Identification and Characterization of Gene(s) with Stage/Growth Regulated Expression in *Leishmania donovani* Isolated from Indian Kala-azar Patients

THESIS

Submitted in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

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Under the Supervision of

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BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE

PILANI (RAJASTHAN) INDIA

2003

BIRLA INSTITUTE OF TECHNOLOGY & SCIENCE

PILANI RAJASTHAN

CERTIFICATE

This is to certify that the thesis entitled '*Identification and Characterization of Gene(s) with Stage/Growth Regulated Expression in Leishmania donovani Isolated from Indian Kala-azar Patients*' and submitted by G Sreenivas ID No. 2000PHXF004 for award of Ph.D. Degree of the Institute, embodies original work done by him under my supervision.

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Date — 16th May 2003

Acknowledgement

The thesis work has followed the pattern motivated by the ideas, principally of my mentor Dr. Poonam Salotra. My preparative years with her are studded with myriad take away lessons of enduring value. I take this opportunity to thank her for giving me infinite amount of freedom, for lending clarity to my ideas and her phenomenal tolerance to my shortcomings.

I would not have succeeded in this effort, had it not been for the ideas, discussions and resources at several crucial stages from Dr Hira L Nakhasi and Dr A Selvapandiyan, CBER, FDA, USA. I would like to express my sincere thanks for all the help they have extended to me.

My thanks are due to Dr Sunita Saxena, Dr. Ashok Mukherjee and Dr. Bhanu Iyengar, who have facilitated my work in a great deal. I wish to thank Dr N V M Rao, Asst Prof, and Prof L K Maheshwari, Deputy Director, BITS, Pilani for being instrumental in shaping my career.

I sincerely acknowledge the help extended to me by Dr R K Bhatnagar, Mr. Naresh Arora, ICGEB for giving me unrestricted access to their lab resources. The isoenzyme analysis was done at IICB, Kolkata and I thank Dr Dwijen Sarkar for allowing me to work in his lab.

I could always rely on Mrs Saratha for her help in countless instances and express my deep felt thanks to her. My thanks are due to Mrs Seema, Mr. Rajesh, Mr. Rajender and Mr. Jagdish who have helped me in my work in various ways.

The nitty-gritty of experimental work would have been tedious without the monumental help offered by my labmates. Raju, Nasim and Ruchi. Their timely help made things easy for me and I profusely thank all of them. I thank Mr. PD Sharma, Mr. Anish Saxena and Mr. R amchandra Chhetri for their sincere support in everyday lab work.

My parents provide me the greatest strength. My wife Hina has been my strongest source of support all through and the deep affection of Sireesha, Radha and Vivek has seen me through ups and downs. My family is the unseen contributor in this effort.

May 2003

Sreenivas G

A_{280}	Absorbance at 280 nm
A_{600}	Absorbance at 600 nm.
Amp	Ampicillin
AP-PCR	Arbitrarily primed polymerase chain reaction
APS	Ammonium persulfate
bp	base pair
BSA	Bovine Serum Albumin
^{o}C	Degree Celsius
Ci	Curie
CL	Cutaneous Leishmaniasis
Cpm	Counts per minute
C terminal	Carboxy terminal
DAB	Diamino Benzidine
ddH_2O	Double Distilled water
ddNTP	Di-Deoxyribose nucleotide
DNA/RNA	Deoxyribose/Ribose nucleic acid
dNTP	Deoxyribose nucleotide triphosphate
dpm	Disintegrations per minute
EDTA	Ethylene diamine tetra-acetic acid
FCS	Fetal Calf Serum
g	Gravitational force
Gms	Grams
HBSS	Hank's balanced salt solution
HRP	Horseradish peroxidase
HEPES	N-2-hydroxyethgylpiperazine-N-2 ethane sulfonic acid
hr/hrs	Hour/hours
IPTG	Isopropyl-β-D-thio-galactosidase
KA	Kala-azar
kb	Kilo base

kDa	Kilo Dalton
LB	Luria Bertani medium
M	Molarity
mci	Millicurie
mg	Milligram
ml	Milliliter
min/mins	Minute/Minutes
mM	Milli Molar
MOPS	3-[N-Morpholino] propanesulfonic acid
N	Normailty
ng	Nanogram
Ni-NTA	Nickel-nitrilotriacetic acid
N terminal	Amino-terminal
O/N	Over night
OD	Optical Density
ORF	Open Reading Frame
PAGE	Poly Acrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PKDL	Post Kala-azar Dermal Leishmaniasis
PMSF	Phenyl Methyl sulfonyl fluoride
rpm	Revolution per minute
RAPD	Random amplified polymorphic DNA
RNase	Ribonuclease
RPMI	Roswell Park memorial Institute
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Poly acrylamide Gel Electrophoresis
Sec	Second
SSC	Sodium chloride Sodium citrate
TAE	Tris acetate EDTA
TEMED	N,N,N',N' tetramethyl ethylene diamine
Tris.	Tris (hydroxymethyl) amino acid

TBS-T	Tris buffered Saline with Tween 20
U	Unit
UV	Ultra Violet
VL	Visceral Leishmaniasis
xg	Times gravity (centrifugal force)
X-gal	5-bromo-4-cholro-3-indoyl-β-D-galactopyroanoside
β-ME	Beta mercaptoethanol
µg	Microgram
µl	Microliter
µM	Micromolar
%	Percentage
~	Approximately
ΔC	C' terminus deletion
ΔN	N' terminus deletion
ΔNC	N' and C' termini deletion

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Introduction

Introduction

Visceral leishmaniasis, fatal if not treated, is caused by *Leishmania* parasites of *L. donovani* complex comprising of *L. donovani, L. infantum,* and *L. chagasi.* More than 90% of the visceral cases in the world are reported from Bangladesh, Brazil, India and Sudan (Bora, 1999). In India, the official estimate of 430,000 VL cases in Bihar state over the past 11 years may represent only a fraction of the real numbers. The actual number is believed to be at least 5 times as great (Monograph, 1991-1996). Significant advances have been made in the development of anti-*Leishmania* drugs. In addition to the traditional pentavalent antimonials, new drugs such as aminosidine and liposome delivered amphotericin B have been introduced. The difficulties of treatment are exacerbated by the spread of resistance to antimony in India (Sundar et al, 2000) and the intractability of the disease to all drugs in patients co-infected with HIV. In most endemic countries the use of some excellent treatments, notably liposomal amphotericin for visceral leishmaniasis, is limited by patients' inability to afford it (Sundar et al, 2001a).

Unlike chemotherapy, vaccination is usually a one-shot affair. This makes it cheaper, and the easier logistics of administration lead to much better compliance. Vaccines have the advantage that they can be administered both in the prophylactic and therapeutic modes. An additional advantage of vaccination is its long-lasting effect and the fact that it avoids problems with drug resistance.

Historically, cutaneous leishmaniasis has been the focus of vaccination attempts, probably because it has been known since antiquity that individuals who had healed their skin lesions were protected from further infections. Bedouin or

some Kurdistani tribal societies traditionally exposed their babies' bottoms to sandfly bites in order to protect them from facial lesions. Another ancient technique practised in the Middle East has been the use of a thorn to transfer infectious material from lesions to uninfected individuals. Current molecular techniques have led to the development of recombinant antigen vaccines, though they have not protected to the level of live attenuated parasites (Rivier et al, 1999). Attenuated parasites, though effective, have been unacceptable for human use due to the absence of a clear genetic profile and danger of reversion (Handman, 2001). The current techniques for discovery of new genes that determine parasite survival and the possibility of manipulation of the *Leishmania* genome reinforce the potential of a live attenuated parasite vaccine.

Due to a large spectrum of drug resistance and emergence of Kala-azar as an opportunistic infection in HIV infected persons, the incidence of the disease is increasing at an alarming proportion throughout the world. Chemotherapy and vector control are the only effective measures for combating the disease, which is nearly always fatal if left untreated. For vaccination studies, cutaneous leishmaniasis caused mainly by *L. major* has remained the main focus. With the exception of antisense RNA, very few successful experimental live attenuated vaccination attempts were reported for *L. donovani.* Therefore, there is an urgent need to develop an effective vaccine against *L. donovani.*

Past efforts for development of vaccines have clearly shown the need for a better understanding of the mechanism of *Leishmania* pathogenesis. To date, there

is no vaccine against *Leishmania* in routine use anywhere in the world though several vaccine preparations are in advanced stages of testing.

In the *Leishmania* life cycle, motile promastigote forms that reside in the alimentary canal of the sandfly vector are transmitted to a mammalian host during a blood meal. Human macrophages ingest the parasites, which then differentiate into the non-motile amastigote form to persist in the macrophage's lysosomal compartment (Molyneux and Killick-Kendrick, 1987). This differentiation event is associated with dramatic morphological and biochemical changes that have been correlated with changes in gene expression in several species (Shapira et al, 1988; Cairns et al, 1989; Kidane et al, 1989; Coulson and Smith, 1990; Turco and Sacks, 1991; Joshi et al, 1993; Charest and Matlashewski, 1994; Pogue et al, 1995a,b). It is of crucial importance to unravel biochemical pathways or discover genes that may be expressed transiently at a critical point of differentiation. Further such genes products that regulate differentiation into the intracellular amastigote form have great potential as targets to block the infection process. These genes may only be expressed at the time of differentiation and at very low abundance. Therefore, very sensitive, unbiased methods and approaches that specifically sample this developmental stage must be employed to identify them.

A number of studies have been conducted searching for changes in gene expression amongst *Leishmania* stages, using methods such as differential or subtractive hybridization, AP-PCR, differential display and cDNA microarrays derived from spliced leader libraries (Coulson and Smith, 1990; Charest and Matlashewski, 1994; Pogue et al, 1995b; Heard et al, 1996; Liu et al, 2000; Wu et al,

2000; Bellatin et al, 2002; Almeida et al, 2002). The observation that in kinetoplastid protozoa differentially expressed genes are often associated with unique 3' untranslated regions has also been exploited (Coulson et al, 1997). However, low copy RNAs which in many cases have been shown to code for essential biological functions are often overlooked. The ability of polymerase chain reactions primed by oligonucleotides of arbitrary sequence to produce a unique DNA fingerprint (Welsh and McClelland, 1990) has been exploited to differentiate parasitic organisms (Waitumbi and Murphy, 1993; Tibayrenc and Neubauer, 1993). Arbitrary primed-polymerase chain reaction (AP-PCR) was found to produce unique DNA fingerprints differentiating several *Leishmania* species or species isolates (Pogue et al, 1995a). Due to its low complexity, the *Leishmania* genome allows AP-PCR technique to be used in DNA fingerprinting to survey the genome for gene sequences of interest. The ability of AP-primers to amplify DNA from diverse portions of an organism's genome allows screening of genomic DNA from a wide range of gene sequences with little bias. To that end, Pogue et al (1995a,b;1996) have identified several classes of differentially expressed RNAs in *L. donovani* which indicates that this system may be a particularly useful technique in the identification of novel genes and their products unique to Indian Kala-azar.

To understand the mechanism of parasitic infection, characterizing the genes, which might be involved in parasite differentiation is of fundamental importance. Specifically, it is proposed to use AP-PCR technique for genomic fingerprinting of Indian isolates and to identify unique DNA sequences, which are differentially expressed in promastigote and amastigote stages. The advantage of this technique

over conventional techniques, to identify stage-specific genes, is that it is rapid and can identify genes even with low copy messages. It is aimed to clone and sequence some of these genes selected on the basis of their level of expression in *in vitro* grown amastigote form of the parasite. Outcome of these studies would lead us to define genes that may have potential as live, attenuated vaccines.

Review of literature

Leishmania is a member of the trypanosomatid protozoa belonging to the order kinetoplastida. It is the causative agent of leishmaniases, diseases transmitted by the blood sucking phlebotomine sandfly. Early accounts of the occurrence of human leishmaniasis were recorded around 1824AD in Asia near the Indian border of Bangladesh. Subsequently epidemics were described in the Gangetic plains where it still has a major presence.

