCP analysis. Samples from Kenya, India and China had the patterns X, Y and Z, respectively.

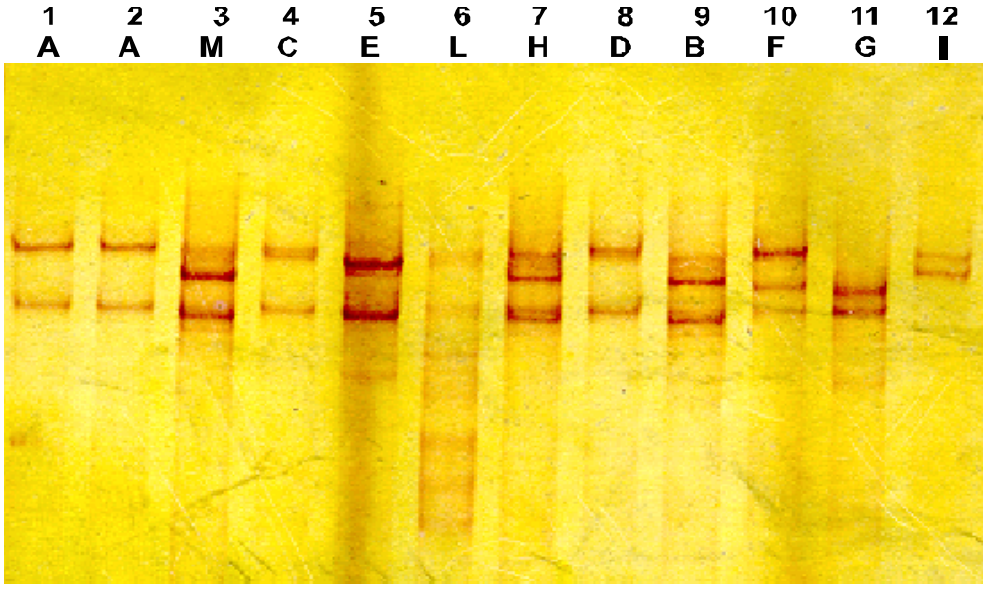


Figure 8a: SSCP analysis of ITS1 regions amplified from different clinical Leishmania donovani isolates. Each SSCP pattern is designated by a capital letter. Patterns A, C and D were very similar but the size of the upper fragment in pattern D was slightly larger than in pattern A. The upper fragment in pattern C had two very close bands. Lane 1: L. donovani MHOM/SD/62/LRC-L61 (positive control). Lane 2-12: strains of L. donovani from eastern Sudan.

ITS-PCR products of Leishmania donovani samples representing different SSCP banding patterns were sequenced in both directions to determine the underlying nucleotide polymorphisms.

The complete dominant ITS sequence (A) is shown in figure (10). The alignments of the variable parts of the ITS1 and ITS2 sequences, which showed variation from the dominant ITS sequence (A) are shown in figures (11&12), respectively. All the variations in the ITS1 SSCP banding patterns were due to deletion of AT pairs or

adenine bases from stretches in the first part of the ITS1 (first 192 nucleotides) with exception of the Chinese isolate which has in addition 2 substitutions at positions 27 and 89 (sequence LdN, Figure 11). In the patterns E, H and L sequences between the nucleotides 73 to 110 were not readable because different nucleotides were found in the same position (2 or 3 and sometimes all 4). These findings were confirmed by repeating sequencing using forward and reverse primers.

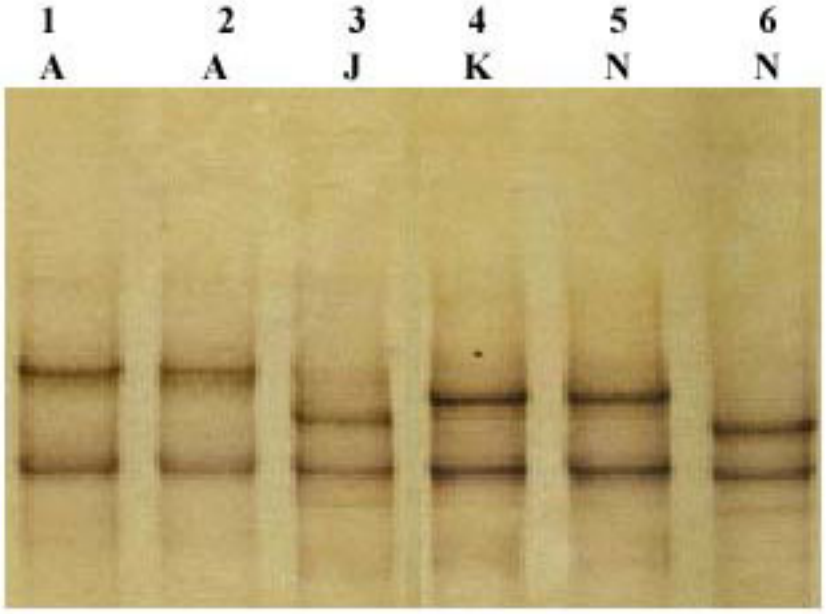


Figure 8b: SSCP analysis of ITS1 regions amplifyed from different clinical Leishmania donovani isolates. Each SSCP pattern is designated by a capital letter. Lane 1: L. donovani MHOM/SD/62/LRC-L61 (positive control). Lanes 2&3: L. donovani strains from eastern Sudan; Lane 4: L.donovani MHOM/KE/85/NLB323 (Kenya); Lane 5: L.donovani MHOM/IN/71/LRC-L51a (India); Lane 6: l.donovani MHOM/CN/??/Wangjiel (China).

No sequence variation was observed in the ITS2A region (positions 320-602) in all isolates. The Sudanese isolates had the same ITS2B sequence (positions 603-1042)

with the exception of one sample, which had a deletion of one guanine base from a G stretch, at position 831 (LdJ sequence).

Isolates from Kenya and India (LdX and LdY sequences, respectively) differed in a single nucleotide, at position 762 in their ITS2B sequence, but showed many nucleotide differences when compared with the Sudanese isolates (Sequences, LdA, J and D) and to the Chinese isolate (sequence LdZ).

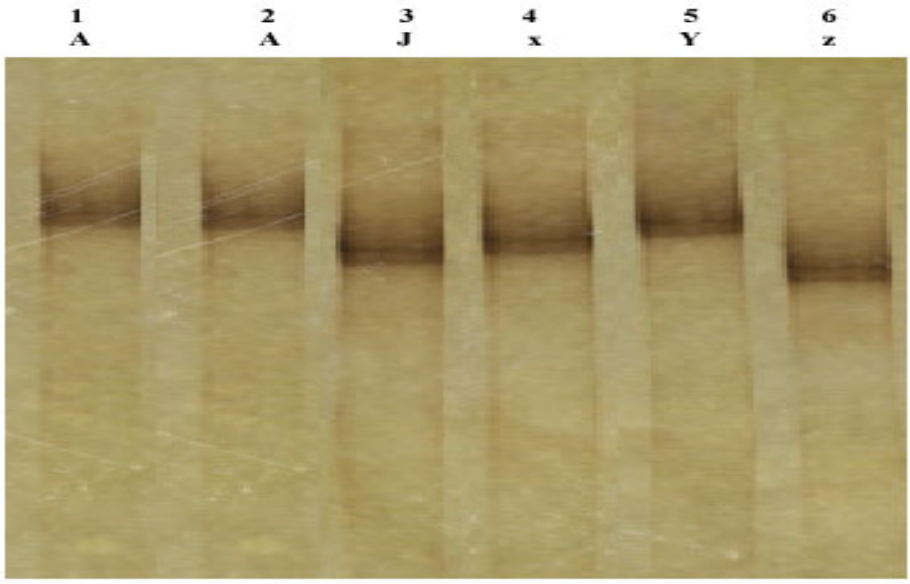


Figure 9: SSCP analysis of ITS2 regions amplified from different clinical Leishmania donovani isolates. Each SSCP pattern is designated by a capital letter. Lane1: L.donovani MHOM/SD/62/LRC-L61 (positive control); Lanes 2&3: L.donovani strains from eastern Sudan; Lane 4: L.donovani MHOM/KE/85/NLB323 (Kenya); Lane 5: L.donovani MHOM/IN/71/LRC-L51a (India); Lane 6: l.donovani MHOM/CN/??/Wangjiel (China).

Figure 10: The complete dominant ITS sequence amplified from clinical *L.donovani* sample from eastern Sudan Primers are represented by bold letters. Sequence of the 5.8S rRNA gene is indicated in red.

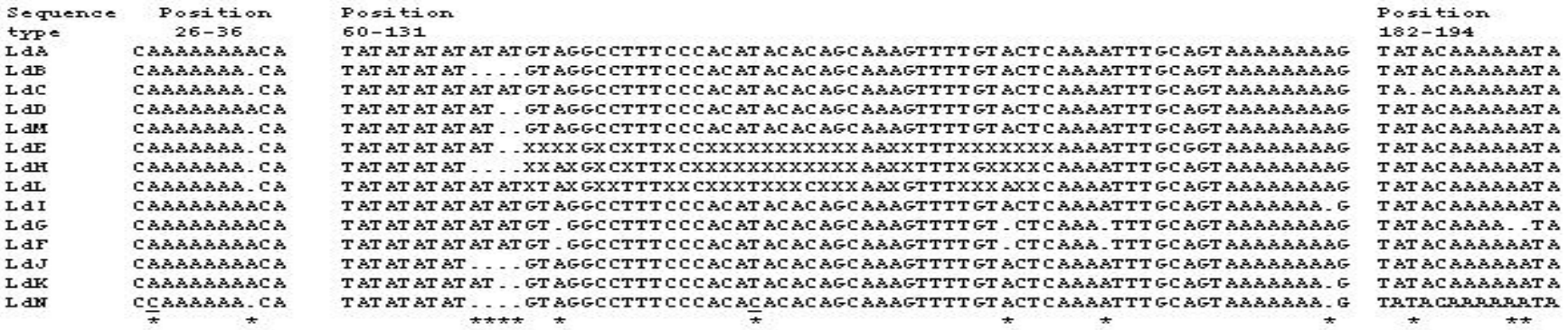


Figure 11: Alignments of the variable parts of the ITS1 sequences amplified from clinical *L. donowani* isolates, representing the 14 different SSCP patterns (LdA...LdN). Dots represent the absence of nucleotides; Bold underlined letters represent point mutations (substitutions); X: A/C/G/T .

Figure 12: Alignments of the variable parts of the ITS2 sequences amplified from clinical *L. donovani* isolates, representing the 5 different SSCP patterns (LdA…LdZ). Dots represent absence of nucleotides; Bold underlined letters represent point mutations (substitutions).

3.4 Detection of DNA polymorphisms in the gp63 sequences:

When part of the coding region of gp63 (XYZ) was amplified from leishmania clinical samples, PCR products of about 1350bp were obtained. When X, Y and Z were amplified separately, PCR products of about 400bp, 350bp and 600bp respectively, were obtained (Figure 13; Appendix2).