The leishmaniases afflict the world's poorest populations. Among the two million new cases each year in the 88 countries where the disease is endemic, it is estimated that 80% earn less than $2 a day (Davies et al, 2003). Human infections with *Leishmania* protozoan parasites, transmitted via the bite of a sandfly, cause visceral, cutaneous, or mucocutaneous leishmaniasis.

Leishmania currently infects about 12 million people in 88 countries, with 600,000 new clinical cases reported annually and many more unreported. Globally leishmaniasis is responsible for approximately 59,000 deaths annually with 350 million at risk. The global burden of leishmaniasis is estimated at 2.4 million disability adjusted life years lost (WHO, 2002). The two major clinical forms of leishmaniasis, cutaneous and visceral are the result of infection by different species of the parasite.

Visceral leishmaniasis (VL), the most severe in the disease spectrum, being fatal if left untreated, is caused by the members of *Leishmania donovani* complex that includes *L.d donovani, L.d. infantum,* and *L.d. chagasi* and is characterized by irregular fever, weight loss, swelling of the liver and spleen and anaemia. The annual incidence and prevalence of VL cases worldwide is 0.5 million and 2.5 million

respectively. More than 90% of the VL cases in the world are reported from Bangladesh, Brazil, India and Sudan (Bora, 1999).

The problem of VL has become more serious because of coinfection with HIV that is becoming increasingly frequent with cases reported in 25 countries (Desjeux, 1998; Pintado and Lopez-Velez, 2001). Control of leishmaniasis is complicated by the multiplicity of *Leishmania* species and their diverse clinical manifestations (Amaral et al, 2002).

Leishmaniasis in India

VL or kala-azar (KA) as it is known in India, is a significant infectious disease in the developing world and of late in the developed world because of increased international travel and HIV infection. KA is a symptomatic infection of the liver, spleen and bone marrow caused by organisms of *Leishmania donovani* complex. The causative organism of VL in the Indian subcontinent is *L. d. donovani* (Sundar et al, 2001b) where it is endemic in the Northeastern regions of the country primarily in the state of Bihar. The disease often turns epidemic claiming lives of thousands and causing severe morbidity to hundreds of thousands. The official estimate of 430,000 VL cases in Bihar state of India over the past 11 years may represent only a fraction of the real numbers. The actual number is believed to be at least 5 times as great (Monograph, 1991-1996). The causative organism in the Indian subcontinent and Africa is *L. d. donovani*, while in the Mediterranean basin and South America it is *L. d. infantum.*

After recovery from VL nearly 10-20% of the patients develop chronic Post Kala-azar Dermal Leishmaniasis (PKDL) in India that requires long and expensive treatment. In the absence of any animal hosts PKDL patients are deemed singular

source of *L donovani* in India (Thakur and Kumar, 1992). PKDL is an unusual dermatosis that develops as a sequel of KA, producing gross cutaneous lesions in the form of hypopigmented macules, erythema and nodules. The disease is relatively common in the Indian subcontinent and less frequent in East Africa, but exceptional in the American and European continents (Ramesh and Mukherjee, 1995).

Leishmania transmission

Leishmania are transmitted between long-lived vertebrate hosts by short lived phlebotomine sand flies (*Phlebotomus spp., Lutzomyia spp. and Psychodopygus spp.)* and have a cycle of development in each host. In the sand fly the parasites are in the promastigote form (1.5-3μmx10-20 μm) with an anterior flagellum and in the vertebrate host they reside intra- and extracellularly as oval, non-motile cells with only a very short flagellum and a maximum diameter of 2.5x6.8μm, which are called amastigotes (Fig1.1). Multiplication of each form is by binary fission (Bryceson, 1996).

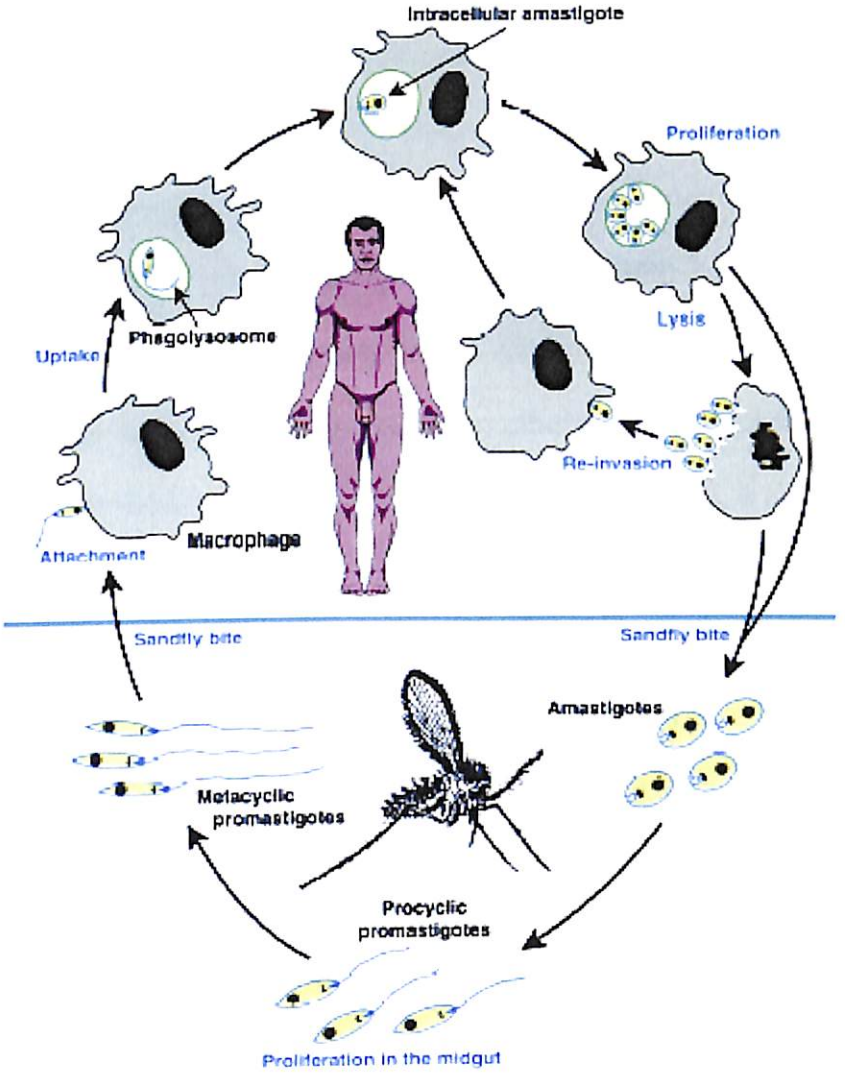


Fig 1.1 Schematic diagram of the *Leishmania* digenic life cycle.

Life Cycle of the *Leishmania* parasite

In the sand fly

After ingestion of a blood meal from an infected host, the parasites initially reside within the peritrophic membrane inside the sand fly's mid-gut. Amastigotes are then released from the macrophages of the blood meal and differentiate into the promastigote stage. They now synthesize an increasingly dense coat of a glycocalyx, which is composed of a variety of glycoconjugates that are bound to the surface of the parasite by a glycophosphatidylinositol (GPI) anchor (McConville and Ralton, 1997; Turco and Descoteaux, 1992). Underneath the glycocalyx, a dense layer of low molecular-weight glycoinositolphospholipids (GIPLs) is found, which is thought to have barrier functions. Among the glycoconjugates, a lipophosphoglycan (LPG), which contains a repeating polymer of disaccharide-phosphate repeating units, is the most abundant with 1-5 X 10^6 molecules/ parasite (Beverley and Turco, 1998; McConville and Ralton, 1997). Following rupture of the peritrophic membrane after about 2 days, the promastigotes attach to the mid-gut wall through specific binding of LPG and rapidly divide. After a further 4-7 days, the parasites cease dividing and differentiate into infective metacyclic promastigotes, which have a structurally altered LPG that is incapable of binding to the mid-gut wall (Sacks et al, 1995b). They migrate to the foregut and esophagus, where they are suspended in the sand fly's saliva and are ready to be inoculated during the next blood meal. This process involves enzymatic damage of the insect cardie valve that normally prevents reflux from the gut to the pharynx (Schlein et al, 1992). In the sand fly's gut, the saliva probably promotes survival and development of the promastigotes, since feeding of *Phlebotomus argentipes* with *L. donovani* suspended in serum containing

antisaliva antibodies led to death of a significant number of vectors and in the survivors to an inhibition of promastigote development and a concomitant reduction of migration to the foregut (Ghosh and Mukhopadhyay, 1998).

In the mammalian host

In order to get into their host, *Leishmania* take advantage of the feeding habits of the vector. The sand fly rips up the epidermis and eventually gains access to dermal capillaries. During this process, parasites are regurgitated into the bite wound. By using forced feeding techniques with *L. major* infected *Phlebotomus papatasi* sand flies, it was shown that the majority (approx. 75%) egested less than 100 parasites, approx. 20% egested 100-1000 parasites and the remainder egested 1000-3000 parasites each (Warburg and Schlein, 1986). Thus, under natural conditions, sand flies transmit very low numbers of promastigotes, which are able to induce disease. Under experimental conditions, when promastigotes usually are suspended in saline and inoculated by syringe into the skin of inbred mouse strains, this low number of parasites will never cause disease even in mouse strains like BALB/c, which are extremely susceptible to *L. major* infection when 10^5-10^7 parasites are inoculated (Bretscher et al, 1992; Doherty and Coffman, 1996; Menon and Bretscher, 1998).

Several changes individually and collectively allow the metacyclic promastigotes to withstand complement activation and to infect macrophages successfully. The lipophosphoglycan binds to serum-mannan binding protein which has a complement activating C1q domain (Green et al, 1994). This results in the lysis of procyclic but not metacyclic promastigotes. *L donovani* and *L. major* metacyclics are protected by a thick glycocalyx augmented by elongation of

lipophosphoglycan through an increase in the number of phosphorylated disaccharide repeat units (Sacks et al, 1995b). As a result the components of the membrane attack complex C5-C9 are shed from the metacyclic surface (Puentas et al, 1991). gp63 which is upregulated in metacyclic also inhibits complement mediated lysis and promoted parasite uptake by cleaving C3b to C3bi (Briitingham et al, 1995; Brttingham and Moser, 1996). Opsonisation of parasites with C3b and more particularly with C3bi, which bind to the macrophage receptors CR1 and CR3 respectively, provides the predominant means by which metacyclics bind to and access the host macrophage. Other receptors for uptake of promastigotes by macrophages that have been identified include mannose-fucose receptor, CR4, the fibronectin receptor, the receptor for advanced glycosylation end products, the Fc receptor and the C-reactive protein receptor (Alexander et al, 1999).

Presence of multiple receptors allows the parasite easy access into macrophages and Langerhans cells where the parasites transform into amastigotes. Langerhans cells are thought to provide a safe haven for the parasite by their failure to produce inducible nitric-oxide synthase. More significantly, although parasites fail to replicate in Langerhans cells, they are not rapidly killed and might save the host cells from apoptosis (Moore and Matlashewski, 1994). Otherwise, the sites of *Leishmania* infection are characterized by a marked increase in the number of macrophages because they are unable to migrate from these sites. This, in part, may be facilitated by LPG: LPG reduces monocyte trans-endothelial migration by modulating expression of the cell-adhesion molecules ICAM-1 and VCAM-1, junctional proteins CD31 and VE-cadherin, and inhibits the induction and release of MCP-1, a chemo-attractant that plays an essential role in the recruitment of

monocytes to the site of inflammation by providing a chemotactic gradient (Lo et al, 1998).

The low number of metacyclic promastigotes that the sandfly introduces into the skin (possibly as few as 100 to 1,000) in natural infections, compared to laboratory infections where thousands to millions of culture-derived promastigotes or tissue-derived amastigotes are injected, has lead to identification of important components in sandfly saliva that are powerful immunomodulators. Recent studies have demonstrated that in addition to parasite products and virulence factors that facilitate the entry of metacyclic promastigotes, components in sandfly saliva suppress macrophage leishmanicidal activity, inhibits nitric oxide (NO) production (Hall and Titus, 1995) and accelerate lesion development (Lima and Titus, 1996). This activity has been attributed to the sandfly salivary peptide maxadilan, a selective agonist of the pituitary adenylate-cyclase activating polypeptide type 1 receptor, which inhibits tumour necrosis factor-α production by lipopolysaccharide-stimulated macrophages (Bozza et al, 1998; Soares et al, 1998) and diminishes their ability to produce NO and kill *Leishmania in vitro* (David et al, 1997). Consequently, administration of maxadilan together with *L. major* promastigotes significantly exacerbated disease in resistant mice, which is associated with diminished NO production in draining lymph nodes (David et al, 1997).