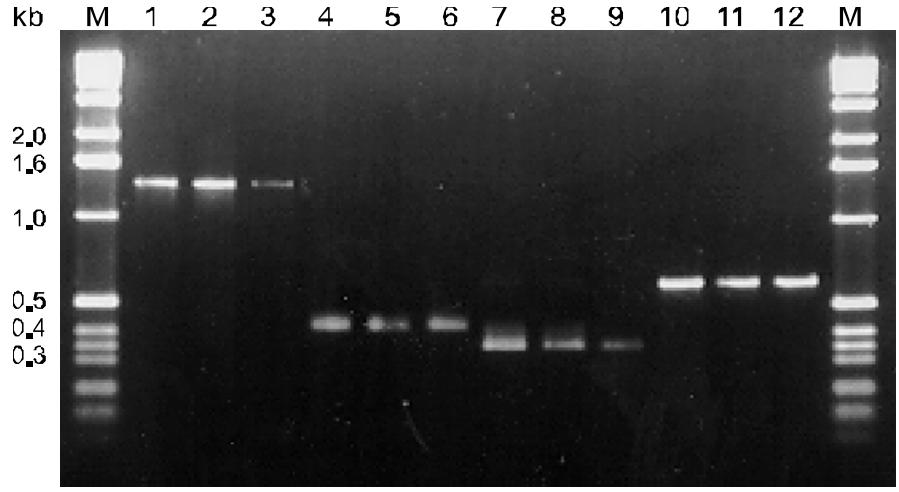


Figure 13: PCR products amplified from 3 different clinical isolates of Leishmania donovani. Lanes 1,2&3: XYZ gp63 PCR products (1350); Lanes 4,5&6: X gp63 PCR products (450bp); Lanes 7,8&9: Y gp63 PCR products (350bp); Lanes 10,11&12: Z gp63 products (600bp); M: Molecular size (weight) standard marker; Kbp: Kilobase-pairs.

In total 31 clinical Leishmania samples spotted on filter papers were investigated. When X and Z regions were used as a target for SSCP analysis, no differences were observed, all the samples had the same SSCP profiles. When Y region was used as a target, 3 polymorphic patterns were observed (Figure 14; Appendix 2). The dominating pattern A was represented by 27 samples. The other patterns B and C were represented by 1 and 3 samples, respectively.

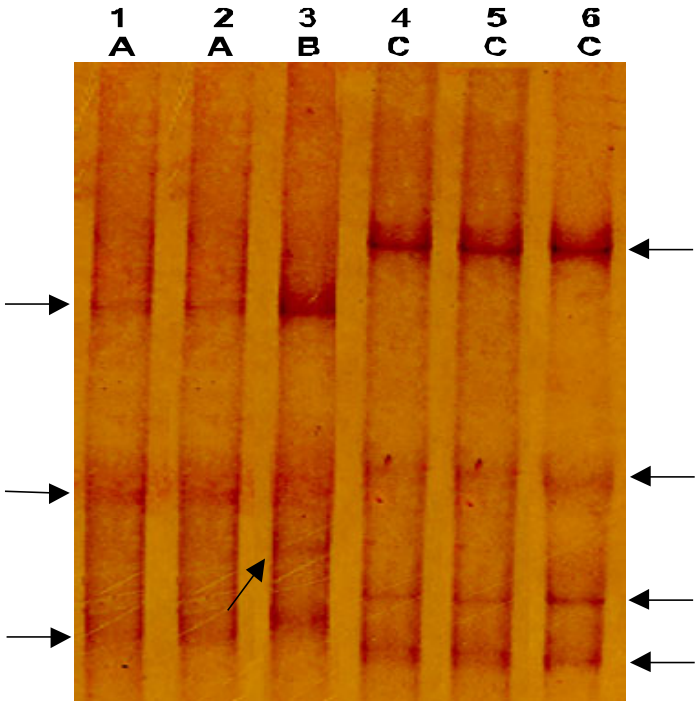


Figure 14: SSCP analysis of Y regions of gp63 gene amplified from different clinical Leishmania donovani isolates from eastern Sudan. Each SSCP pattern is designated by a capital letter. Patterns A and B were very similar but the size of the upper fragment in pattern A was slightly larger than in pattern B. Also the size of the lower fragment of pattern A is slightly smaller than in pattern B and there is very faint additional band. Lane 1: L. donovani MHOM/SD/62/LRC-L61 (positive control). The position of the bands are indicated by arrows.

Nucleotide polymorphisms, obtained by sequencing of part (Y) of the coding region of gp63, and representing the 3 different SSCP banding patterns were due to single base exchanges (one transition and 2 transversions) as shown in figure (15).

Figure 15: Alignments of part (Y) of the coding region of the gp63 gene sequences amplified from clinical samples of *L.donovani* from eastern Sudan representing the 3 different SSCP patterns (A,B,C). Dots: represent absence of nucleotides. Bold underlined letters represent point mutations (substitutions). Primers are represented by bold letters.

3.5 Detection of DNA polymorphisms in different anonymous DNA fragments:

When anonymous DNA fragments, namely L0110, L510, L413, LK413, L0308 and L0114 were amplified from leishmanial genomic DNA, PCR products that were about 580, 250, 300, 350,120 and 230 bp respectively, were obtained (Figure 16). Unfortunately we succeeded to get good PCR products only for DNA extracted from the 8 successful cultures. Only for the fragment 110 we were able to get good PCR products from 31 samples spotted on filter papers as well. For the other anonymous fragments only, weak, faint and sometimes no PCR products could be amplified directly from clinical samples spotted on filter papers.

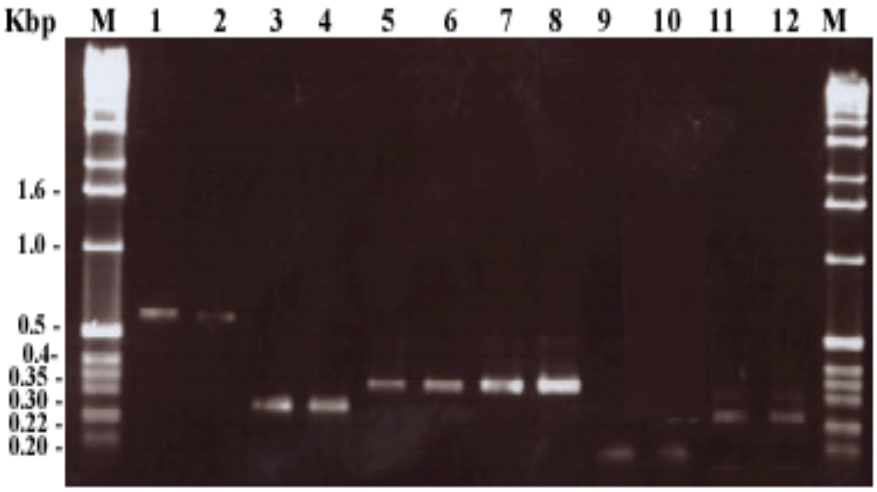


Figure 16: DNA fragments amplified from 2 different clinical isolates of *Leishmania donovani*. Lanes 1&2: PCR products obtained for anonymous fragment (110); Lanes 3&4: PCR products obtained for anonymous fragment (510); Lanes 5&6: PCR products obtained for anonymous fragment (413); Lanes 7&8: PCR products obtained for anonymous fragment (K413); Lanes 9&10: PCR products obtained for anonymous fragment (308); Lanes 11&12;

PCR products obtained for anonymous fragment (114); M: Molecular size (weight) standard marker; Kbp: Kilobase-pair.

SSCP was performed for the 6 anonymous DNA fragments namely, L0110, L510, L413, LK413, L0308 and L0114. Concerning the region L0110, 31 samples were investigated and no differences in SSCP patterns were observed. For the other 5 fragments the 8 cultured samples were investigated and also no differences in SSCP patterns were observed with exception of 114 region, which showed two patterns. Pattern A was represented by 7 samples. The other pattern B was represented by one sample (Figure 17; Appendix 2). When SSCP was applied, for the anonymous DNA fragments obtained from faint PCR products amplified from samples spotted on filter papers, no reliable results were obtained**.**

Figure 17: SSCP analysis of anonymous fragment (114) amplified from different clinical *Leishmania donovani* isolates from eastern Sudan. Each SSCP pattern is designated by a capital letter. Lane 1: *L. donovani* MHOM/SD/62/LRC-L61 (positive control).

Figure 18: Anonymous DNA loci sequences amplified from clinical *L. donovani* samples from eastern Sudan. Primers are represented by bold letters. Polymorphism in locus 114 is represented by bold underlined letters.

The sequences of the 6 amplified anonymous loci in the L.donovani DNA were shown in figure (18). Only locus 114 sequence showed 2 polymorphic patterns A&B which differed by one A/C exchange in position 127 and a small insertion/deletion in position 111.

3.6 PCR fingerprinting:

PCR fingerprinting technique using M13 and T3B primers revealed no differences in profiles of all 26 studid *L. donovani* strains (8 successful cultures from eastern Sudan plus the 12 obtained from the collection of the Royal Tropical Institute, Amsterdam and the 6 WHO reference strains).

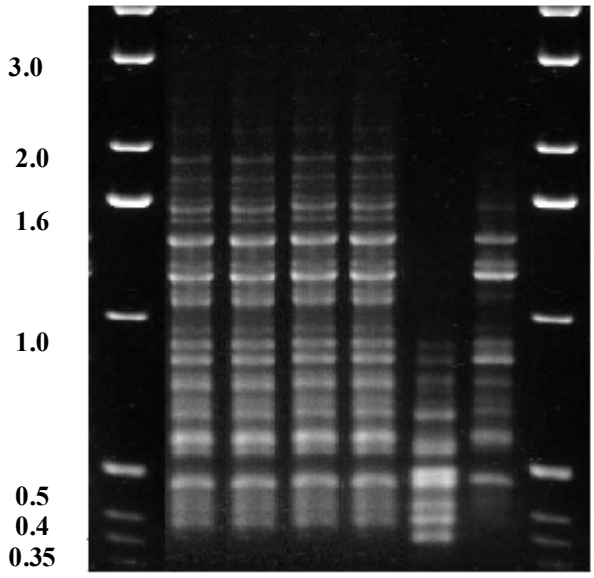


Figure 19: Results of the PCR fingerprinting using the $(GTG)_5$ primer. Lane 1-3: Sudanese strains of *L. donovani* MHOM/SD/62/LRC-L61, MHOM/SD/98/GCI, and Don 13. Lane 4: *L.donovani* MHOM/KE/85/NLB323. Lane 5: *L. donovani* MHOM/IN/71/LRC-L51a. Lane6: *L.donovani* MHOM/CN/??/Wangjie1. M; molecular size (weight) standard marker. Kbp: Kilobase-pairs.

PCR fingerprinting technique using M13 and T3B primers revealed no differences in profiles of all 26 studid *L. donovani* strains (8 successful cultures from eastern Sudan plus the 12 obtained from the collection of the Royal Tropical Institute, Amsterdam and the 6 WHO reference strains).

When the (GTG)5 primer was used, L. donovani from India showed a unique profile (Fig.19, lane 5); the fingerprint of the Chinese strain (Fig.19, Lane 6) was very similar to those of the Sudanese strains; however, the high molecular weight bands appeared very faint, which might be due to partial degradation or

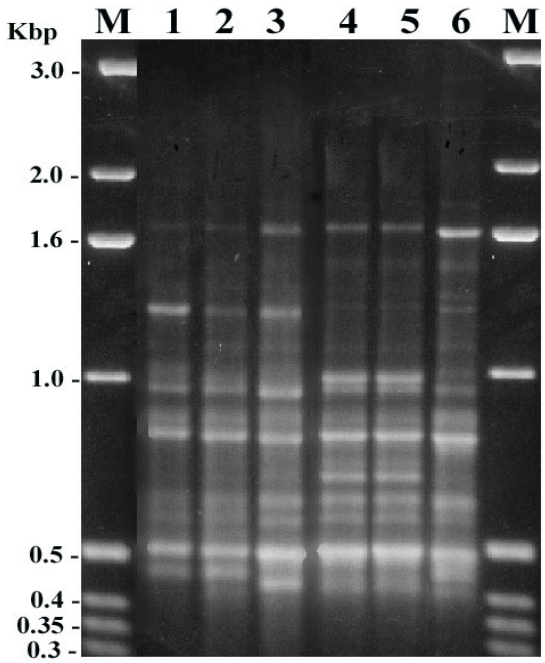


Figure 20: Results of the PCR fingerprinting using the $(GACA)_4$ primer. Lane 1-3: Sudanese strains of *L. donovani* MHOM/SD/62/LRC-L61, MHOM/SD/98/GCI, and Don 13. Lane 4: *L.donovani* MHOM/KE/85/NLB323. Lane 5: *L. donovani* MHOM/IN/71/LRC-L51a. Lane6: *L.donovani* MHOM/CN/??/Wangjie1. M; molecular size (weight) standard marker. Kbp: Kilobase-pairs.

lower concentration of DNA in this sample. With the primer $(GACA)_4$ identical fingerprinting patterns were obtained for all isolates from Sudan with the exception of the one ITS2 pattern J isolate (Fig. 20, Lane 3). This Isolate differed slightly from the other Sudanese isolates as did the isolate from China (Fig.20, Lane 6). The Kenyan and the Indian isolates (Fig.20, lanes 4&5, respectively) showed the same profiles with this primer.

4 Discussion

In this study we evaluated the use of nested PCR utilizing the ITS1 and ITS2 regions as targets for diagnosis and direct differentiation of *Leishmania donovani*. Also PCR products of gp63 and anonymous regions were attempted for the characterization and identification of intraspecific polymorphisms of the causative agent of visceral leishmaniasis (VL) in eastern Sudan from clinical samples spotted on filter papers using SSCP technique. Also we assessed DNA polymorphisms among 23 cultured *L. donovani* isolates from eastern Sudan, one from Kenya, one from India and one from China with 4 different PCR based approaches as tools to detect polymorphisms.

The present study showed that PCR of both inguinal lymph node and bone marrow aspirates is significantly more positive than microscopy (tables 4 & 5). No difference was detected between microscopy and PCR when the clinical materials were taken from patients with confirmed cases of VL.This is in agreement with Osman *et al*. (1997). However, *Leishmania* DNA was demonstrated in 48.3% of VL suspect cases, thereby confirming the clinical diagnosis. This indicates that the main advantage of using PCR as a diagnostic tool is for VL suspects.

The sensitivity of microscopic examination of smears from lymph node varies from 78% (Siddig *et al*., 1989) to 58.3% (Zijlstra *et al.,* 1992), while the sensitivities of microscopy of splenic and bone marrow aspirates were 96.4 and 70.2% (Zijlstra *et al*., 1992). This is congruent with the findings of this study, where 30% of the clinical samples from lymph node aspirates that were found to be negative on basis of microscopy, were positive when the aspirates were taken from bone marrow. This

observation indicates that the significance of microscopy results should be judged carefully against the types of the clinical material.

When 3 clinical samples were taken from lymph node, bone marrow and skin from post kala-azar dermal leishmaniasis (PKDL) patient and PCR was performed for these samples, a positive result was obtained only from the skin sample. Although one sample is not enough to draw a conclusion, the same result was obtained by Osman *et al.* (1998) who found that only 2 of 18 bone marrow aspirates from PKDL patients to be positive in PCR. This is probably due to the clearance of parasites from the lymph node and bone marrow as cell-mediated immunity develops during or after treatment of VL.

PCR of the entire ITS region of *L. donovani* was found to be less sensitive than PCR of the 18S ribosomal RNA described by Osman *et al*., (1997 & 1998). In spite of this, ITS region is targeted in a nested PCR (ITS1 & ITS2) because sequences of this region are more variable and allow clear species identification by RFLP or SSCP and also strain differences could be expected.

Various DNA techniques have been valuable for the study of genetic variation, and their application has provided a wealth of molecular data (McManus & Bowles, 1996). Intraspecific variation in SSCP banding patterns was clearly observed in the ITS1 region with 12 profiles detected among Sudan isolates, with profile A being dominant (76.7%), and 2 ITS1 –SSCP profiles observed among the samples from Kenya, India and China. On the other hand, all but one of the Sudanese samples had the same ITS2 – SSCP pattern (A). Isolates of the same species (*L. donovani*) but of different

geographical origin had showed varying ITS2-SSCP patterns. We may conclude that ITS2-SSCP pattern analysis could be a useful method for differentiation of *L. donovani* strains from different geographical areas. To consolidate this observation, more samples from different geographical origins are required.

Iwahana *et al.* (1992) claim that PCR-SSCP analysis is only effective for DNA fragments of less than 300 to 400 bp. Therefore the ITS2 region was further divided into 2 regions (ITS 2A and ITS 2B), using internal primers. Again the same above result was obtained. This means that ITS2 sequence seems to be highly conserved in all investigated *L. donovani* isolates from Sudan, with the exception of only one sample. In contrast, ITS1 region appears to undergo more rapid evolutionary changes and may vary among populations of the same species of the same geographical origin. The polymorphisms in the ITS1 observed in this study were independent of the clinical symptoms, of the stage of the disease and of the tribe of the patient.

DNA fragments showing different SSCP patterns were further characterized by radioactive cycle sequencing to determine the underlying nucleotide polymorphisms. Different patterns in the ITS1 region were mainly due to deletion of Adenine residues from A stretches or AT pairs (Fig.11). This may be attributed to the weak AT double bond. Patterns L, H and E have a unique part in their sequences where different nucleotides were located at the same position. This is already clearly indicated in the SSCP analysis where patterns L and H consist of multiple bands (Fig.8a). This may be attributed to the fact that the ITS region is a multi-copy gene and heterogeneity may also arise between individual copies of the ribosomal operon, but mixed infections or

hybrids by different strains of *L. donovani* can not be excluded. The results obtained by SSCP for ITS2 were also confirmed by DNA sequencing.

Intraspecies variations in SSCP banding patterns were clearly observed in the coding region of gp63 with 3 profiles detected among 31 Sudanese *L. donovani* isolates. These results were also confirmed by DNA sequencing. The gp63 molecule participates in the parasites initial attachment to mammalian macrophages and likely contributes to their survival within the macrophage phagolysosomes (Bordier, 1987; Chang *et al*., 1990). This finding indicates that gp63 could be a potential useful marker for study of polymorphisms. Those polymorphisms can possibly correlated to the severity of the disease The same result was obtained by Victoir *et al.* (1998) who illustrates the very high genomic and genetic plasticity of gp63 genes revealed by gp63-RFLP and PCR-RFLP in 4 species of subgenus *viannia* and the possibility to relate these genetic differences to phenotypic properties, such as pathogenicity.

Six amplified anonymous regions of DNA were screened for polymorphisms with the SSCP technique from isolates spotted on filter papers. Unfortunately good SSCP results were obtained from only one region. In contrast to this finding, good PCR products and SSCP polymorphic patterns were observed among cultured Sudanese *L. donovani* (DON12- DON23) using the same anonymous primers (Lewin, 2000). This difference may be due to the fact that, these anonymous regions represent single copy genes and hence the SSCP was hampered by the little amount of PCR products obtained from samples spotted on filter papers. At the same time good results were obtained when SSCP was performed for 8 of the same above isolates but from cultured parasites. We may conclude that anonymous regions could be good for population

genetics studies taking into consideration, they are single copy genes and thus, can address for genetic diploidy and sexual reproduction of *Leishmania* provided that there is large amount of PCR products.

No intra-specific variation was observed in the RFLP patterns when the amplified ITS regions of different strains of *L donovani*, were digested with a panel of different frequently cutting restriction enzymes (Fig. 7). This may be explained by the fact that sequence variation may go undetected by RFLP analysis since the restriction enzymes survey only a subset of the total variable sites (Stothard *et al*., 1997).

In contrast to this observation, considerable heterogeneity was found within the ITS-RFLP of strains *of L. tropica* and *L. aethiopica* (Schönian *et al*., 2000 & 2001) and of New World species of *Leishmania* whereas different species showed different levels of variation (Guevara *et al.* 1992; Cupolillo, *et al.* 1995). These findings reveal that different levels of heterogeneity occur within different species of *Leishmania*.

When PCR-fingerprinting approach was done on cultured parasites, highly similar PCR profiles were observed between all tested isolates (Figs. 19&20); thus confirming previous results (Schönian *et al*., 1996; Oskam *et al.*, 1998; Lewin, 2000). This may be attributed to the fact that the fingerprinting technique is less discriminatory because mutations can be detected only if they affect primer-binding sites or if they lead to bigger insertions/deletions that would change the fragment size. Gasser (1997) also reported that fragments of the same size but differing in sequence will co-migrate within one band in a gel, and may be misinterpreted as the same sequence.

We can conclude that SSCP is advantageous relative to both PCR-RFLP and PCR-fingerprinting approaches for the detection of sequence variation in rRNA genes within the *L. donovani* species. The SSCP technique is sensitive to detect genetic diversity that differed by only one nucleotide (Fig. 12 sequences, LdX & LdY).

In addition, ITS-SSCP is a technique that can be performed easily and rapidly, directly from blotted clinical samples infected with *Leishmania* parasites without prior cultivation of the parasite, unlike PCR-fingerprinting. Samples collected under field conditions where cultures are vulnerable to contamination can be analysed directly by PCR-SSCP methods in specialized laboratories. Further more, by cultivating the *leishmania* parasite it is possible that mixed infections will be missed due to different growth rates of different strains in blood agar culture (Ibrahim *et al*., 1994). Thus SSCP can be an excellent method for the screening of genetic polymorphisms within the genus *Leishmania* because it can be used with low parasite numbers and contaminated samples. Samples showing different SSCP patterns could be subsequently sequenced. Automated direct sequencing of the PCR product without prior screening by the SSCP technique might be profitable for the detection of polymorphic sequences in single copy genes. However with multicopy genes, SSCP is of advantage because direct sequencing is hampered by possible sequence differences among the individual copies (see patterns E, H, and L in this study).