Host-parasite interactions

Protection of promastigotes from the killing and degradative activities of macrophages is provided by the surface structures LPG and gp63 on the metacyclic promastigote. By preferentially accessing macrophages via CR3 and CR1, the

promastigotes fail to trigger the macrophage respiratory burst (Brittingham and Mosser, 1996). LPG also transiently inhibits phagosome-endosome fusion (Desjardins and Descoteaux, 1997), scavenges oxygen radicals generated during the respiratory burst (Chan et al, 1989), inhibits protein kinase C activity (Giorgione et al, 1996) and suppresses macrophage NOS2 expression and NO production (Proudfoot et al, 1996). gp63 has also been associated with suppression of the oxidative burst (Sorensen et al, 1994), and compelling evidence suggests that its protease activity protects the parasite from lysosomal cytolysis and degradation (Seay et al, 1996). Glycoinositolphospholipids (GIPLs) and non-inositol-containing glycosphingolipids constitute a dense glycocalyx immediately adjacent to the parasite surface through which LPG and gp63 project. GIPLs downregulate PKC activity (McNeely et al, 1989) and strongly inhibit NOS2 expression (Proudfoot et al, 1995).

Fusion of phagosome-lysosome takes place as the metacyclic transforms into the small ovoid amastigotes, and the parasites are able to survive and multiply within the acidic, hydrolase-rich parasitophorous vacuole (Alexander and Vickerman, 1975; Chang and Dwyer, 1976; Antoine et al., 1990; Russell et al, 1992). This transformation is associated with downregulation of LPG (McConville and Blackwell, 1991; Turco and Sacks,1991) and gp63 (Medina-Acosta et al, 1989; Bahr et al, 1993) expression on the parasite surface. Although little or no LPG is synthesized by amastigotes (with the possible exception of *L. major;* Moody et al, 1993; Ferguson, 1997), gp63-like molecules are found, in amastigote lysosomes and the flagellar pocket (Medina-Acosta et al, 1989). Consequently, the surface of amastigotes is dominated by GIPLs (Ferguson, 1997).

Amastigotes rely to a great extent on opsonization using the macrophage Fc receptor as indicated by studies on *L. major* (Guy and Belosevic, 1993) and *L. mexicana* (Peters et al, 1995) amastigotes. Further studies on amastigotes suggest that *L. major* (Guy and Belosevic, 1993) and *L. donovani* (Blackwell et al, 1985) can use CR3, that *L. amazonensis* can attach to heparan sulphate (Love et al, 1993) and a fibronectin receptor (Wyler et al, 1985), that *L. donovani* can attach to the mannose-fucose receptor (Blackwell et al, 1985) and that *L. major* can bind to a lectin-like receptor that recognizes LPG (Kelleher et al, 1995). However, although amastigotes adhere to and invade macrophages by mechanisms other than those characterized for promastigotes, current knowledge of the mechanisms by which this takes place is fairly limited (Mosser and Rosenthal, 1993; 1994). Evidently, promastigotes and amastigotes can enter host cells by multiple routes and this redundancy indicates that the route of entry is ultimately not the deciding factor in determining intracellular parasite survival.

Determinants of *Leishmania* virulence

Leishmania possess infection-related molecules, which allow them to establish successful intracellular parasitism in phagolysosomes or parasitophorous vacuoles of macrophages (Chang and Dwyer, 1976; Chang et al, 1990). In human leishmaniasis, these mononuclear phagocytes are invariably seen as the only parasitized cells. They are thus undoubtedly the principal host cells of *Leishmania*, although experimental evidence exists for leishmanial infection of other cell types, e.g. dendritic cells (Qi et al, 2001) and fibroblasts (Chang, 1978; Bogdan et al, 2000). The association of *Leishmania* with macrophages at the cellular level is characterized as akin to symbiosis (Requena et al, 2000). This notion was proposed

from observations of host-parasite cellular interactions in the *L. amazonensis*-J774 macrophage *in vitro* system (Chang, 1980). In that case, the infection produces no acute cytopathology or rapid cytolysis of the host cells. It is essentially a self-renewable or self-sustainable host-parasite *in vitro* system. Since human disease does occur with infection of macrophages *in vivo*, it is likely that the pathology and clinical symptoms may originate from interactions of these infected macrophages with other elements in the host.

It has been proposed that some parasite-specific antigens, derived from the infected cells, interact with the host immune system in a negative way, directly responsible for the immunopathology manifested as the clinical symptoms seen in leishmaniasis. Consistent with this notion are findings that kala-azar patients produce large amounts of *Leishmania*-specific, but non-protective antibodies against certain intracellular antigens of these organisms (Requena et al, 2000). A hypothetical model is thus proposed accordingly, depicting that *Leishmania* virulence results from interactions of different *Leishmania* determinants with separate compartments of host immune system, namely those for infection and those involved in immunopathology (Chang and McGwire, 2002).

The determinants that help the parasite to enter the host cell have been considered as virulence factors and extensively studied. All these molecules appear to play certain roles in *Leishmania* infection of macrophages. These determinants help *Leishmania* successfully establish intracellular parasitism in the following sequential events:

(A) evasion of humoral lytic factors

(B) attachment of parasites to macrophages followed by their intracellular

entry into these phagocytes

(C) intracellular survival of the endocytized parasites

(D) promastigote-to-amastigote differentiation and

(E) replication of the amastigotes.

The categorization of the host-parasite cellular interactions into sequential events pertains to the primary infection of macrophages in the mammalian hosts by promastigotes. Events (A) to (C) and (E) are relevant as well to the secondary infection of macrophages by amastigotes from already infected cells. The spreading of amastigotes to infect additional cells must be considered as crucial for the development of leishmaniasis. However, it may be mechanistically a rather simple event in considering normal functions of macrophages. One of their functions is to scavenge damaged or dying cells and their cellular debris, which may include degenerating cells (due to heavy parasitization), parasitophorous vacuoles and even released amastigotes with adherent host molecules in leishmaniasis. Much attention has been thus devoted to the infection of macrophages by promastigotes, although the manner, by which their molecules actually function in infection remains to be elucidated. Data obtained from different host-parasite systems are also not always consistent even for the molecules more extensively studied, like gp63 and LPG. gp63 is an ecto-metalloprotease that is especially abundant in the surface of promastigotes and also released by this stage of *Leishmania* (McGwire et al, 2002). gp63 is known to help promastigotes in event (A) by rendering them resistant to complement-mediated cytolysis (Brittingham et al, 1995). It also appears to act, perhaps, together with LPG in event (B), namely infection of macrophages by promastigotes via receptor- mediated endocytosis (Chang and Chang, 1986; Russell

and Wilhelm, 1986; Liu and Chang, 1992; McGwire and Chang, 1994). Both may be important in event (C) for their intraphagolysosomal survival. Some of these molecules may be involved directly or indirectly in these and/ or the remaining events. Parasite molecules involved in events (D) and (E) for differentiation and replication of intracellular *Leishmania* may have additional functions beyond infection, especially in the latter case. Regardless of the functional and definitional ambiguity associated with these determinants, there is no evidence that they directly cause the clinical symptoms seen in leishmaniasis. For example, repeated injections of susceptible animals with gp63 or LPG do not reproduce the typical symptoms of leishmaniasis, e.g. various types of cutaneous lesions or hepatosplenomegaly and other clinical signs related to kala-azar (Chang and McGwire, 2002).

Leishmania genome organization

The *Leishmania* genome differs from the typical eukaryotic genome. The *Leishmania* nuclear genome contains approximately $3.5x10^7$bp and is organized into 34-36 chromosomes ranging in size from 0.3 to 3 Mb, which possess repetitive telomeric sequences but do not condense during the mitotic cycle. Visualization of chromosomes is only possible using pulsed field gel electrophoresis. Under the conditions of nutritional stress or drug selection, *Leishmania* spp. can amplify specific segments of the genome as mini-chromosomal amplicons. The *Leishmania* is a diploid organism and functionally asexual. Variations in the relative sizes of homologous chromosomes have been reported in the Old World species of *Leishmania* (Britto et al, 1998). The chromosomal organization of *Leishmania* is similar to many protozoan parasites; a compartmentalization into conserved core domains and polymorphic chromosome ends (Lanzer et al, 1995). In terms of

structure and maintenance of chromosomal termini, *Leishmania* conforms to those described in other eukaryotes. Telomerase activity, the activity of the ribonucleoprotein enzyme complex responsible for addition of deoxyribonucleotide triphosphate to the 3' ends of chromosomal strands, has been demonstrated in *Leishmania* with primer recognition and elongation properties similar to those of other eukaryotes (Cano et al, 1999).

Although *Leishmania* organisms multiply by binary fission, an assumption of sexual reproduction naturally arises from the existence of diploid genome. Several lines of evidence such as gene-knockouts, restriction-site polymorphism, genetic recombination and karyotype alterations point to the diploid state. However, in contrast to the well documented genetic exchange strategies in *T. brucei* (Bingel et al, 2001) the issue of genetic exchange mechanism in *Leishmania* is much debated (Gibson and Stevens, 1999). Recently, it has been shown that *T. cruzi* also possesses an extant capacity for genetic exchange and follows a mechanism distinct from that proposed for *T. brucei* (Gaunt et al, 2003). The existence of self-fertilization mode of replication has been suggested to account for the contrast between the high number of variants in the chromosome 1 (Blaineau et al, 1992). In addition, natural *Leishmania* hybrids have been described in isolates from Old and New Worlds (Banuls et al, 1999; Gibson and Stevens, 1999). Despite this evidence, sexual exchange has not been shown experimentally in *Leishmania* and the available data indicate that in the wild genetic exchange might be infrequent at best. Therefore, sexual crosses for identifying phenotypes of interest are feasible in trypanosomes subject to the limited availability of tsetse fly colonies, but are poorly suited to applications in *Leishmania* (Beverley, 2003). Population genetics studies

indicate that the two genetic consequences of sexual reproduction, segregation and recombination are absent from *Leishmania*, and a clonal, asexual model has been proposed for *Leishmania* populations (Tibayrenc, 1995). The clonality versus sexuality debate underlines the significant diversity of most parasites, a parameter that is increasingly taken into account in clinical surveys, vaccine and drug design (Tibayrenc and Ayala, 2002).

Genome plasticity in Leishmania

A significant level of interspecific and intrastrain chromosomal polymorphism was noted in early PFGE based studies of *Leishmania* (Bastien et al, 1992). This polymorphism was largely due to variations in chromosome size, of up to 25% of the chromosome length. The results of more recent PFGE studies, used to establish the chromosomal karyotype of *Leishmania,* reveal that homologous chromosomes vary in size not only between species but even within clones of one species (Wincker et al, 1996; Britto et al, 1998). Extensive karyotypic polymorphism has also been noted in the size of the gp63-containing chromosomes in 58 field isolates of *L. peruviana,* in the rRNA-gene containing chromosomes of *L.* (*Viannia*) spp., and in the homologous chromosomes containing the H-region associated with resistance to methotrexate in some strains of *L. tropica* (Espinoza et al, 1995; Olmo et al, 1995; Inga et al, 1998). Although most laboratory strains of *Leishmania* exhibit a stable karyotype, chromosomal karyotypes can be unstable during laboratory culture and size variation can arise rapidly. Chromosomal karyotypes were altered during subcloning of an *L. infantum* strain and occur spontaneously or after nutrient stress or subcloning in *L. tarentolae* (Bastien et al, 1990; Rovai et al, 1992). Long term (4-year) cultivation also induces rearrangement of the gp63 locus (Victior et al, 1995).