In this study, of 40 cultures attempts in the field only 8 were successful. This was due to problems with contamination (aseptic techniques in the field were not ideal as in the equipped laboratory), problems with the conservation of adequate media, and problems with maintenance of the optimal temperature under field conditions. Simultaneously,

we were able to apply ITS-SSCP for these 8 samples directly from collections obtained by spotting of the lymph node or bone marrow aspirates on filter papers. Although SSCP has been used to detect sequence variation in ITS, gp63 and anonymous regions of *L. donovani,* this method could be a powerful analytical tool also for other parasitic organisms. SSCP can also be used for the detection of strain-specific polymorphisms not only in the above mentioned targets but also in any already published sequence and thus lessen the dependence on reference laboratories for identification of *Leishmania* strains.

No correlation was however discerned between the PCR-SSCP polymorphic patterns and the clinical manifestation of the human disease (6PKDL, 83 VL and 1 CL patients). Also Schönian *et al*., (2000, 2001) found no correlation between different ITS-RFLP, fingerprinting patterns and the clinical manifestation of the disease caused by the *L. aethiopica* and also the disease caused by *L. tropica*. It would be interesting to determine whether *L. donovani* causing mucosal leishmaniasis, PKDL and VL in Sudan could be correlated with specific SSCP patterns, may be other gene (i.e. virulent genes that still have to be identified) could be used as a target in further study.

There was male predominance among the patients in this study area. This may be due to the male greater outdoor exposure to sandfly and the greater value placed on male lives, which bring them in close contact with the forest. The high infection rate among children may indicate that some infections are domestic or peridomestic. Most of the patients in this area were from low socio-economic class, anaemic and this may indicate that leishmaniasis has characteristics of an opportunistic disease too.

Lastly we can emphasise that, it is a high time in Sudan to find a quick accurate tool for differential diagnosis between endemic diseases resembling clinically Leishmaniaasis, like chronic malaria, typhoid, brucellosis, trypanosomiasis. PCR diagnosis evaluated in this study can be used as a supplement to the existing gold standard microscopic method, especially for leishmaniasis suspect cases. This will help in prescribing accurate treatment, improving quality of the care of the patient and reduce the morbidity and mortality rates. Also it can be applied to samples collected from vector or reservoir host to support control program.

The advantage of the PCR products investigated in this study is that can be used for epidemiological, population genetics studies and for typing of the organism. Because this method is expensive to be done as a routine work in Sudan, it can be recommended for diagnosis of unusual clinical presentation as a second line confirmation in an algorithm of diagnosis.

4.1 Conclusions:

1. PCR is especially useful for the confirmation of cases suspected of VL. Also PCR can be performed successfully, directly from clinical samples spotted on filter papers with no prior cultivation.
2. Nested ITS- PCR is a more sensitive method than microscopy for the detection of *leishmania* parasites. In addition ITS sequences are variable and hence allow strain differences.
3. SSCP has the advantage over both RFLP and PCR fingerprinting that it can detect DNA polymorphisms and point mutations at a variety of positions in DNA fragments. Also it has higher resolution power for strain identification, which is required for understanding of the epidemiology of *leishmania.*
4. Anonymous regions, which are single copy genes, could be good markers especially for population genetics studies provided that we have large amount of PCR products.
5. Gp63 could be a potential useful marker for study of polymorphisms and the possible correlation of these polymorphisms to the severity of the disease.
6. Different levels of heterogeneity occur among different species of *Leishmania* with *L. tropica* being most variable (Schönian *et al*.; 2001) followed by *L. aethiopica* (Schönian et al., 2000) and then *L. donovani* (this study, Lewin, 2000)
7. It would be interesting to determine whether *L. donovani* causing mucosal leishmaniasis, VL and PKDL in Sudan could be correlated with specific SSCP patterns, may be other relevant genes could be used in further study using SSCP technique.

4.2 References

Abdallah, R. E.; Ali, M.; Wasfi, A. I. & El-Hassan, A. M. 1973. Cutaneous leishmaniasis in the Sudan. *Trans. R. Soc. Trop. Med. Hyg.*, **67**:549-559.

Abdallah, R. E. & Sherif, H.1978. Epidemics of cutaneous leishmaniasis in northern Sudan. *Ann. Trop. Med. Parasitol.*, **72**:349-352.

Abdallah, R. E. 1980. Serodiagnosis of visceral leishmaniasis in an endemic area of the Sudan. *Ann. Trop. Med. Parasitol.* **74**:415-419.

Addy, M.& Nandy, A. 1992. Ten years of kala-azar in West Bengal, part 1. Did post kala-azar dermal leishmaniasis initiates the outbreak in 24-Parganas? *Bull. WHO,* **70**:431-346.

Ali, S.; Müller, C. R. & Epplen, J. T. 1986. DNA fingerprinting by oligonucleotide probes specific for simple repeats. *Hum. Genet.,* **74**:239-243.

Andresen, K.; Ibrahim, M.E; Theander, T.G. & Kharazmi, A. 1996. Random amplified polymorphic DNA for the differentiation of Leishmania donovani isolates from Sudan. *Trans. R. Soc. Trop. Med. Hyg.,* **90**: 204-205.

Andresen, K.; Gasim, S; El-Hassan, A. M.; Khalil, E. A. G.; Barker, D. C.; Theander, T. G., 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. *Trop. Med. Int. Health,* **2**(5):440-444.

Ashford, R.W; Desjeux, P. & de Raadt, P. 1992. Estimation of population at risk of infection and number of cases of leishmaniasis. *Parasitol. Today,* **8**: 104-105.

Badaro, R; Reed, S. G. & Carvalho, E. M. 1983. Immunofluorescent antibody test in American visceral leishmaniasis: sensitivity and specificity of different morphological forms of two leishmania species. *Am. J. Trop. Med. Hyg.*, **32**(3):480-484.

Baily, G. G. & Nandy, A. 1994. Visceral leishmaniasis: more prevalent and more problematic. *J. Infec.*, **29**:241-247.

Bard, E. 1989. Molecular biology of Leishmania. *Biochem.Cell Biol.*, **67**: 516-524.

Barker, D. C. 1989. Molecular approaches to DNA diagnosis. *Parasitol.*, **99**(S), 125-146.

Bastien. P; Blaineau, C; Taminh, M; Rioux, J.A; Roizes, G. & Pages, M. 1990. Interclonal variation in molecular karyotype in Leishmania infantum imply a mosaic strain structure. *Mol. Biochem. Parasitol.*, **40**, 53-62.

Berman, J. D. 1997. Human leishmaniasis: clinical, diagnostic, and chemotherapeutic development in the last 10 years. Clin. Infect. Dis., **24**(4): 684-703.

Bevan, I. S.; Rapley, R. & Walker, M. R. 1992. Sequencing of PCR -amplified DNA. *PCR Methods and Applications*, **1**:222-228.

Beverley, S.M.; Ismach, R.B.; & McMahon-Pratt, D. 1987. Evaluation of the genus Leishmania as revealed by comparison of nuclear DNA restriction fragment patterns. *Proc. Natl. Acad. Sci*. USA, **84**, 484-488.

Beverley, S.M.; Ellenberger, T. E.; Lovannisci, D. M.; Kapler, G. M.; Petrillo-Peixoto, M. & Sina, B. J. 1988. Gene amplification in Leishmania. In: Englund, P. T. & Sher, *A. (eds.). Vol. 9. Biology of Parasitism MBL Lectures in Biology*, New York, 431-448.

Bishop, R.P., & Miles, M. A. 1987. Chromosome size polymorphisms of Leishmania donovani. *Mol. Biochem. Parasitol.*, **24**:263-272.

Bishop, R. P.; & Akinsehinwa, F. 1989. Characterization of Leishmania donovani stocks by genomic DNA heterogeneity and molecular Karyotype. *Trans. R. Soc. Trop. Med. Hyg.,* **83**:629-634.

Blaineau, C.; Bastien, P.; Rioux, J. A.; Roizés, G. & Pagés, M. 1991. Long-range restriction maps of size-variable homologus chromosomes in Leishmania infantum. *Mol. Biochem. Parasitol.,* **46**(2): 292-302.

Bordier, C. 1987. The promastigote surface protease of Leishmania. *Parasitol. Today,* **3**:151-153.

Chang, K. P., Fong, D. & Bray, R. S. 1985. Biology of Leishmania and leishmaniasis. *In: Chang, K.P. & Bray, R. S. (eds.). Vol.1. Leishmaniasis*. Elsev. Biomed. Press, Amsterdam, pp. 1-30.

Chang, K. P.; Chaudhuri. G. & Fong, D. 1990. Molecular determination of Leishmania virulence. *Annu. Rev. Microbiol.,* **44**:449-529.

Cotton, R. G. H. 1993. Current methods of mutation detection. *Mutation Res.,* **285**: 125-144.

Cupolillo, E.; Grimaldi, Jr. G.; Momen, H.& Beverley, S. M. 1995. Intergenic region typing (IRT): a rapid molecular approach to the characterization and evolution of leishmaniasis. *Mol. Biochem. Parasitol.,* **73**:145-155.

Davidson, R. N. 1999. Science and clinical practice: visceral leishmaniasis in clinical practice. *J. Infect.,* **39**:112-116.

de Beer, P.; Harith, A. E.; van Grootheest, M. & Winkler, A. 1990. Outbreak of kala-azar in the Sudan. *Lancet,* **335**: 183-224.

Desjeux, P. 1996. Leishmaniasis: public health aspects and control. *Clin. Dermatol.,* **14**:417-423.

Elbihari, S. & El-Hassan, A. M. 1987. The histopathology of naturally-occuring cutaneous leishmaniasis in the reservoir host Psammomys obesus. *Bull. Soc. Pathol. Exot. Filiales,* **80** (4): 615-623.

El-Hassan, A.M.; Ghalib, W.H.; Zijlstra, E.E.; Eltoum, I. A., Satti, M.; Ali, M. S. & Ali, H. M. A. 1992. Post kala-azar dermal leishmaniasis in the Sudan: clinical features, pathology and treatment. *Trans. R. Soc .Trop. Med. Hyg.,* **86**:245-248.

El-Hassan, A. M.; Zijlstra, E .E.; Ismael, A. & Ghalib, W.H. 1995. Recent observations on the epidemiology of kala-azar in the eastern and central states of the Sudan. *Trop. Geograph. Med.,* **47**:151-156.

Elnaiem, D. A.; Hassan, H. K. & Ward, R. D. 1997. Phlebotomine sandflies in a focus of visceral leishmaniasis in a border area of eastern Sudan. *Ann. Trop. Med. Parasitol.,* **91** (3):307-318.

El-Safi, S. H. & Evans, D. A. 1989. A comparison of the direct agglutination test and enzyme-linked immunosorbent assay in the sero-diagnosis of visceral leishmaniasis in the Sudan. *Trans. R. Soc. Trop. Med. Hyg.,* **83**:334-337.

El-Safi, S. H. & Peters, W. 1991. Studies on the leishmaniasis in the Sudan.1. Epedemics of cutaneous leishmaniasis in Khartoum. *Trans. R. Soc. Trop. Med. Hyg.,* **85**:44-47.

El –Sibae, M. M.; Esa, N. M. & Morsy, T. A. 1993. Rodents and cutaneous leishmaniasis in Qasim, Saudi Arabia. *J. Egypt Soc. Parasitol.,* **23** (3):667-673.

Evans, D.; Lanham, S.M, Baldwin, C. L. & Peters, W. 1984. The isolation and isoenzyme characterization of Leishmania braziliensis sp. from patients

with cutaneous leishmaniasis acquired in Belize. *Trans. R. Soc. Trop. Med. Hyg.,* **78**,35-42.

Evans, T. G. & Pearson, R. D. 1988. Identification of leishmanial antigens in the sera of patients with American visceral leishmaniasis. *Infect. Immune.,* **56**(12):3139-3144.

Evans, D. (ed.). 1989. Handbook on Isolation, Characterization and cryopreservation of Leishmania. *WHO,* Geneva, Switzerland, 15-17.

Frommel, T. O.; Button, L. L.; Fujikura, Y. & Mc Master, W. R. 1990. The major surface glycoprotein (Gp63) is present in both life stages of Leishmania. *Mol. Biochem. Parasitol.,* **38**: 25-32.

Gasser, R. B. & Chilton, N. B. 1995. Characterization of taeniid cestode species by PCR-RFLP of ITS2 ribosomal DNA. *Acta Tropica,* **59**:31-40.

Gasser, R. B. 1997. Mutation scanning methods for the analysis of parasite genes. *Internat. J. Parasitol.,* **27**,1449-1463.

Gasser, R.B. & Zhu, X. Q. 1999. Sequence-based analysis of enzymatically amplified DNA fragments by mutation detection techniques. *Parasitol. Today*, **15** (11):462-465.

Ghalib, H. W.; El-Toum, I. A.; Kroon, C. C. M. & El-Hassan, A. M. 1992. Identification of Leishmania from mucosal leishmaniasis by recombinant DNA probes. Trans. R. Soc. *Trop. Med. Hyg.,* **86**:158-160

Guevara, P., Alonso, G., da Silveira, J. F., de Mello, M., Scorza, J. V., Anez, N. & Ramirez, J.L. 1992. Identification of new world Leishmania using ribosomal gene spacer probes. *Mol. Biochem. Parasitol.,* **56**, 15-26.

Harith, A. E.; Kolk, A. H. Kager, P. A.; Leeuwenburg, J.; Muigai, R. Kiugn, S. & Laarman, J. J. 1986. A simple and economical direct agglutination test for

serodiagnosis and seroepidemiological studies of visceral leishmaniasis. *Trans. R. Soc. Trop. Med. Hyg.,* **80**:583-586.

Harith, A. E.; Kolk, A. H. J; Leeuwenburg, J.; Muigai, R.; Huigen, E.; Jelsma, T. & Kager, P. A. 1988. Improvement of a direct agglutination test for field studies of visceral leishmaniasis. *J. Clin.Microbiol.,* **26**: 1321-1325.

Hassan, M. D. Q; Ghosh, A.; Ghosh, S. S.; Gupta, M, Basu, D.; Malik, K. K, Adhya, S. 1993. Enzymatic amplification of mini-exon-derived RNA gene spacers of Leishmania donovani: primers and probes for DNA diagnosis. *Parasitol.,* **107**:509-517.

Henriksson, J., Solari, A., Rydaker, M., Sousa, O. E. & Pettersson, U. 1996. Karyotype variability in Trypanosoma rangeli. *Parasitol.,* **112**: 385-391

Hiss, R. H.; Norris, D. E.; Dietrich, C. H.; Whitcomb, R. F.; West, D. F.; Bosio, C .F.; Kambhampati, S; Piesman, J.; Antolin, M. F. & Black, W. C. 1994. Molecular taxonomy using single strand conformation polymorphism (SSCP) analysis of mitochondrial ribosomal DNA gene. *Insect Mol. Biol.,* **3**: 171-182.

Hommel, M.; Peters, W.; Ranque, J; Quilici, M . & Lanotte, G. 1978. The micro-ELISA technique in the serodiagnosis of visceral leishmaniasis. *Ann. Trop. Med. Parasitol.*, **72**(3):213-218.

Hommel. M.; Attar, Z.; Fargeas, C.; Dourado, C.; Monsigny, M.; Mayer, R. & Chance, M. L. 1997. The direct agglutination test: a non-specific test specific for the diagnosis of visceral leishmaniasis. *Ann. Trop. Med. Parasitol.,* **91**(7):795-802.

Hommel. M. 1999. Visceral leishmaniasis: biology of the parasite. *J. infect.,* **39**: 101-111.

Hoogstraal, H. & Heyneman, D. 1969. Leishmaniasis in the Sudan Republic. 30. Final epidemiological report. *Am. J. Trop. Med. Hyg.,* **18**:1091-1210.

Huey, B. and Hall, J. 1989. Hyper variable DNA fingerprinting in Escheriechia coli: minisattelite probe from bacteriophage M13. *J. Bacteriol.* **171**:2528-2532.

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

Iwahana, H.; Yoshimoto, K. & Itakura, M. 1992. Detection of point mutations by SSCP of PCR-Amplified DNA after endonuclease digestion. *Bio. Feedback,* **12**, 65-66.

Jackson, P. R.; Wohlhieter, J. A.; Jackson, J. E.; Sayles, P.; Diggs, C. L. & Hockmeyer, W. T. 1984. Restriction endonuclease analysis of Leishmania Kinetoplast DNA characterizes parasites responsible for visceral and cutaneous diseases. *Am. J. Trop. Med. Hyg.,* **33**, 808-819.

Kar, K.1995. Serodiagnosis of Leishmaniasis. *Curr. Rev. Microbiol.,* **21**:123-152.

Kirk, R. & Sati, M. H. 1947. Observations on the use of sodium antimony gluconate (sodium stibogluconate) in the treatment of kala-azar. *Ann. Trop. Med. Parasitol.,* **41**:1421.

Lainson, R. & Shaw, J. J .1987. Evolution, classification and geographical distribution. In: Peters, W. & Killick-Kendrick, R.. *The Leishmaniasis in Biology and Medicine*. London: Academic press, pp.1-120.

Le Blancq, S. M., & Peters, W. 1986. Leishmaniasis in the Old World: 4. The distribution of Leishmania donovani senso lato zymodemes. *Trans. R. Soc. Trop. Med. Hyg.,* **80** (3): 367-377.

León, A. M.; Shaw, J. J. & Tapia, F. J. 1996. A guide for the cutaneous leishmaniasis connoisseur. In: Tapia, F.J.; Ditimar, G. C. & Sànchez, M. A., *Molecular and Immune Mechanisms in the Pathogenesis of Cutaneous leishmaniasis*, Landes comp., pp.1-23.

Lessa, E. P. & Applebaum, G. 1993. Screening techniques for detecting allelic variation in DNA sequences. *Mol. Ecology,* **2**:119-129.

Lewin, S. 2000. Entwicklung molekularer Marker fur populations genetische Untersuchungen bei Leishmanien. *Ph. D. thesis, Faculty of Biology,* Humboldt University, Berlin, Germany. Pp. 103.

Lighthall, G. K. & Giannini, S. H. 1992. The chromosomes of Leishmania. *Parasitol. Today,* **8** (6): 192-198.

Lovannisci, D. M., Goebel, D.; Allen, K.; Kaur, K. & Ulman, B. 1984. Genetic analysis of adenine metabolism in Leishmania donovani promastigotes: evidence for diploidy at the adenine phosphoribosyl transferase locus. *J. Biol. Chem.,* **259**: 14617-14623.

Macedo, A. M.; Melo, M. N.; Gomes, R. F. & Pena, S. D. J. 1992. DNA fingerprints: a tool for identification and determination of the relationships between species and strains of Leishmania. *Mol. Biochem. Parasitol.,* **53**, 63-70.

Mauricio, I. L., Stothard, J. R. & Miles, M. A. 2000. The strange case of Leishmania chagasi. *Parasitol.Today,* **16** (5): 188-189.

McClelland, M.; Petersen, C. and Welsh, J. 1992. Length polymorphisms in tRNA intergenic spacer detected by using the polymerase chain reaction can distinguish streptococcal strains and species . *J. Clin. Microbiol.,* **30**: 1499-1504.

McMahon-Pratt, D. and David, J. R. 1981. Monoclonal antibodies that distinguish between New World species of Leishmania. *Nature,* **291**:581-583.

McManus, D. P. & Bowles, J. 1996. Molecular genetic approaches to parasite identification: their value in diagnostic parasitology and systematics. *Int. J. parasitol.,* **26**: 687-704.

Meredith, S. E. O.; Zijlstra, E. E.; Schoone, G. J.; Kroon, C. C. M.; Van Eys, G. J. J. M.; Schaeffer, K. U.; El Hassan, A. M. & Lawyer, P. G. 1993. Development and application of the polymerase chain reaction for the detection and identification of Leishmania parasites in clinical material. *Arch. Inst. Pasteur Tunis.,* **70**: 419-431.

Meredith, S. E. O.; Kroon, N. C. M.; Sondrop, E; Seaman, J.; Garis, M. G. A.; Van Ingen, C. W.; Oosting, H.; Schoone, G. J.; Terpstra, W. J. & Oskam, L. 1995. Leishmania-kit, a stable direct agglutination test based on freeze dried antigen for serodiagnosis of visceral leishmaniasis. *J. Clin. Microbiol*. **33**:1742-1745.

Miles, M. A, Povoa, M. M., Sousa, A. A., Lainson, R L. & Shaw, J. J. 1980. Some methods for the enzymatic characterization of Latin American Leishmania with particular reference to Leishmania mexicana amazonensis and subspecies of Leishmania hertigii. *Trans. R. Soc. Trop. Med. Hyg.,* **74**: 244-253.

Miniter, D. M.; Wijers, D. J. B.; Heisch, R. B. & Manson-Bahr, P. E. C. 1962. Phlebotomus martini-a probable vector of kala-azar in Kenya. *Br. Med. J.,* **2**:835.

Molyneux D.H. & Killick-Kendrick, R. 1987. Morphology, ultrastructure and life cycles. In: Peters, W. & Killick-Kendrick, R. The Leishmaniasis in Biology and Medicine. London: Academic Press, 121-176.