The conservation of linkage groups in chromosomes of different *Leishmania* species and the identical linear organization of individual markers in chromosome V of *L. major* and *L. infantum* (Wincker et al, 1996; Ravel et al, 1999) indicate that the observed genome plasticity in *Leishmania* is probably not caused by interchromosomal rearrangements. Rather, amplification/deletion events in subtelomeric regions as shown for *L. infantum* chromosomes, within the repeated arrays of mini-exon genes on *L. major* chromosome II, and rRNA gene clusters and gp63 genes of several *Leishmania (Viannia)* spp. are responsible for variability in chromosomal size (Iovannisci and Beverley, 1989; Blaineau et al, 1992; Victior et al, 1995; Ravel et al, 1995, 1996; Inga et al, 1998). As an explanation for this heterogeneity, Bastien et al (1990) proposed a 'mosaic' structure for *Leishmania* strains, with different cells containing differently sized chromosomal homologues because of frequent amplification/deletion.

Genomic arrangement

A considerable degree of heterogeneity exists in the organization and expression of genes in *Leishmania.* The *Leishmania* genome contains repetitive DNA sequences and has 58-60% G/C content. *Leishmania* genes exhibit a distinctive codon bias, lack introns, occur as single genes, paired genes or may be repeated in tandem arrays, and exhibit considerable divergence in flanking sequences, even between closely related genes. Short, repetitive, DNA-sequence motifs are common elements of most eukaryotic genomes and are present as tandem repeats, known as mini-or micro-satellites, interspersed throughout the genome in non-protein coding regions, or characterize chromosome ends in telomere associated sequences.

Hybridizations of PFGE gels using whole-chromosome and chromosome-fragment probes indicate that the repetitive sequences *of Leishmania* are usually not chromosome-specific and are not necessarily shared by all chromosomes (Bastien et al, 1992). Microsatellite DNA including di-, tri- and tetra-nucleotide repeat motifs is present on the chromosomes of several *Leishmania* spp., with (CA)n repeats predominating (Rossi et al, 1994). These microsatellites are associated in clusters containing different repeat elements and, although present in similar numbers, their distribution in the *Leishmania* genome appears more irregular than that in the genomes of higher eukaryotes.

Ravel et al (1995) described the presence of a G/C-rich, 81-nucleotide repeat minisatellite- DNA sequence on four *L. infantum* chromosomes. On chromosomes I and V the minisatellite clusters are located in the subtelomeric regions and their involvement in chromosomal size instability has been proposed. Mini- and micro-satellite DNA sequences have also been utilized as PCR amplification primers, to assess DNA polymorphism in *Leishmania* (Schonian et al, 1996). The arrangement of repeated DNA sequences can be specific to the genus or complex level. Rodriguez et al (1997) demonstrated the existence of an *L. braziliensis*-complex specific genomic fragment of 1.8kb, consisting of homonucleotide runs of 6 to 25 nucleotides, microsatellites (GT)s, (AG)s and $(GATC)_4$, and additional repeats of six to 16 nucleotides in length. In addition to intergenic repetitive elements, many peptides of parasitic protozoa contain repetitive amino-acid domains, often not present in their higher eukaryotic homologues, which provoke high levels of antibody response. Repetitive motifs are common to molecules exposed on the surface membranes of *Leishmania,* such as LPG2, gp46, A2, PSA, PSA-2 (Lohman et al,

1990; Murray and Spithill, 1991; Charest and Matlashewski, 1994; Flinn et al, 1994; Descoteaux et al, 1995). Repetitive motifs are also present in the kinesin of *L. chagasi* (39-aminoacid repeat), the secreted acid phosphatases of *L. donovani* (SacP-1 and SacP-2; two types of eight-residue, serine/threonine-rich repeats), the Lm20 and Lm39 peptides of *L. major* and the *L. mexicana* LmSap2 protein (Burns et al, 1993; Wiese et al, 1995; Shakarian et al, 1997). Regardless of their function, the host response to these repetitive elements can serve as a basis for serological or molecular diagnosis.

Gene composition in Leishmania

Leishmania and *Crithidia* were found to be more G/C rich (58% and 57%, respectively) than *T. brucei* (51%) or *T. cruzi* (44%) (Alonso et al, 1992). Further, coding regions had a higher G/C content than non-coding regions (NCR) and 3' NCR were more G/C rich than 5' NCR. It was speculated that the high G/C content of *Crithidia* and *Leishmania* might be a reflection of the more primitive nature of these organisms. Moreover, *Crithidia* and *Leishmania* were found to share a similar base-utilization scheme at all three codon positions. Within a codon, there is a strong preference (about 85%) for G or C in the third, or 'wobble', position of *Leishmania* amino-acid codons, a slight A/T bias (about 55%) in codon position 2 and a G/C bias (about 60%) in codon position 1 (Alvarez et al, 1994).

Gene transcription and splicing

Nuclear (non- ribosomal) genes in *Leishmania* are co-transcribed by a pol-II type polymerase, to yield polycistronic precursor RNA which is processed in a bimodal fashion. A common, 39-nucleotide, mini-exon sequence containing a 'cap' structure is *trans*-spliced to the 5' end of all mature messages, and 3'

polyadenylation gives rise to individual, monocistronic mRNA. The mini-exon splicing is controlled by polypyrimidine (CT) tracts and occurs at a downstream AG dinucleotide. The processes of *trans*-splicing and polyadenylation are linked at sites separated by 200-500 nucleotides. The selection of polyadenylation sites is an imprecise event, with a strong preference for polyadenylation at CA_n, GA_n, or TA_n sites. Non-ribosomal and non-histone mRNA are characterized by long 5' and 3' UTR.

Leishmania genome sequencing

With the gene density of one gene/~3.7kb, the total number of genes is predicted to be in the range of 8,500. Of the total 36, chromosomes 1, 3, 4, 5 and 24 have been completely sequenced and the other chromosomes are at an advanced level of completion at the three collaborating centers, Sanger Institute, UK, Seattle Biomedical Research Institute, USA and Beowulf Center, Brazil. (http://www.sanger.ac.uk/Projects/L_major/progress.shtml).

At the genome level, *Leishmania* is an unusual unicellular eukaryote. Its genes are often organized in tandem repeats. Most interestingly, the genes are organized into large (>100-500kb) polycistronic clusters of adjacent genes on the same strand. The chromosomes range in size from 0.3 to 2.8 Mb (Bastien et al, 1998). Non-repeated genes of related function can also occur in long transcription units. The density of protein coding genes appears to be constant. The genes do not cluster into prokaryote like operons of genes with similar function, but some regions appear to have higher than expected concentration of large genes. Extensive posttranscriptional processing is required to yield mature mRNAs including trans-splicing of a small RNA spliced leader sequence measuring 39nt in length onto 5'

ends of all mRNA molecules (Ivens and Blackwell, 1996). Gene expression is also controlled at the level of translation (Landfear and Wirth, 1984).

Unusual features in *Leishmania* biology

Organisms of the order *Kinetoplastida* have a unique organelle called the kinetoplast, an appendix of their single mitochondrion located near the basal body of the flagellum that contains a network of thousands of small interlocking circular DNAs. Kinetoplastids are among the most ancient eukaryotes, with rRNA lineages extending farther back than those of animals, plants, and even fungi (Beverley, 1996; Fernandes et al, 1993). The kinetoplast is only one of their many distinctive features. Several fundamentally important biological phenomena that were first discovered in *Leishmania* and trypanosomes include- 'programmed' antigenic variation of surface glycoproteins (Bridgen et al, 1976), glycosylphosphatidylinositol anchors of membrane proteins (Holder and Cross, 1981; Ferguson and Cross, 1984), expansion and contraction of telomeric DNA repeats (Bernards et al, 1983), bent DNA helices (Marini et al, 1983), eukaryotic polycistronic transcription (Johnson et al, 1987), trans-splicing of precursor RNAs (Boothroyd and Cross, 1982; Walder et al, 1986), mitochondrial RNA editing (Benne et al, 1986), other unique organelles such as glycosomes (Opperdoes and Borst, 1977), and distinctive metabolic pathways (Bachi et al, 1980). Several of these phenomena, first uncovered because of their prominence in kinetoplastids, have subsequently been found in higher eukaryotes and have become the focus of intense research interest in those systems. In addition, the many novel mechanisms used by *Leishmania* and trypanosomes to thwart immune defenses thrown at them by their mammalian hosts

have led to a deep appreciation of the diversity and complexity of host parasite interactions (Donelson et al, 1999).

Previous studies have established that 30% of the genome is composed of repeated elements, about half of which are telomeric and subtelomeric repeats and the rest of which are dispersed transposons, repeated genes, and other simple repeated sequences. None of the protein-encoding genes of *Leishmania* studied to date contain introns, simplifying the identification of these genes in the genomic DNA. Most, if not all, of these genes are initially transcribed into large polycistronic precursor RNAs of 60 kb or more in length that are cleaved into monocistronic mRNAs by the action of two intergenic RNA cleavage reactions, trans-splicing of a 39-nt "spliced leader" to generate the 5' ends of all mRNAs, and 39 cleavage and polyadenylation to create the 3' ends. In contrast to most other eukaryotes, no consensus 3' poly(A) sites have been described, but efficient 5' trans-RNA splicing typically occurs at a short consensus sequence preceded by a polypyrimidine tract (Matthews et al, 1994). Little is known about the protein components of the putative trans-spliceosome, although a number of small nuclear RNAs are known to participate in the process. Because cis-splicing of introns in yeast and mammals and trans-splicing of the spliced leader in *Leishmania* and trypanosomes are mechanistically similar, however, it seems likely that similar proteins participate in these two processes.

In summary, evolutionarily ancient *Leishmania* and trypanosomes are placed at the genetic border between prokaryotic and eukaryotic organisms. They share some features with prokaryotes (genes without introns, polycistronic transcription), other features with eukaryotes (pre-mRNA splicing), and have some features found

in neither (protein-encoding genes without promoters) making this organism a very interesting model.

Gene structure in Leishmania

Introns (characteristic of genomic organization in higher eukaryotes) and cis-splicing mechanisms have not been demonstrated in any *Leishmania* gene. The presence of pseudogenes, also common to higher eukaryotes, has been noted, downstream of the *L. mexicana* pyruvate-kinase gene and *cpb* Cysteine proteinase tandem array (Ernest et al, 1994; Mottram et al, 1997). The organization of ribosomal RNA genes in *Leishmania* resembles that, of other eukaryotes: multiple, head-to tail repeats separated by non-transcribed spacers (Leon et al, 1978; Requena et al, 1997). Investigations of non-ribosomal genes in *L. donovani* and *L. major* have identified 40 single-copy genes, eight loci containing linked pairs (16 genes) of closely related genes, and 32, tandemly repeated, multi-copy gene loci. Paired genes are characterized by a high degree of conservation in peptide sequence, although there is considerable divergence in the 5' and 3' untranslated regions (UTR) of each gene transcript, a feature perhaps related to differences in their regulation. The duplicate gene loci characterized to date produce a differently sized transcript for each gene, which are either differentially expressed during the life-cycle (as for *L. donovani* ATP-ase and *L. mexicana* secreted-acid phosphatase transcripts) or exhibit markedly different levels of transcript abundance in promastigotes as reported for *L. infantum* acidic-ribosomal- protein *LiP, LiF* and *LiPO* loci, and *L. major* phosphoglycerate-kinase genes (Meade et al, 1989; Soto et al, 1993a,b; Wiese et al, 1995; McKoy et al, 1997). Highly expressed proteins such as α-and β- tubulins, heat-shock proteins, proteases, transporters, flagellar proteins

and surface antigens are present in multiple copies in the *Leishmania* genome. Multi-copy genes are usually organized as direct tandem repeats. However, they may also alternate with other repeated genes (such as the *A2-A2rel* and gp63 *mspS-mag* gene arrays), include unlinked copies located elsewhere in the genome (as for α-tubulin, *hsp70,* P-glycoprotein and *psa-2* genes), or exist as scattered individual genes, and some genes with related function can exist as single-copy and multi-copy variants (Spithill and Samaras, 1987; Stein et al, 1990; Hanekamp and Langer, 1991; Murray and Spithill, 1991; Langford et al, 1992; Bock and Langer, 1993; Roberts et al, 1993; Legare et al, 1994; Hubel and Clos, 1996; Quijada et al, 1997; Soto et al, 1997; Ghedin et al, 1998; McCoy et al, 1998). A tandemly repeated organization may function as a mechanism, independent of transcript stability, to increase the transcript abundance of highly expressed proteins. There is some correlation between copy number of the heat-shock-protein genes in promastigotes and the intracellular concentration of the corresponding peptide. The multi-copy *hsp70* and *hsp83* code for peptides forming 2.1% and 2.8% of cellular protein, respectively, whereas the peptide coded by the single-copy *hsp*100 accounts for only 0.25% (Brandau et al, 1995; Hubel et al, 1995). However, other mechanisms must also be involved in regulating concentrations of HSP, as *hsp*100 transcripts are more abundant than *hsp70* transcripts in lesion-derived amastigotes (Hubel et al, 1997). Individual genes in tandem arrays are often expressed as similarly sized transcripts which are coordinately regulated, although in some instances repeated gene arrays produce multiple transcripts. The abundance of these multiple transcripts is differentially regulated in the *psa-2,* α-tubulin, *hsp70, LmcDNA16, cpb* Cysteine proteinase, paraflagellar-rod-protein *pfr- 2* and gp63 loci (Spithill and