Morales, G.; Carrillo, G.; Requena, J. M.; Guzman, F.; Gomez, L. C.; Patarroyo, M. E. & Alonso, C. 1997. Mapping of the antigenic determinants of the Leishmania infantum gp63 proteins recognized by antibodies elicited during canine visceral leishmaniasis. *Parasitol.,* **114**:507-516.

Morsy, T. A.; Sabry, A. H.; Riffat, M. M. & Wahba, M. M. 1996. Psammomys obesus Cretzschmar, 1828 and zoonotic cutaneous leishmaniasis in Sinai Peinsula, Egypt. *J. Egypt. Soc. Parasitol.* **26** (2): 375-381.

Neave, S. A. M. 1904. Leishmania donovani in the Sudan. *Br. Med. J.,* **1**:1252.

Neva, F. & Sacks, D. 1990. Leishmaniasis. In: Warren, K. S. & Mahmoud, A. A. F. (eds.). Tropical and Geographical Medicine. McGraw-Hill Information Services Co., New York, 296-309.

Noyes, H. A.; Belli, A. A. & Maingon, R. 1996. Appraisal of various random amplified polymorphic DNA-polymerase chain reaction primers for Leishmania identification. *Am. J. Trop. Med. Hyg.* , **55**:98-105.

Orita, M; Wahana, H.; Kanazawa, H.; Hayashi, K. & Sekiya, T. 1989. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc. Natl. Acad. Sci.* USA, **86**, 2760-2770.

Oskam, L.; Pratlong, F.; Zijlstra, E. E.; Kroon, C. C. M. Dedet, J. P.; Kager, P. A.; Schönian, G.; Ghalib, H. W., El-Hassan, A. M. & Meredith, S. E. O.1998. Biochemical and molecular characterization of Leishmania parasites

isolated from an endemic focus in eastern Sudan. *Trans. R. Soc. Trop. Med. Hyg.*, **92**:120-122.

Osman, O. F.; Oskam, L.; Zijlstra, E. E.; Kroon, N. C. M.; Schoone, G. J. Khalil, E. A. G.; El-Hassan, A. M. & Kager, P. A. 1997. Evaluation of PCR for diagnosis of visceral leishmaniasis. *J. clin. Microbiol.*, **35**:2454-2457.

Osman, O. F.; Oskam, L.; Kroon, N. C; Schoone, G. J; Khalil, E. T.; El Hassan, A. M.; Zijlstra, E. E. & Kager, P. A. 1998. Use of PCR for diagnosis of post – kala-azar dermal leishmaniasis. *J. Clin. Microbiol.*, **36** (6): 1621-1624.

Pappas, M. G.; Hajkowski, R.; Tang, D. B. & Hockmeyer, W. T. 1985. Reduced false positive reactions in the Dot-enzyme linked immunosorben assay for human visceral leishmaniasis. *Clin. Immunol. Immunopathol.*, **34**: 392-396.

Piarroux. R. R.; Azaiez, R.; Lossi, A. M.; Reynier, P.; Muscatelli, F.; Gambarelli, F.; Fontes, M; Dumon, H. & Quilici, M. 1993. Isolation and characterization of a repetitive DNA sequence from Leishmania infantum: development of a visceral leishmaniasis polymerase chain reaction. *Am. J. Trop. Med. Hyg.*, **49**:364-369.

Piarroux, R.; Gambarelli, F.; Dumon, H.; Fontes, M.; Dunan, S.; Marcy, C.; Toga, B. & Quilici, M. 1994. Comparison of PCR with direct examination of bone marrow aspiration, myeloculture, and serology for diagnosis of visceral leishmaniasis in immunocompromised patients. *J. Clin. Microbiol.*, **32** (3):746-749.

Pogue, G. P; Koul, S.; Lee, N. S.; Dwyer, D. M.; & Nakhasi, H. L 1995a. Identification of intra and interspecific Leishmania genetics polymorphisms by arbitrary primed polymerase chain reaction and use of

polymorphic DNA to identify differentially regulated genes, *Parasitol. Res.,* **81** (4): 282-290.

Pogue, G. P; Lee, N. S; Koul, S.; Dwyer, D. M.; & Nakhasi, H. L 1995b. Identification of differentially expressed Leishmania donovani genes using arbitrarily primed polymerase chain reaction. *Gene,* **165**:31-38.

Ramamoorthy, R.; Donelson, J. E.; Paetz, K. E.; Maybodi, M.; Roberts, S. C. and Wilson, M. E. 1992. Three distinct RNAs for the surface protease gp63 are differentially expressed during development of Leishmania donovani chagasi promastigotes to an infectious form. *J. Biol. Chem.,* **267**:1888-1895.

Ravel, C.; Debussay, P.; Blackwell, J. M.; Ivens, A. C.; Bastien, P. & The Leishmania Genome Network. 1998. The complete chromosomal organization of the reference strain of the Leishmania genome project, L. major 'Friedlin'. *Parasitol today*, **14** (8)**:** 301-303.

Reddy, G. R. 1995. Determining the sequence of parasite DNA. *Parasitol. Today,* **11**:37-42.

Reithinger, R. & Davies, C. R. 1999. Is the domestic dog (Canis familiaris) a reservoir host of American cutaneous leishmaniasis? A critical review of the current evidence. *Am. J. Trop. Med. Hyg.,* **61**(4):530-541.

Rioux, J. A.; Dereure, J.; Kiami, A.; Pratlong, F.; Sirdar, K. and Lambert, M. 1990. Ecoepidemiology of leishmaniasis in Syria: 1.Leishmania major. Yakimoff and Schokhar (Kinetoplastida- Trypanosomatidae) infestation of Psammomys obesus Cretzschmar (Rodentia-Gerbillidae). *Ann.Parasitol. Hum.Comp.,* **65**(5-6)**:**203-207.

Roberts, S. C.; Swihart, K. G., Agey, M. W.; Ramamoorthy, R.; Wilson, M. E. & Donelson, J. E. 1993. Sequence diversity and organization of the msp gene family encoding gp63 of Leishmania Chagasi. *Mol. Biochem. Parasitol.,* **62**:157-172.

Roberts, J. L.; Handman, E. & Foote, S. J. 2000. Science, Medicine, and the future leishmaniasis. *BMJ.,* **321**:801-804.

Rogers, W. O. & Wirth, D. F. 1987. Kinetoplast DNA minicircles: region of extensive sequence divergence. *Proc. Nat. Acad. Sci.* USA, **84**:565-569.

Rolland, L.; Zilberfarb, V.; Furtado, A. & Gentilini, M. 1994. Identification of a 94-Kilodalton antigen on Leishmania promastigote forms and its specific recognition in human and canine visceral leishmaniasis. *Parasite Immunol.,* **16**(11):599-608.

Saiki, R. K.; Gelfard, D. H.; Stoffel, S.; Scharf, S. J.; Higuchi, R.; Horn, G. T.; Mullis, K. B. & Erlich, H. A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase, *Science,* **239** (4839): 487-491.

Sati, M. H. 1958. Early phase of an outbreak of kala-azar in the southern Fung. *Sud. Med. J.,* **1**:98-111.

Schönian, G.; Schweynoch, C.; Zlateva, K.; Oskam, L.; Kroon, N.; Gräser, Y. & Presber, W. 1996. Identification and determination of the relationship of species and strains within the genus Leishmania using single primers in the polymerase chain reaction. *Mol. Biochem. Parasitol.,* **77**, 19-29.

Schönian, G.; Akuffo, H.; Lewin, S; Maasho, K.; Nylen, S.; Pratlong, F.; Eisenberger, C. L.; Schnurl, F. & Presber, W. 2000. Genetic variability within the species Leishmania aethiopica does not correlate with clinical variations of cutaneous leishmaniasis. *Mol. Biochem. Parasitol.,* **106**:239-248.

Schönian, G.; Schnur, L.; El Fari, M.; Oskam, L, Kolesnikov, A. A.; Sokolowska-Köhler, W. & Presber, W. 2001. Genetic heterogeneity in the species Leishmania tropica revealed by different PCR-based methods. *Trans. R. Soc. Trop. Med. Hyg.,* **35** (35):1-8.

Seaman, J.; Meereer, A. J. & Sandrop, E. 1996. The epidemics of visceral leishmaniasis in western Upper Nile, southern Sudan: course and impact from 1984 to 1994. *Int. J. Epidemiol.,* **25**:862-871.

Shaw, J. J. 1994. Taxonomy of the genus Leishmania: present and future trends and their implications. *Mem. Ins. Oswaldo. Cruz., Rio de Janeiro,* **89**(3): 471-478.

Siddig, M.; Ghalib, H. W.; Shillington, D. C. & Petersen, E. A. 1989. Visceral leishmaniasis in the Sudan: comparative parasitological methods of diagnosis. *Trans. R. Soc. Trop. Med. Hyg.,* **82**:66-68.

Simpson, L. 1987. The mitochondrial genome of Kinetoplastid protozoa: genomic organization, transcription, replication and evolution. *Annu. Rev. Microbiol.,* **41**:363-382.

Singh, N. & Rastogi, A. K. 1999. Kinetoplast DNA minicircles of Leishmania donovani express a protein product. *Biochem. Biophys. Acta,* **1444** (2):263-268.

Stothard, J. R.; Frame, I. A. & Miles, M. A. 1997. Use of polymerase chain reaction-based single strand conformational polymorphism and denaturing gradient gel electrophoresis methods for detection of sequence variation of ribosomal DNA of Trypanosoma cruzi. *Internat. J. parasitol.,* **27** (3): 339-343.

Sturm, N. R. & Simpson, L. 1990. Kinetoplast DNA minicircles encode guide RNAs for editing of cytochrome oxidase subunit 111 mRNA. *Cell,* **61** (5):879-884.

Tibayrenc, M.; Neubauer, K.; Barnabe, C.; Guerrini, F.; Skarecky, D. & Ayala, F. J. 1993. Genetic characterization of six parasitic protozoa: Parity between random -primer DNA typing and multilocus enzyme electrophoresis. *Proc. Natl. Acad. Sci.* **90**: 1335-1339.

Uliana, S. R.; Affonso, M. H.; Camargo, E. P. & Floster-Winter, L. M. 1991. Leishmaniasis: genus identification based on a specific sequence of the 18s ribosomal RNA sequence. *Exp. Parasitol.,* **72**:157-163.

Van Eys, G. J. J. M.; Schoone, G. J.; Ligthart, G. S.; Alvar, J.; Evans, D. A. & Terpstra, W. J. 1989. Identification of 'old world' leishmaniasis by DNA recombinant probes. *Mol. Biochem. Parasitol.,* **34**: 53-62.

Van Eys, G. J. J. M.; Guizani, I.; Ligthart, G. S. & Dellagi, K. 1991. A nuclear probe for the identification of strains within the Leishmania donovani complex. *Exp. Parasitol.,* **72**: 459-463.

Van Eys, G. J. J .M.; Schoone, G. J.; Kroon, N. C. M. & Ebelling, S. B. 1992. Sequence analysis of small subunit ribosomal RNA genes and its use for detection and identification of Leishmania parasites. *Mol. Biochem. Parasitol.,* **51**: 133-142.

Victoir, K.; Banuls, A. L.; Arevalo, J.; Llanons-Cuentas, A.; Hamers, R.; Noel, S.; DeDoncker, S.; Le Ray, D.; Tibayrence, M. & Dujarden, J. C. 1998. The gp63 locus, a target for genetic characterization of Leishmania belonging to subgenus viannia. *Parasitol.,* **117**:1-13.

Webb; J. R.; Button, L. L. & Mc Master, W. R. 1991. Heterogeniety of the genes encoding the major surface glycoprotein of Leishmania donovani. *Mol. Biochem. Parasitol.,* **48**:173-184.

Weiss, J. B. 1995. DNA probes and PCR for diagnosis of parasitic infections. *Clin. Microbiol. Rev.,* **8**(1):113-130.

Welsh, J. & McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucl. Acids Res.,* **18**: 7213-7218.

WHO.1984. Expert committee: the leishmaniasis. *WHO Techn. Rep.* Ser.701

WHO.1990. Expert committee: control of the leishmaniasis. *WHO Techn.* Ser.793. Geneva: World Health Organization.

WHO. 1991. Information on the epidemiological and control of the leishmaniasis by country on territory. *WHO/leish/91.30.* Geneva: World Health Organization.

WHO. 1996. The leishmaniasis and leishmania/HIV co-infections. *Inf. Circ.-WHO Mediterr. Zoon. Control Cent.* **40**:11-13.

WHO. 1998. Leishmania and HIV in gridlock. *WHO/UNAIDS report.*. Geneva: World Health Organization. 15-25.

WHO. 2000. Division of control of tropical diseases. Leishmaniasis control home page WWW.who.int/health-topics/leishmaniasis.htm (updated 2000).

Williams, J.G.K.; Kubelik, A.R.; Livak, K.J.; Rabalski, J.A. & Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucl. Acids Res.,* **18**: 6531-6535.

Wilson, M.E.; Hardin,K. K. & Donelson, J. E. 1989. Expression of the major surface glycoprotein of Leishmania donovani chagasi in virulent and attenuated promastigotes. *J. Immunol.,* **143**:678-684.

Wilson, S. M. 1991. Techniques: Nucleic acid techniques and the detection of parasitic diseases, *Parasitol. Today,* **7**(9)**:**255.

Wincker, P. Ravel, C.; Blaineau, C; Pages, M.; Jauffret, Y., Dedet, J. P. & Bastien, P. 1996. The Leishmania genome comprises 36 chromosomes conserved across widely divergent human pathogenic species. *Nucl. Acids Res.,* **24**(9)**:** 1688-1694.

Wincker, P.; Ravel, C.; Britto, C.; Dubessay, P.; Bastien, P.; Pages, M. & Blaineau, C. 1997. A direct method for the chromosomal assignment of DNA markers in Leishmania. *Gene,* **194**:77-80.

Wong, A. K-C., 1995. Molecular genetics of the parasitic protozoans Leishmania. *Biochem. Cell Biol.* **73**:235-240.

Zeese, W. & Frank, W. 1987. Present epidemiological situation of kala-azar in the Republic of Sudan. *Zentralbl. Bakt. Mik. Hyg.,* **A-264**: 414-421.

Zijlstra, E. E.; Ali, M. S.; El-Hassan, A. M.; El-Toum, I. A.; Satti, M.; Ghalib, H. W.; Sandrop, E. & Winkler, A. 1991. Kala-azar in displaced people from southern Sudan: epidemiology, clinical and therapeutic findings. *Trans. R. Soc. Trop. Med. Hyg.,* **85**: 365-369.

Zijlstra, E. E.; Siddig, A. M., El-Hassan, A. M, El-Toum, I. A., Satti, M, Ghalib, H. W; Kager, P. A. 1992. Kala-azar: a comparative study of parasitological methods and the direct agglutination test in diagnosis. *Trans. R. Soc. Trop. Med. Hyg.* **86**:505-507.

Zijlstra, E. E.; Ali, M. S.; El-Hassan, A. M.; Hofland, H. W. C.; El-Toum, I.; Satti, M. & Ghalib, H. W. 1993. The treatment of kala-azar in the Sudan with sodium stibogluconate: a randomizes trial of three dosage regimens. *Trans. R. Soc. Trop. Med. Hyg.,* **87**: 307-309.

Zijlstra, E. E., El-Hassan, A. M.& Ismael, A. 1995. Endemic kala-azar in eastern Sudan: post kala-azar dermal leishmaniasis. *Am. J. Trop. Med. Hyg.,* **52**:299-305.

4.3 Appendices

Appendix 1: Demographic and clinical data for112 visceral leishmaniasis patients from eastern part of Sudan around Gedaref State (April 1997-November 1998).

Serial number	Identification number	Sex	Age in complete Years	Site of parasite isolation	Diagnosis	*Tribe or origin
1	1131	M	9	LN, BM	VL	W. Sudan
2	1146	F	7	LN, BM	N.N	E. Sudan
3	32	M	12	LN	VL	W. Sudan
4	33	M	25	LN, BM	N. N	Falata
5	17	M	10	LN	VL	Eretria
6	20	M	39	LN, BM	VL	Falata
7	44	M	13	LN	VL	Falata
8	41	M	17	LN, BM	N. N	Falata
9	45	M	20	LN, BM	VL	Arab
10	47	M	18	LN, BM	N. N	W. Sudan
11	1161	F	9	LN, BM	VL	Falata
12	1152	M	11	LN, BM	N .N	Falata
13	1150	F	12	LN, BM	VL	Falata
14	1129	F	11	LN, BM	VL	Falata
15	18C	M	20	LN, BM	N. N	W. Sudan
16	39	M	17	LN, BM	VL	Arab
17	1110	M	10	LN, BM	N.N	W. Sudan
18	1117	F	7	LN, BM	VL	W. Sudan
19	36	M	18	LN	VL	W. Sudan
20	31	M	14	LN	VL	W. Sudan
21	30	M	4	LN	VL	W. Sudan
22	29	M	18	LN	VL	Falata

23	28	M	28	LN, BM	VL	Falata
24	26	M	23	LN	VL	W. Sudan
25	22	M	19	LN	VL	W. Sudan
26	19	M	11	LN, BM	VL	Falata
27	16	F	13	LN	VL	W. Sudan
28	15	M	20	LN	VL	Falata
29	13	M	6	LN, BM	VL	Falata
30	12	M	27	LN	VL	W. Sudan
31	11	M	8	LN, BM	VL	W. Sudan
32	10	F	11	LN	VL	W. Sudan
33	9	F	6	LN	VL	W. Sudan
34	8	F	5	LN, BM	VL	W. Sudan
35	7	M	18	LN	VL	Falata
36	3	M	21	LN	VL	W. Sudan
37	2	F	13	LN, BM	VL	Falata
38	19C	M	24	LN, BM	VL	Eretria
39	13C	F	18	LN	VL	W. Sudan
40	9C	M	11	LN	VL	Eretria
41	60	M	7	LN, BM	VL	Falata
42	103	M	18	LN	VL	W. Sudan
43	100	M	4	LN	VL	W. Sudan
44	76	M	17	LN	VL	W. Sudan
45	71	M	14	LN	VL	W. Sudan
46	61	M	14	LN	VL	Falata
47	102	M	23	LN	VL	Falata
48	70	M	35	LN	VL	S. Sudan
49	90	M	25	LN, BM	VL	C. Sudan
50	101	M	25	LN, BM	VL	W. Sudan
51	18	M	22	LN, BM	VL	W. Sudan
52	104	M	22	LN	VL	Eretria

53	4	M	12	LN	VL	Falata
54	1	M	21	LN	VL	W. Sudan
55	5	F	9	LN	VL	W. Sudan
56	1140	F	8	LN	VL	W. Sudan
57	1135	M	29	LN, BM	VL	W. Sudan
58	72	M	16	LN	VL	W. Sudan
59	1116	M	4	LN	VL	Falata
60	6	M	13	LN, BM	VL	W. Sudan
61	42	M	17	LN	VL	W. Sudan
62	40	F	12	LN, BM	VL	Falata
63	13N	M	28	LN	VL	W. Sudan
64	46	M	7	LN	VL	W. Sudan
65	3N	M	1	LN, BM	N. N	W. Sudan
66	2N	M	5	LN, BM	N. N	W. Sudan
67	1149	F	12	LN	VL	Falata
68	24	M	13	LN	VL	W. Sudan
69	14	M	35	SKIN, LN, BM	PKDL	W. Sudan
70	25	M	9	LN	VL	W. Sudan
71	15C	M	2	LN	VL	W. Sudan
72	21	M	8	LN	VL	W. Sudan
73	1147	M	9	LN	VL	Falata
74	35	M	14	LN, BM	VL	Falata
75	5C	M	18	LN	VL	Arab
76	16C	M	24	LN, BM	VL	W. Sudan
77	79	M	24	LN, BM	VL	W. Sudan
78	4C	M	18	LN, BM	VL	Arab
79	27	F	12	LN, BM	VL	W. Sudan
80	8C	F	12	LN, BM	VL	W. Sudan
81	3C	M	18	LN, BM	VL	Falata
82	7C	M	10	LN, BM	VL	W. Sudan

83	20C	M	15	LN, BM	VL	W. Sudan
84	2C	F	20	LN, BM	VL	Falata
85	10C	F	7	LN, BM	VL	W. Sudan
86	66	M	14	LN	VL	W. Sudan
87	86	M	17	LN, BM	VL	Arab
88	67	M	13	LN	VL	Arab
89	C	M	20	LN	VL	W. Sudan
90	E	M	13	LN	VL	Falata
91	B	M	14	LN, BM	VL	Falata
92	A	M	30	LN	VL	Falata
93	D	M	25	LN	VL	W. Sudan
94	62	M	20	LN	VL	W. Sudan
95	75	M	17	LN, BM	VL	W. Sudan
96	63	M	31		N. N	Eretria
97	81	M	9	LN, BM	N. N	Falata
98	97	F	13	LN, BM	N. N	W. Sudan
99	10N	M	20	LN, BM	N. N	Falata
100	11N	F	16	LN, BM	N. N	W. Sudan
101	DON12	-	-	BM	VL	N. N
102	DON13	-	-	BM	VL/PKDL	N. N
103	DON14	-	-	BM	VL	N. N
104	DON15	-	-	BM	VL	N. N
105	DON16	-	-	BM	VL	N. N
106	DON17	-	-	BM	VL	N. N
107	DON18	-	-	LN	PKDL	N. N
108	DON19	-	-	BM	PKDL	N. N
109	DON20	-	-	BM	VL	N. N
110	DON2I	-	-	BM	VL	N. N
111	DON22	-	-	BM	PKDL	N. N
112	DON23	-	-	LN	PKDL	N. N

Samples from 101 to 112 were obtained from the collection of the Royal Tropical Institute, Amsterdam.