Samaras, 1987; Bhaumik et al, 1991; Flinn and Smith, 1992; Ramamoorthy et al, 1992; Handman et al, 1995; Coulson et al, 1996; Moore et al, 1996; Mottram et al, 1997; Quijada et al, 1997;Voth et al, 1998). However, multiple mRNA from the *L. donovani A2* and *L. enriettii* histone *H2B* genes exhibit similar developmental regulation (Genske et al, 1991; Charest and Matlashewski, 1994; Zhang et al, 1996).

In many paired and repeated gene loci there is a conservation in gene number and/or organization between different *Leishmania* spp., including the ATPase, *hsp83,* acidic ribosomal PO protein and *lmstII* loci (Meade et al, 1991; Soto et al, 1993a; Skeiky et al, 1994; Hubel and Clos, 1996; Webb et al, 1997). However, in other loci, particularly those encoding surface antigens, a greater degree of heterogeneity is evident. The gp63 and *hsp70* loci both differ in organization and gene-copy number between species (Hanekamp and Langer, 1991; Webb et al, 1991; Bock and Langer, 1993; Roberts et al, 1993; Steinkraus et al, 1993; Gonzalez- Aseguinolaza et al, 1997; Quijada et al, 1997). Gene sequence also differs between the gp63 genes of *L, chagasi* and *L. major* (Ramamoorthy et al, 1992; Voth et al, 1998). Aamastigote-specific A2 genes are also not well conserved among *Leishmania* spp., as antibody to A2 is only detected in *L. donovani* and *L. mexicana* infections and not in infections with other species (Ghedin et al, 1997). There are 14 tandemly arrayed *psa* (promastigote-surface-antigen) genes in *L. major* which produce multiple transcripts that are differentially regulated, but the six *psa* genes in *L. infantum* produce a single transcript (Murray and Spithill, 1991; Handman et al, 1995; Jimenez Ruiz et al, 1998). With few exceptions, the UTR of *Leishmania* transcripts from duplicate and tandemly repeated genes are unique. Conservation in proximal 5' and3'UTR has been observed within the different

classes (constitutive, stationary and log) of *L. guyanensis* gp63, among the *L. chagasi* gp63 classes (about 200 nucleotides in all 5' UTR and 397 in the *mspL* and *mspS 3'* UTR) and in the *L. donovani* ATPases (73 nucleotides in the 5' UTR and 15 in the 3' UTR), but this conservation does not include the entire UTR for these genes (Meade et al, 1989; Ramamoorthy et al, 1992; Roberts et al, 1993; Steinkraus et al, 1993). The purpose of this limited conservation is unknown, as similar conservation is not apparent in comparisons between unrelated genes nor even between the different classes of gp63.

Gene transcription and splicing

Nuclear (non- ribosomal) genes in *Leishmania* are co-transcribed by a pol-II type polymerase, to yield polycistronic precursor RNA which is processed in a bimodal fashion. A common, 39-nucleotide, mini-exon sequence containing a 'cap' structure is *trans*-spliced to the 5' end of all mature messages, and 3' polyadenylation gives rise to individual, monocistronic mRNA. The mini-exon splicing is controlled by polypyrimidine (CT) tracts and occurs at a downstream AG dinucleotide. The processes of *trans*-splicing and polyadenylation are linked at sites separated by 200-500 nucleotides. The selection of polyadenylation sites is an imprecise event, with a strong preference for polyadenylation at CA_n, GA_n, or TA_n sites. Non-ribosomal and non-histone mRNA are characterized by long 5' and 3' UTR (Stiles et al, 1999b).

5' trans-splicing of Transcripts

Synthesis of mRNA from polycistronic transcripts in trypanosomatid protozoa necessarily requires additional 5' and 3' end-processing events. A common, 39-

nucleotide spliced leader (SL) sequence, also known as a mini-exon, is attached to the 5' end of all messages in a bimolecular process known as *trans*-splicing (Nilsen, 1995; Vanhamme and Pays, 1995). The SL segment is donated from the 5' end of a precursor-spliced leader RNA encoded separately in the genome. The 39-nucleotide SL and SL-intron sequences are highly conserved in *Leishmania* but differences are noted in the length and sequence of non-transcribed regions as well as in the size of the precursor SL RNA: 86 nucleotides in *L. donovani* 89 in L. *enriettii* and 96 in *L. tarentolae* (Miller et al, 1986; Fernandes et al, 1994; Fleischmann and Campbell, 1994; Lamontagne and Papadopoulou, 1999). The SL gene in *Leishmania* is present as a tandem array of about 150 copies, which group into three classes based on sequence and comprise 0.1% of the parasite genome (Miller et al, 1986; Lamontagne and Papadopoulou, 1999). Ligation of the SL to polycistronic pre-mRNA occurs via a two-step, phosphodiester-exchange reaction involving the formation of a branch Y intermediate. The presence of a highly modified 'cap 4' structure on the spliced leader is required for in-vivo *trans*-splicing, thus providing a 5' cap structure on mature transcripts as is seen in all eukaryotic cellular mRNA. The precise function of the cap structure in *Leishmania* is not known, although antisense oligonucleotides complementary to the SL inhibit *in-vitro* translation in *L. amazonensis,* by invading a hairpin in the SL RNA (Compagno et al, 1999). Involvement of the SL in protein binding, mRNA export and stability, and translation has also been proposed.

Recently, *trans*-splicing has been suggested to play an important role in the control of gene expression (Lamontagne and Papadopoulou, 1999). SL RNA is shown to be developmentally regulated in *L. donovani.* The existence of a third

stem-loop structure (a potential substrate for RNA- binding proteins) is therefore predicted. The amastigote specific SL transcript is polyadenylated, comprises 12-16% of the SL RNA pool under acidic growth conditions, and is 2.5- to 3.0-fold more stable than poly(A-) SL transcripts. The poly(A+) SL RNA is derived from only one of the three SL-RNA gene classes in *L. donovani.* Polyadenylation and increased stability may serve to control levels of the SL transcript during parasite development. However, evidence from the *L. donovani* ATPase locus indicates that *trans*-spliced, developmentally regulated messages are detectable by RT-PCR in different *Leishmania* stages (Stiles et al, 1999a). If SL activity mediates differential gene regulation, it therefore probably involves the efficiency of *trans*-splicing rather than the presence/absence of splicing.

In *T. brucei,* control of *trans*-splicing resides in 5' UTR sequences and is mediated by polypyrimidine (CT) tracts upstream of an AG-dinucleotide splice acceptor site (Matthews et al, 1994; Schurch et al, 1994; Vassella et al, 1994). A study using transfected chimeric molecules containing *L. chagasi* gp63 5' sequences linked to a luciferase gene demonstrated that maximal gene activity requires the presence of a CT-rich region between 31 and 69 nucleotides upstream of the splice site (Ramamoorthy et al, 1996). Sequential replacement of three adenosine residues in this region, which may function in the formation of a branched intermediate structure in the *trans*-splicing reaction, also reduced luciferase expression in an additive manner. A 30-nucleotide, pyrimidine-rich region immediately upstream of the splice acceptor site did not, by itself, maintain luciferase expression, although it is not clear whether a larger CT tract is required or that the CT tract is not involved.

In a variety of transfection studies, *Leishmania* reporter-gene expression has also been found to be dependent on the presence of flanking sequences containing polypyrimidine tracts and an AG dinucleotide. Accurate splicing at the wild-type splice-acceptor site, the initial AG downstream of the CT tract in gp63-luciferase experiments, occurred despite the presence of nearby alternative splice sites (Ramamoorthy et al,1996). Examination of the sequences at *L. major* and *L. donovani trans*-splicing sites, as reported in the literature or stored in sequence databases, confirms a strong preference for SL addition at the first AG dinucleotide downstream of a polypyrimidine tract.

The CT tract can be immediately upstream of the AG splice-acceptor site (as in the A2 antigen, gp63, and ATPase *ldhlB* genes) or lie more distantly (e.g. 107 nucleotides away in ATPase *ldhlA* and 97 away in *lmdrl* (Hendrickson et al, 1993; Roberts et al, 1993; Charest et al, 1996; Stiles et al, 1999a). Several exceptions to this observation occur when the initial AG dinucleotides are close to the polypyrimidine tract, such as for the D2 glucose transporter (second AG), *metal* (second or third AG), and *lmdrl* (third AG) transcripts. These use a downstream AG acceptor site, perhaps indicating that there has to be a certain spacing between the polypyrimidine tract and the branch A residues for the formation of the Y intermediate and the *trans*-splicing site (Langford et al, 1992; Hendrickson et al, 1993; Nourbakhsh et al, 1996). However, several instances of non-consensus SL acceptor sites have been reported (Myler et al, 1993; Taylor et al, 1994).

5' and 3' Untranslated Regions

The 5' and 3' UTR of *Leishmania* transcripts, exclusive of ribosomal and histone messages, are longer than those characteristic of other eukaryotes. Analysis

of the UTR reported for *L. donovani* and *L. major* transcripts (52 for 5' and 50 for 3'), excluding the spliced leader and poly(A) tail, demonstrated that the UTR in ribosomal and histone messages are short, with mean lengths of 39 nt for 5'UTR (average for nine genes) and 233 nt for 3'UTR (average for 15 reported genes). In contrast, the mean UTR lengths of the other *L. donovani* and L. *major* messages are 197nt for 43 reported 5' UTR-and 1021nt for the 35 reported 3'UTR. The reported range for 5' UTR is 688nt for ATPase *ldhlA* and the shortest non-ribosomal 5'UTR are 11nt for *mag* and 13nt for a thiol-specific antioxidant protein (Ghedin et al, 1998; McCoy et al, 1998; Webb et al, 1998; Stiles et al, 1999a).

The longest 3' UTR-2973nt for *FesodB* message, 2185-2214nt for ATPase *ldhlB* transcripts, and 2020 and 2035 for A2 antigen transcripts-are seen in genes expressed exclusively or predominantly in amastigotes, indicating an association with regulation (Charest and Matlashewski, 1994; Charest et al, 1996; Paramchuk et al, 1997; Stiles et al, 1999a). However, the length of 5' and 3' UTR might also reflect the genomic organization and spacing of genes within *Leishmania,* with the coupling of 5' and 3' splicing reactions determining UTR length (LeBowitz et al, 1993).