* M: male; F: female. ** LN: lymph node; BM: bone marrow

*** VL: visceral leishmaniasis, PKDL: post kala-azar dermal leishmaniasis

*** VL/PKDL: simultaneous VL and PKDL, N.N: not known

**** W: western, E: eastern, C: central, S: southern

Appendix 2: Strains of *Leishmania donovani* analysed in this study and the results of analyses.

Serial number	Identification number	SSCP ITS1 pattern	SSCP ITS2 pattern	SSCP gp63 (Y) pattern	SSCP 114 marker pattern
1	2C	A	A	A	A
2	20C	A	A	C	B
3	3C	A	A	A	A
4	15C	A	A	A	A
5	8C	A	A	C	A
6	4C	A	A	A	A
7	16C	A	A	A	A
8	5C	A	A	A	A
9	DON 12	A	A		n.d
10	DON 13	J	J	n.d.	n.d.
11	DON 14	D	A	n.d.	n.d.
12	DON15	A	A	n.d.	n.d.
13	DON 16	A	A	n.d.	n.d.
14	DON 17	D	A	n.d.	n.d.
15	DON 18	D	A	n.d.	n.d.
16	DON 19	A	A	n.d.	n.d.
17	DON 20	A	A	n.d.	n.d.
18	DON 21	A	A	n.d.	n.d.
19	DON 22	A	A	n.d.	n.d.
20	DON 23	D	A	n.d.	n.d.
21	MHOM/SD/75/LV139	A	A	n.d.	n.d.
22	MHOM/SD/62/LRC-L61	A	A	A	A
23	MHOM/SD/68/1S	A	A	n.d.	n.d.

24	MHOM/KE/85/NLB323	K	X	n.d.	n.d.
25	MHOM/IN/71/LRC-	K	Y	n.d.	n.d.
26	L51a	N	Z	n.d.	n.d.
27	MHOM/CN/??/Wangjiel	A	A	A	n.d.
28	10C	C	A	A	n.d.
29	25	C	A	A	n.d.
30	1140	D	A	A	n.d.
31	1147	A	A	A	n.d.
32	24	A	A	A	n.d.
33	1135	A	A	A	n.d.
34	104	M	A	A	n.d.
35	70	C	A	A	n.d.
36	4	C	A	A	n.d.
37	1149	A	A	A	n.d.
38	3	A	A	A	n.d.
39	79	B	A	A	n.d.
40	35	A	A	A	n.d.
41	27	A	A	A	n.d.
42	21	A	A	B	n.d.
43	40	A	A	A	n.d.
44	1	A	A	A	n.d.
45	7C	A	A	A	n.d.
46	5	A	A	A	n.d.
47	42	A	A	C	n.d.
48	1116	A	A	A	n.d.
	14				
49	72	A	A	A	n.d.
50	102	A	A	n.d.	n.d.
51	97	A	A	n.d.	n.d.

52	D	H	A	n.d.	n.d.
53	46N	A	A	n.d.	n.d.
54	103	A	A	n.d.	n.d.
55	44	A	A	n.d.	n.d.
56	10N	A	A	n.d.	n.d.
57	100	A	A	n.d.	n.d.
58	11N	A	A	n.d.	n.d.
59	32	A	A	n.d.	n.d.
60	75	A	A	n.d.	n.d.
61	36	A	A	n.d.	n.d.
62	62	A	A	n.d.	n.d.
63	9C	A	A	n.d.	n.d.
64	16	A	A	n.d.	n.d.
65	E	A	A	n.d.	n.d.
66	12	A	A	n.d.	n.d.
67	15	A	A	n.d.	n.d.
68	10	A	A	n.d.	n.d.
69	9	A	A	n.d.	n.d.
70	7	A	A	n.d.	n.d.
71	C	A	A	n.d.	n.d.
72	B	E	A	n.d.	n.d.
73	13N	A	A	n.d.	n.d.
74	26	A	A	n.d.	n.d.
75	!7	A	A	n.d.	n.d.
76	86	L	A	n.d.	n.d.
77	A	H	A	n.d.	n.d.
78	66	H	A	n.d.	n.d.
79	67	A	A	n.d.	n.d.
80	76	F	A	n.d.	n.d.
81	71	G	A	n.d.	n.d.

82	61	I	A	n.d.	n.d.
83	30	A	A	n.d.	n.d.
84	31	A	A	n.d.	n.d.
85	29	A	A	n.d.	n.d.
86	18	A	A	n.d.	n.d.
87	22	A	A	n.d.	n.d.
88	13C	A	A	n.d.	n.d.
89	39	A	A	n.d.	n.d.

n.d.: not done

4.4 Acknowledgements

I would like to thank many individuals for their generous help and support to complete this thesis.

I am deeply indebted to Dr. Gabriele Schönian (Dept. of Microbiology and Hygiene, Humboldt University) for her continued valuable suggestions, useful scientific comments, enthusiasm, guidance, constructive criticism throughout this work. Indeed thank you very much for your unflagging efforts in making it possible to defend this thesis at the Humboldt University (Berlin, Germany). Really it has been a privilege and honours to work with you both as a scientist and a person.

I am also most grateful to Professor Wolfgang Presber (Dept. of Microbiology and Hygiene, Humboldt University) for his valuable comments, support and encouragement. Many thanks to Professor Richard Liucius (Faculty of Biology, Humboldt University) for his supervision

I also acknowledge Dr. M. El fari for his general help and his wife Fayza who really made me feel at home. Also I would like to express my thanks to Dr. Wanda Sokdowska-Kohler for her help during my stay at the Institut für Mikrobiologie und Hygiene (Berlin, Germany). My thanks also extend to the technical staff at the same institute for their excellent help during the practical work.

Also I would like to thank Mr. Hamed El Neel and his technical staff (Gedaref Clinic, Sudan) for collection of clinical samples and to Khalid O. El Tai (Faculty of Agriculture, University of Khartoum) for his help during the fieldwork.

I am also grateful to Professor Michael A. Miles and to Dr. Isabel Mauricio for their assistance, provision of useful comments, and for the experience I gained during my stay at London School of Hygiene and Tropical Medicine, Dept. of Infectious and Tropical Diseases, Pathogen Molecular Biology and Biochemistry Unit.

Also I would like to thank Zuhier M. Elzubier for his assistance in the layout of the illustrations.

I also gratefully thank my husband for his encouragement, support, help and his tolerance of the prolonged disruption of normal social life.

Last but not least, I would like to thank my parents for their meticulous moral support and encouragement from primary education until I finished my Ph. D.

This work is generously financed by German Academic Exchange Service (DAAD), WHO (TDR Research Training Grant) and a grant from the Institut für Mikrobiologie und Hygiene, Faculty of Medicine, Humboldt University, Berlin.

Lebenslauf:

Name:	Nahla Omer Ahmed El Tai
Geschlecht:	weiblich
Geburtsdatum:	10.12.1969
Geburtsort:	Khartoum, Sudan
Familienstatus:	verheiratet, ein Sohn
Nationalität:	Sudan
Adresse zu Hause:	Dept. of Zoology, Faculty of Science, University of Khartoum, P.O. Box 321, Khartoum 11115, Sudan
Gegenwärtige Adresse:	Institüt für Mikrobiologie und Hygiene, Medizinsche Fakultät der Humboldt Universität Zu Berlin Dorotheenstr. 96, D-10098, Berlin

Studium:

1985:	Sudan general secondary school certificate
1986-1991:	University of Khartoum, Faculty of Science (B.Sc. Honours Degree in Zoology, second class, division one with the university prize).
1992	1994: M.sc. in Zoology, University of Khartoum
1998	Promvend an den Medizinischen und Math Naturwiss. Fakultäten der Humboldt-Universität zu Berlin

Berufstätigkeit:

Zur Zeit**:**	Lecturer, Department of Zoology, University of Khartoum

Stipendien

1991-1994:	Incountry DAAD-Stipendium zur Erlangung des M.Sc.
1991-1994:	Incountry DAAD-Stipendium zur Erlangung des M.Sc.
1997-1999:	Incountry DAAD-Stipendium zur Erlangung des Ph.D
Nov.97-March 98:	WHO-Stipemdium zur Ausbildung in Molekularbiologie Im Rahmen der Promation in der London School of Hygiene and Tropical Medicine, University of London
April 98-Nov.98:	DAAD - Sipendium zur Durchführung der Molekularbiologischen experimentellen Arbeiten im Rahmen der Promotion an der Humboldt-Universität, Berlin

Trainingkurse:

Nov.-Dec. 1995	Datenverabeitung und Biostatistik: SPSS für windows, Tropical Medicine Research Institute (TMRI), Sudan and the Danish Bilharziasis Laboratotry (DBL), Denmark durchgeführt am Zentrum für Datenverarbeitung und Statistik, Khartoum, unterstützt von Danish International Development Assistance (Danida), Dänemark.
Aug.-Sep. 1996	Dawin field course in Tropical Ecology, Kibale forest, Unganda, unterstützt vom British Council, Khartoum.

Mitgliedschaften:

1. Sudanese Environmental Conservation Society.
2. Sudanese Biological Society.
3. Sudanese Ornithological Society.

Wissenschaftliche Leistungen

1. Survey of vertebrae fauna of four selected Nile Island and one sand bar in Khartoum. Verteidigt alTeilleistung für den B.Sc. Zoologie.
2. African water fowl census 1993 and Jan. 1995 the part carried out in Sudan, organised by the international water fowl and wet lands research bureau (IWRB) UK.
3. Chemical composition and quality grading of three commercial fishes from the Nile. FAO 6th experts consultation on technology in Africa, August, 1996 Nairobi.

Publikationen, die aus der vorliegenden Arbeit entstanden sind:

1. El Tai, N.O., Osman, O.F., El Fari, M., Presber, W., and Schönian, G. 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. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* (2000) **94**, 575-579.
2. El Tai, N.O., El Fari, M., Mauricio, I. Miles, M.A., Oskam, L. El safi, S.H.,
3. Persber, W.H., and Schönian, G. *Leishmania donovani*: intraspecific
4. polymorphisms of Sudanese isolates revealed by PCR-based analysis and DNA sequencing. *Experimental Parasitology*, (2001) **97**, 35-44.

5. 3- Lewin, S.; Schönian, G.; El Tai, N.; Oskam, L.; Bastien, P.; Presber, W. Strain typing in *Leishmania donovani* by using sequence confirmed amplified region analysis. *International Journal for Parasitology*. (2002) **32:** 1267-1276.

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides Statt, daß ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen literatur und Hilfsmittel angefertigt habe und ich diese Arbeit in gleicher oder ähnlicher From noch keiner anderen Prüfungsbehörde vorgelegt habe.

Berlin, den

Nahla O. A. El Tai