Gene expression regulation

The exact mechanisms of regulation of message abundance in *Leishmania* are not clear, several salient points have emerged. Regulation of transcript abundance appears to be post-transcriptional and is dependent on 3' UTR and intergenic sequences. It is not clear if these function via their effects on mRNA processing or by binding of stability/degradation factors. In the absence of the characteristic eukaryotic modulation of primary transcription via individual promoter

activity, post-transcriptional mechanisms are the major determinants in the regulation of mRNA abundance in *Leishmania.* The change in steady-state levels of regulated transcripts during *Leishmania* development is not accompanied by increased transcription initiation. The rate of nascent transcript production by isolated nuclei in nuclear run-on assays is unchanged in different developmental forms, in spite of dramatic differences in mRNA abundance for the *L. donovani* A2 antigen and spliced leader RNA, *L. chagasi* gp63 and gp46 and *L donovani* and *L. mexicana* glucose transporters, as well as *L. infantum hsp70* and *L. amazonensis hsp83* genes under heat-shock conditions (Argaman et al, 1994; Ramamoorthy et al, 1995; Charest et al, 1996; Beetham et al, 1997; Quijada et al, 1997; Burchmore and Landfear, 1998; Lamontagne and Papadopoulou, 1999). Post-transcriptional regulation of steady-state transcript levels ultimately involves a process of message stability degradation and, by inference, the existence of protein factors and their recognition sequences within the transcripts. The interplay between these factors and their binding sites during the *Leishmania* life-cycle could be regulated at the level of *trans-* splicing and polyadenylation of primary transcripts into mRNA, or by differential expression of the protein factors affecting the degradation/stability of precursor or mature transcripts.

Differentially expressed genes in *Leishmania*

Differentiation of the *Leishmania* into the amastigote form is a prerequisite for its intracellular survival. Several environmental factors including acidic pH, elevated temperature, and the harmful phagolysosomal milieu trigger cytodifferentiation accompanied by the differential expression of a variety of genes (MacFarlane et al, 1990; Glaser et al, 1991; Turco and Descoteux, 1992; Zilberstein and Shapira, 1994;

Garlapati et al, 1999). Such stage-specific gene expression is crucial for adaptation because *Leishmania* differentiates from an extracellular to an intracellular parasite. Hence, such developmentally regulated genes have been the main focus of many studies. The spliced leader RNA gene (Lamontagne and Papadopoulou, 1999), meta 1 gene (Uliana et al, 1999), the system C for proline transport (Mazareb et al, 1999), the glucose transporter gene *LmGT2* (Burchmore and Landfear, 1998), the gene for protein KMP-11 (Berberich et al 1998), the gene for mitogen activated protein (MAP) kinase homologue LMPK (Krobitsch et al, 1998), the heat shock protein Hsp100 (Krobitsch et al, 1998) the A2 gene family (Charest and Matlashewski, 1994), Cysteine proteinase (Souza et al, 1992) and *Lm*cDNA 16 gene family (Flinn and Smith, 1992). One bottleneck for identifying such genes was the non-availability of continuous source of parasites from amastigote stage. Whereas the promastigotes are relatively easy to grow in vitro, in recent years cytodifferentiation into axenic amastigotes was successfully adapted.

Treatment for VL

Chemotherapy offers several advantages in the control of leishmaniasis. Drugs are not affected by parasite heterogeneity, they can be administered orally, and, most importantly, once they are developed, better formulations are relatively easy to produce. Unfortunately, drugs are much less effective in immunocompromised individuals, and drug resistance, is emerging as a major problem.

Conventionally, long parenteral courses of pentavalent antimonial (Sbv) drugs have been used for both visceral and cutaneous leishmaniasis. The second line drugs amphotericin and the less frequently used pentamidine are toxic. The

difficulties of treatment are exacerbated by the spread of resistance to antimony in India (Sundar et al, 2000) and the intractability of the disease to all drugs in patients co-infected with HIV. In most endemic countries the use of some excellent treatments, notably liposomal amphotericin for visceral leishmaniasis, is limited by the cost (Sundar et al, 2001a).

Concerns about treatment failure for VL are exacerbated by geographical variations in antimonial treatment regimens, severity of disease, and sensitivity of *Leishmania* species. In north Bihar in India there is clear evidence of acquired resistance of *L. donovani* for visceral leishmaniasis to antimonial drugs with up to 60% failure rate with treatment. Amphotericin B has been a standby treatment during this developing crisis; the drug can be used in short courses and gives > 90% cure rate (Sundar et al, 2001b). In addition, the aminoglycoside paromomycin, is likely to be approved after necessary trials are completed. However, it is the alkylphosphocholine miltefosine, first developed as an anticancer drug and which can be taken orally, that offers the most hope. In a series of trials this drug achieved a 94% cure rate even among patients with antimony resistant disease (Jha et al, 1999). However, teratogenic potential of this drug requires that it should be used with caution in women of childbearing age. Another potential oral drug sitamaquine, lacked a linear correlation between dose and cure rates and had an unsatisfactory safety and efficacy profile (Davies et al, 2003).

Progress in vaccine development

Of all the parasitic diseases, leishmaniasis is considered the most likely to succumb to vaccination. The parasite has a particularly simple life cycle, resolution of primary cutaneous leishmaniasis usually results in resistance to re-infection, and

studies in experimental models have suggested simple CD4 Th1-type, cell mediated resistance involving activation of macrophage killing mechanisms by T lymphocyte-derived interferon-γ. In experimental models of cutaneous leishmaniasis, in which CD4 Th1 responses are driven towards a polarized Th1 response, protection can indeed be achieved by vaccination, although this rarely results in complete protection from development of lesions. Such vaccines, however, stimulate only poor memory, and protection wanes after a few weeks (Handman, 2001). In primate studies and clinical trials they show immunogenicity but rarely give appreciable protection (Kenney et al, 1999; Belkaid et al, 2002). In the past three to four years our view of the requirements for vaccine induced immunity has changed. A major paradigm shift reflects the role of CD8 T cells. New models of cutaneous leishmaniasis indicate that CD8 cells are vital for primary resistance (Belkaid et al, 2002). It has also been recognized that CD8 cells are required for the maintenance of long term vaccine induced immunity. Although the capacity to induce CD8 cell responses is a feature of DNA vaccines, this has also been shown for some protein-based vaccines (Rhee et al, 2002). Recent advances in combining vaccine delivery systems in so called prime-boost schedules are also being tested in models of cutaneous leishmaniasis. Rapid progress in vaccine development is also hindered when natural challenge is the only means of validation. For visceral leishmaniasis, the situation remains less promising, and, although there are concerns about whether the same vaccine will work for all leishmaniases, human trials of vaccines against visceral leishmaniasis are likely to follow only from successful outcome of those against cutaneous leishmaniasis.

Current issues in vaccine design

The observations regarding the need for both CD4+ and CD8+ T cells for acquired resistance, as well as the requirements for sustained IL-12 production and parasite persistence to maintain immunity, have important implications with regard to vaccination strategies. The concern that non-living, protein-based vaccines will elicit poor CD8+ T-cell responses and be less potent and durable than live vaccines has, to some extent, been substantiated in human trials. Live vaccination, or leishmanization as it is known- which involves the inoculation of virulent organisms in the arm to protect against the development of severe or multiple lesions, particularly on the face- provides virtually complete and life-long protection (Modabber, 1989). By contrast, a safe, non-living vaccine made up of whole-cell killed *Leishmania* inoculated with *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) as an adjuvant failed to confer substantial protection to humans against cutaneous disease (Sharifi et al, 1998; Momeni et al, 1999). The underlying problems with this vaccine are indicated by recent results from the *L. major* mouse model; mice immunized with killed promastigotes or recombinant proteins plus IL-12 as an adjuvant had a high level of protection when challenged 2–4 weeks after vaccination, but they had already lost a substantial degree of protection when challenged after 12 weeks (Gurunathan et al, 1998; Mendez, 2001). Immunity could be maintained by repeated administration of antigen or IL-12 or by antigen and/or IL-12 delivered by plasmid DNA. Immunization using plasmid DNA encoding single or multiple *Leishmania* antigens is a particularly effective approach to generate strong and long-lasting protection against *L. major*, owing to its ability to induce CD4+ and CD8+ T-cell responses, its sustained delivery of antigen and its provision of a strong

Th1-promoting adjuvant in the form of unmethylated CpG dinucleotide motifs. As more natural challenge models have revealed differences in the immune mechanisms that are required to control infection, it will be important to evaluate candidate vaccines using sandfly challenge. In addition to the inoculum size and site of challenge, sandfly-transmitted infections differ from those initiated by a needle in that infected sand flies also inoculate small amounts of saliva. Many studies have reported that the co-injection of parasites with salivary-gland homogenates of vector sand flies produces a substantial increase in lesion size and/or parasite burden that is due, in large part, to an up-regulation of type-2 responses by components in the salivary gland lysate (Gillespie et al, 2000). These results have prompted the use of defined salivary antigens, delivered as either recombinant proteins or as plasmid DNA, to vaccinate mice against a challenge inoculation containing *L. major* plus sandfly saliva (Valenzuela et al, 2002; Morris et al, 2001).

Development of gene transfection protocols in *Leishmania* enabled a number of innovative approaches in attempts to generate vaccines. These include the inactivation of genes encoding enzymes thought to be important for parasite intracellular survival, including the dihydrofolate reductase gene (Titus et al; 1995), the cysteine proteinase genes (Alexander et al, 1998), the HSP100 heat shock protein (Hubel et al, 1997), the trypanothione reductase (Dumas et al, 1997) and Biopterin Transporter (Papadopoulou et al, 2002).

In most pathogens virulence genes are defined as those important for survival and/or pathogenesis of the parasite within the sand fly or mammalian hosts, but not for growth in routine culture media. This definition is not ironclad and exceptions are known. One obvious example arises from the fact that there are

dozens of 'routine' culture media. It also allows for the possibility that virulence genes are not 'absolute': that is, it includes genes whose loss shows a quantitative but not complete loss of virulence. In evolutionary terms, even relatively small effects in 'fitness' or virulence can have large consequences. On the other hand 'essential' genes, defined as ones required for growth *in vitro*. However, 'essential' genes frequently can play roles in virulence beyond just enabling growth *in vitro*. Given the deadly nature of some *Leishmania* infections, and the desire to control parasite infections through chemotherapy or vaccination, both 'virulence' and 'essential' genes have great potential in efforts oriented towards the identification of new chemotherapeutic targets.

Recombinant leishmanial antigens have been used in experimental models with varying success. A recombinant vaccine (Trifusion, a fusion peptide of the leishmanial antigens LMST11, TSA, and LeIF) is being prepared for clinical trials, and the recent observation that Trifusion, given in combination with immunostimulatory CpG oligodeoxynucleotides, stimulated CD4 and CD8 cells and long term immunity is encouraging (Rhee et al, 2002). Genetic immunization also showed excellent promise in many of the DNA vaccination studies, mainly in cutaneous leishmaniasis. The use of attenuated organisms is very attractive because they are the closest mimic to the natural course of infection and may therefore lead to similar immune responses. Recent advances in the ability to manipulate the *Leishmania* genome by knocking out genes essential for the long term survival of the parasite in the human host has the potential to make live-attenuated vaccines much more feasible.

Aims and Objectives

The aim of the study is to identify and characterize differentially expressed genes in *Leishmania* from Indian kala-azar patients using rapid and sensitive methodologies. The studies will contribute towards understanding the mechanism of *Leishmania* pathogenesis and facilitate development of attenuated *Leishmania* vaccine. The specific objectives of the present study have been defined as under.

1. To set up parasite cultures from patient samples

Leishmania have a tendency to spontaneously lose virulence during *in vitro* culture by processes not clearly understood. Therefore, it is planned to use parasites isolated from Indian KA patients instead of standard WHO isolates that have been cultured *in vitro* for the past several years. Since it is difficult to obtain enough parasites from a clinical sample to perform biochemical and molecular analysis, it is necessary to culture the parasites for few passages to obtain parasite material in sufficient quantity. Cultures of Indian isolates of *L. donovani* will be prepared from bone marrow aspirates of VL patients.

2. To generate axenic amastigotes in culture

In vitro transformation of promastigotes into amastigotes will be done in order to obtain a continuous and homogeneous source of axenic amastigotes unlike hamster or Balb/C spleen derived amastigotes.

3. To characterize parasite isolates and select a representative isolate

Several *L. donovani* isolates from KA patients originating from the endemic region will be prepared for AP-PCR analysis. AP-PCR on a number of isolates from one geographic area will theoretically give the same fingerprint. If the AP-PCR pattern obtained from these isolates is different, the parasite isolate, which is predominant in the region will be used for subsequent studies. All *Leishmania*

isolates will be characterized by standard techniques including isoenzyme analysis and species-specific monoclonal antibodies.

4. AP-PCR analysis for genomic fingerprinting of Indian isolates of Leishmania

Leishmania DNA isolated from Indian KA isolates will be subjected to AP-PCR analysis. It is proposed to use several AP-PCR primers that distinguish the subspecies in the *L. donovani* class of parasites.

5. To identify AP-PCR fragments unique to Indian *L donovani*

AP-PCR patterns from Indian samples will be compared with other Old World *Leishmania donovani* isolates such as those from Sudan, Ethiopia as well as India. Those fragments unique to Indian *Leishmania* will be identified and isolated.

6. To clone and sequence polymorphic fragment(s):

Polymorphic DNA bands will be isolated, cloned into a suitable vector and DNA sequence analysis carried out.

7. Northern hybridization analysis

Individual DNA clones isolated from AP-PCR fragments will be used as probes in Northern analysis with total RNA isolated from pro- and axenic amastigotes. Such an analysis will lead to identification of DNA clones, which represent transcribed sequences and code for genes that are differentially expressed between the two forms of the parasite. Genes that are expressed at a higher level in any of the two stages will be selected for further analysis.

8. To clone and characterize full length gene(s)

Complete copy/copies of the gene(s) will be isolated from cosmid library/genomic DNA using the polymorphic fragments as probe(s). Nucleotide

sequence will be determined and subjected to Genbank search to identify homology with known sequences. Molecular characterization of such genes will be undertaken.

9. Bacterial expression of the gene products

Expression from the full-length copy of gene(s) will be attempted in an appropriate expression vector.

Genomic finger printing of Leishmania donovani isolates

Introduction

Leishmania parasites of different species share a range of specific molecular and biological characters. Nevertheless, they are responsible for a great diversity of tropisms. Understanding *Leishmania* genetic diversity is important for epidemiological and taxonomic studies as well as for precise diagnosis. Further, accurate identification of pathogenic agent at the level of subspecies and strain provides a firm basis for all studies of medical relevance (Banuls et al, 2002).

The characterization of *Leishmania* species and strains can be accomplished by clinical, epidemiological and biochemical criteria. A number of biochemical methods have been developed which can provide diagnostic information or insight into molecular diversity. Isoenzyme profiles have been widely used for identifying taxonomic markers (Kreutzer and Christensen, 1980; Andersen et al, 1996). Alternatively, typing of isolates has also been achieved by using monoclonal antibodies (McMahon-Pratt and David, 1981). In recent years, genetic diversity in *L. donovani* has been assessed using an array of molecular markers. Variability among isolates of *L. donovani* was shown using PCR fingerprinting methods that use single non-specific primers or short random primers in PCR (Noyes et al, 1996; Schonian et al, 1996). This method has the advantage of randomly sampling the genome for sequence polymorphisms. PCR analyses of intergenic spacers such as ribosomal internal transcribed spacers (ITS) and gp63 intergenic spacers have been applied to strain differentiation in *L. donovani* (Mauricio et al, 1999; 2001; El Tai et al, 2001). The gp63 intergenic typing was able to identify five genetic clusters within *L. donovani* (Mauricio et al, 2001). However, restriction polymorphisms were not

detectable either in ITS1 that separates small subunit rRNA and 5.8S rRNA genes or in ITS2 that lies between 5.8S rRNA and large subunit rRNA genes (El Tai et al, 2000). Single strand conformation polymorphism (SSCP) analysis with PCR amplified ITS1 followed by sequencing has shown different SSCP patterns and 11 different sequence types in 64 of the isolates derived from an endemic focus of Sudan (El Tai et al, 2000). Further, strong correlation between PCR-SSCP patterns and geographic origin of the isolates was found on analysis of ITS-2 (El Tai et al, 2001). Similarly, parasite typing with species or complex specific PCR primers could distinguish parasite isolates in Mexico and in Brazil (Amalia and Gustavo, 2002; Ishikawa et al, 2002).

Association between the specific multilocus genotypes and the diverse clinical manifestations of the disease, namely KA and PKDL could not be found in parasites isolated in Africa (El Tai et al, 2001; Lewin et al, 2002). However, no such studies have been reported with isolates of *L. donovani* from Indian population. Despite overall similarities there are many important differences between Indian and African forms of KA and PKDL warranting investigations in this direction.

Leishmania have successfully adapted to varied and heterogeneous environments during its life cycle. These trypanosomatid parasites are submitted to successively rapid changes in

a) temperature- from ambient temperature in sand fly vector to 37°C in the mammalian host,

b) pH- from neutral to very acidic in the sandfly midgut and the macrophage phagolysosome,

c) nutrient and oxygen concentration and

d) immune attack by complement, antibodies and T cells.

In this context efficient mechanisms are essential for the long-term and short-term survival of the parasite. This adaptation is triggered by changes in gene expression in several species (Handman, 1999). Therefore, the products of genes that regulate differentiation into the intracellular amastigote form have great potential as targets to block the infection process. A number of studies have been conducted searching for changes in gene expression amongst *Leishmania* stages, using methods such as differential or subtractive hybridization, arbitrarily primed polymerase chain reaction (AP-PCR), differential display, cDNA microarrays derived from spliced leader libraries and proteomics (Coulson and Smith, 1990; Charest and Matlashewski, 1994; Pogue et al, 1995b; Heard et al, 1996; Liu et al, 2000; Wu et al, 2000; Bellatin et al, 2002; Almeida et al, 2002; El Fakhry et al, 2002). Comparison of the changes in gene expression among different growth stages and isolation of the developmentally regulated genes would help to elucidate the mechanisms of gene regulation so as to provide the information needed to disrupt the progression of the disease. Moreover, these genes may only be expressed at the time of differentiation and in low abundance. Therefore, unbiased methods such as AP-PCR that randomly sample diverse portions of the parasite genome increase the probability of identifying differentially expressed genes.

In the present study, AP-PCR technique was exploited to map genetic differences within the Indian isolates of *L donovani* and to identify DNA polymorphisms that distinguish geographic isolates. Further, we utilized the polymorphic fragments obtained there of to identify, isolate and characterize differentially expressed gene sequences.

Materials

Agarose, Trizol, *Taq* polymerase, dNTPs and $MgCl_2$ were from Invitrogen, USA. Bovine Serum Albumin, Glycine, Glutamine, HEPES, Penicillin, Streptomycin, Gentamycin sulphate, Tris, M-199, RPMI-1640 and other fine chemicals were from Sigma Chemicals, USA. Glycerol, Disodium hydrogen phosphate, Isopropanol, sodium dihydrogen phosphate, Potassium acetate, SDS, Sodium acetate, Sodium chloride, Sodium hydroxide, Sodium bicarbonate were purchased from SRL, India. Charged nylon membrane was from Amersham Biotech, USA. Radioactive ^{32}PdATP was purchased from BRIT, Hyderabad. Cell culture plasticware were obtained from Corning, USA. Media components for bacterial cultures were purchased from Hi-Media Laboratories, India. Goat anti-rabbit IgG-FITC conjugate was obtained from National Institute of Immunology, New Delhi.

Parasites

The reference stocks of *Leishmania* used in this study were; *L. donovani* 1S from Sudan (WHO designation, MHOM/SD/00/1S-C12D), *L. donovani* WR657 from India (MHOM/IN/80/DD8), *L. donovani* WR684 from Ethiopia (MHOM/ET/67/82), *L. donovani* AG83 from India (MHOM/IN/83/AG83) *L. donovani infantum* from Spain (MCAN/SP/00/XXX) *L. major* LV 39 (MRHO/SU/59/P/LV39) from Soviet Union and *L. tropica* WR 664 (MHOM/SU/74/K27) from Soviet Union. *L. donovani* AG83 was obtained from Indian Institute of Chemical Biology, Kolkata and all other cultures were kind gifts from Dr Hira L Nakhasi, CBER, FDA, USA.

Experimental methods

Promastigotes from clinical samples

Bone marrow samples were collected from KA patients originating from Bihar, reporting to Safdarjung Hospital, New Delhi. Prior consent from patients was obtained for collecting the bone marrow samples following the guidelines by the Institutional Ethical committee. The patients presented with clinical symptoms of KA such as fever, hepatosplenomegaly, anaemia and leucopenia. The patients were evaluated clinically and confirmed by demonstration of amastigotes in bone marrow aspirates. The bone marrow samples were collected in medium M199 pH 7.4, and 25mM HEPES supplemented with 10% heat inactivated fetal calf serum with 100µg/ml streptomycin and 100U/ml Penicillin and incubated at 26°C. The promastigotes thus obtained were propagated and used to isolate DNA.

In vitro transformation into amastigote stage

Cytodifferentiation into axenic amastigotes was accomplished by inducing promastigotes to gradually adapt to altered growth conditions (Joshi et al, 1993). Initially the promastigotes were grown at 26°C in RPMI-1640 medium with 200mM glutamine supplemented with 20% fetal calf serum and buffered with 20mM MES (4-morpholino)-ethanesulfonic acid at pH5.5. After several serial passages at 26°C the cells were adapted to grow at higher temperature by gradually increasing the temperature and passaging several times. Under these conditions the parasites transformed into and grew as intermediate forms. Subsequent to several serial transfers in the pH5.5 medium the intermediate form parasites were adapted to growth in the same medium but at 37°C in an atmosphere containing 5% CO_2

environment. Once established as amastigotes these cells could be propagated continuously or cycled between the two stages.

Isoenzyme analysis

Preparation of extracts

Cellulose acetate electrophoresis is a WHO recommended method for isoenzyme characterization of leishmanial strains (Kreutzer and Christensen, 1980). The extracts for electrophoresis were prepared according to the method of Evans (1989). Briefly promastigotes of each isolate were inoculated into 100ml M199 medium with 10% fetal calf serum and allowed to grow for 72hours at 24°C. The promastigote were then washed thrice in Hanks balanced salt solution and centrifuged at 2000 Xg for 20min at 4°C. The supernatant was discarded and the pellet resuspended in an equal volume of stabilizer solution (200mM dithiothreitol and 200mM ε-amino caproic acid in 200mM EDTA, diluted 1:100 prior to use). The material was then freeze-thawed in liquid nitrogen thrice and examined under microscope to confirm complete cell lysis. It was centrifuged at 27,600 Xg for 30min at 4°C. The supernatant was stored as 10µl aliquots.

Enzyme activities

Activities of five soluble enzymes including 6- Phosphogluconic dehydrogenase, nucleoside hydrolase, Glucose 6 phosphate dehydrogenase, malate dehydrogenase and malate esterase were analyzed. An agar underlay was prepared by dissolving the specific components of the individual strain in 10 ml of reaction buffer and combined with 10ml of 1.2% agar at 50°C. The solution was then quickly poured on to prelabelled square petridishes and stored at 4°C.

Electrophoresis

A 10µl aliquot of enzyme extract was added to the well of a zip zone plate (Helena Laboratories, Texas). Labelled and presoaked for 20min Titan III cellulose acetate (CA) membranes were then blotted and placed on the base aligning plate with matrix side being uppermost. Samples were applied in triplicate and the membranes were then placed in the zip zone chamber, CA side downwards. To prepare the gel apparatus, 50ml of buffer was placed in each outer electrode compartment and filter papers aligned longitudinally to ensure electrical contact. Crushed ice was placed in the central compartment to prevent the plate from drying out and to preserve heat-labile enzymes. Following electrophoresis for the specified voltage and time, the membranes were blotted and plated gently lowered onto agar/filter paper, the matrix side being in contact with the agar/filter paper. The plates were incubated at 37°C for 5-10min. Once the bands began to appear, the plates were removed, blotted and fixed in 5 percent acetic acid for 5-10min.

Immunofluorescence assay

The immunofluorescence assay was performed as described by Jaffe and Mc Mahon Pratt (1983) with slight modifications. Monoclonal antibodies G2D10, raised against a promastigote membrane antigen of *Leishmania gerbelli*, is known to recognize all species of *Leishmania* (Kenner et al, 1999) and D2, T-1 and T-10 specific to *L. donovani*, *L. major* and *L. tropica* respectively, were employed. The monoclonals were obtained from WHO.

- Mid log phase *Leishmania* cells were pelleted at 4000 rpm for 10 minutes at 4°C (at an approximate cell density of 8-10X10^6 cells/ml).
- The cells were washed with chilled 1X PBS 3 times at 4000 rpm for 10 minutes

at 4°C.

- The pellet was resuspended in 0.5ml of 4% p-formaldehyde and incubated on ice for 15-20 minutes to allow for membrane permeabilization of parasite.
- Approximately 20µl of cell suspension was spotted on poly L-lysine coated slides and incubated for 10-20 minutes in humid chamber. The glass slide was washed with chilled 1XPBS to remove unbound parasites.
- The adhered cells were fixed by addition of 100% methanol and the slide was incubated on ice for 5 minutes.
- Fixed cells were washed with ice cold 1x PBS three times with 5 minutes incubation and continuous shaking on a rocking platform.
- Cells were blocked with 20µl of 5% BSA in PBS at 37° C for 1 hr. Cells were washed with ice cold 1x PBS for three times with 5 minutes incubation.
- 100µl of primary antibody (1:200 dilution in 1%BSA-PBS) was added and glass slides incubated for 1 hr at room temperature.
- 100µl of secondary antibody (Goat anti-rabbit IgG FITC conjugate1:200 dilution in 1%BSA-PBS) was added after the unbound primary antibody was removed by washing steps as done before and incubated for 1hr in dark.
- Slides were washed with ice cold 1x PBS three times with 3-5 minutes incubation.
- A drop of 10% glycerol in PBS was added on the slide and viewed under confocal microscope (Zeiss).

Isolation of nucleic acids

Genomic DNA and total RNA were prepared from minimally passaged parasites from Kala-azar isolates. Mid-log phase parasites (~$1x10^9$ cells) were washed twice with cold PBS and used for DNA isolation by Wizard Genomic DNA kit (Promega) as per manufacturer's instructions. Total RNA from promastigotes, axenic amastigotes was prepared from parasites at identical time points using Trizol reagent following the protocol supplied. Briefly, the cells (1-$1.5X10^9$) were lysed in Trizol, at room temperature and to the homogenized samples 0.2 volumes of chloroform was added followed by centrifugation at 12,000 xg for 15 min. The aqueous phase was collected and 0.5 volumes of Isopropyl alcohol was added to precipitate total RNA. The total RNA was pelleted by centrifugation and washed in 75% ethanol (in autoclaved DEPC treated water).

AP-PCR reactions

AP-PCR reactions were carried out essentially as described by Pogue et al (1995a). The 50µl reaction mixture contained 200µM each of dNTP, 2mM $MgCl_2$, 100ng of oligonucleotide, 100 ng of template DNA (unless stated otherwise) and 2.5 units of Taq DNA polymerase overlaid with mineral oil. A panel of oligos was initially examined for fingerprinting studies and three selected for further investigations. The oligos were chosen based on the consistent amplification profiles that these oligos yielded.

Oligo	Sequence
AP-6	5' GTG ATC GCA G 3'
AP-9	5' GTT GCG ATC C 3'
AP-16A	5' CAG AGA CCA C 3'

The PCR conditions were as follows; denaturation at 94°C for 1min, annealing at 36°C for 1min and extension at 72°C for 2min for a total of 45 cycles followed by a final 15min extension at 72°C. Amplification products from AP-PCR reactions were analyzed by electrophoresis in 1% agarose gels followed by staining with ethidium bromide to visualize the DNA fragments. The pattern of AP-PCR amplification fragments is sensitive to the amount of genomic DNA template and the amount of AP oligo (Welsh and McClelland, 1990). The amount of template and AP oligo were optimized for obtaining reproducible amplification from all *Leishmania* species tested. In case of oligo AP-9, amplification reactions were also carried out with 50ng and 150ng DNA concentration to obtain additional unique DNA fragments. Polymorphic DNA bands were sliced from the agarose gels and purified.

Extraction of DNA from agarose gel

The PCR reaction mix was fractionated by agarose gel electrophoresis. Polymorphic DNA fragments were excised from agarose gel and were eluted from the gel slice using gel extraction kit (Qiagen) following the protocol supplied. Gel pieces containing the DNA fragment of interest were chopped into fine bits and transferred to a 1.5ml microfuge tube. Three gel volumes of solubilization buffer PQ was added and incubated at 55°C for 10minutes. Tubes were vortexed intermittently for complete solubilization of gel particles. Samples were loaded on to the Qiaquick gel extraction spin column and centrifuged at 10,000 rpm for 2 minutes. The column was washed with 750µl of wash buffer PE followed by a spin at 10,000 rpm for 1minute. To remove residual PE, the column was spun for an additional 1minute. The column was air dried by keeping in 37°C oven for 10minutes. The dried column

was kept in a fresh microfuge tube and the DNA was eluted in 50µl autoclaved Milli-Q H_20 by spinning the column at 10,000 rpm for 5minutes.

Northern hybridization

Northern hybridization was carried out essentially as described by Pogue et al (1995a) as per the following details.

Sample preparation

The total RNA prepared from either promastigotes or axenic amastigotes stored in 70% ethanol were centrifuged at 3000 rpm for 60 secs to completely remove traces of ethanol. After briefly drying under vacuum the samples were rehydrated by addition of 30-40µl DEPC-RNAsin water and incubated in a dry bath at 65°C for 15minutes and immediately shifted to ice bath.

10X MOPS Electrophoresis buffer

10X MOPS	:	50ml
35% Formaldehyde	:	50ml

Made up to 500ml with DEPC treated water

Sample Mixture

Total RNA	:	15µg (1-3µl)
10XMOPS	:	2.0µl
Formamide	:	20µl
Formaldehyde	:	7µl

The mixture was incubated at 65°C in dry bath for 15minutes and stored on ice till loading after a brief spin. To the denatured RNA samples, 2µl of 10X RNA Gel loading buffer (50% glycerol, 10mM EDTA, 0.25% w/v Bromophenol blue 0.25% w/v

Xylene cyanol) and 1μl of ethidium bromide solution (10μg/ml) were added before loading on formaldehyde agarose gel. RNA marker was run along with the samples (New England Biolabs).

Electrophoresis on formaldehyde gels

Gel boats and electrophoresis tank were initially treated with 3% solution of H_2O_2 for 10min and rinsed thoroughly with DEPC treated water to remove contaminating RNases. 0.6 g of agarose was dissolved in 43ml of DEPC treated water and boiled in microwave oven. After letting it cool to 55°C, 6ml of 10X MOPS buffer and 10.8ml of formaldehyde were added. The gel was cast and allowed to polymerize for 30 minutes. Once polymerized, the gel was electrophoresed at 80V for 5 minutes before loading the RNA samples. The denatured RNA samples were electrophoresed for 3 hours at 80V. After the electrophoresis, photograph was taken and the position of the markers noted.

Northern blot

The formaldehyde gel was rinsed briefly with double distilled water followed by soaking in 5 gel volumes of alkaline transfer buffer (0.01N NaOH/3M NaCl) for 20 minutes. Nylon$^+$ membrane of appropriate size was floated in DEPC water for soaking and then in transfer buffer for 5 minutes. The gel containing fractionated RNA was placed on the transfer set up. Nylon membrane was placed on top of the gel and the RNA was allowed to blot by capillary transfer using alkaline transfer buffer. After O/N transfer, the membrane was marked for origin and wells. The side of membrane carrying RNA was irradiated with UV in cross-linker for 5 minutes (1.5J/cm^2 for damp membranes).

Probe Preparation

Random priming reaction:

50ng of the template DNA in nuclease free water was denatured in dry bath for 10 minutes at 95°C and quickly placed on ice. The tube was centrifuged briefly in cold and the reaction was set up as follows

Template DNA	-	50ng
10x labeling buffer including random oligomers	-	5µl
dNTP mixture (dCTP, dGTP, dTTP) each	-	2µl
α^{32} P dATP (3000ci/mM 50uci)	-	5µl
Klenow fragment	-	1µl
Nuclease free water, to	-	50µl

The reaction was incubated at 37°C for 6hr and terminated by addition of 5µl of 0.2M EDTA. The unincorporated α-^{32}P d ATP was removed by cleaning the probe with Qiagen Nucleotide removal kit as per instructions supplied.

Hybridization protocol

Formamide pre-hybridization solution

	Final conc.	Stock	10ml
Formamide	50%	100%	5 ml
20X SSC	5X	20X	2 ml
Sodium Phosphate pH 6.5	0.5M	20mM	0.4 ml
Denhardt's solution	5X	50X	1ml
Glycine	1%	10%	1ml
SDS	1%	10%	1ml

Sonicated salmon sperm DNA (Sigma, USA) was denatured and added at a final concentration of 10μg/ml prior to use.

The probe was labeled to a specific activity of $>10^8$ dpm/μg. The membrane carrying immobilized DNA/RNA was soaked in 5XSSC. The membrane was placed DNA/RNA side up in hybridization bottle and approximately 1ml of formamide pre-hybridization solution was added per $10cm^2$ of membrane. The pre-hybridization was carried out in hybridization oven by incubating at 42°C for 3hr. The radiolabeled probe ($1x10^7$cpm) was denatured by heating at 100°C for 10 minutes and quickly transferred to ice. The denatured probe was added and the hybridization reaction was allowed to continue for 16 hours. The hybridization solution was collected into 15 ml tube and the membrane was washed by the following steps.

Washing protocol

The membrane was washed with 5XSSC 2 times at room temperature for 30 seconds followed by washing with 2XSSC 2 times at 42°C for 15min each. Finally the membrane was washed in 0.2XSSC one time 65°C for 30 minutes.

Autoradiography

After washings, membrane was damped in 2XSSC and exposed to X-ray film and stored at -70°C. After 24-72 hours exposure, X-ray film was developed. Blots were visualized by autoradiography using Biomax films (Kodak). The intensity of the hybridized bands was quantified from autoradiograms using AlphaEase software (Alphaimager). Normalization of the RNA added in each lane was done by the ribosomal RNA quantity.

Nucleotide sequence analysis

The polymorphic AP-PCR DNA fragments that yielded differentially transcribed RNA species between pro- and amastigote parasites on Northern hybridization were cloned into pCRII-TOPO vector system (Invitrogen) and sequenced on automated sequencer (Perkin Elmer).

Results

Parasite isolation and characterization by IFA

Leishmania parasites were isolated from bone marrow aspirates of Indian patients of Kala-azar (n=19) and propagated as promastigotes for 3-5 passages *in vitro* before DNA isolation. Monoclonal antibodies can be used to easily and rapidly

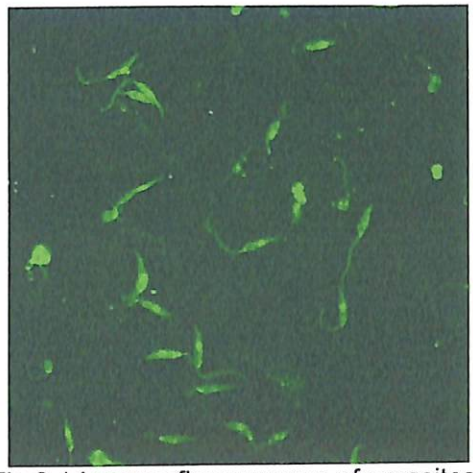


Fig 3.1 Immunofluorescence of parasites with G2D10 monoclonal antibodies

distinguish the species of *Leishmania*. The G2D10 monoclonal antibodies used are known to give positive reaction with members of *Leishmania* genus where as the D2 antibodies are specific to *L. donovani*. All the isolates and the control samples from *L. major* and *L. tropica* displayed the typical fluorescence with G2D10. Immunofluorescence pattern with one of the *Leishmania* isolates is shown (Fig 3.1).

For the purpose of typing the isolates, the IFA analysis was done with the isolates and standard reference strains of *L. donovani*, *L. major, L. tropica*. All the isolates tested exhibited fluorescence pattern characteristic of *L. donovani* species with D2 antibodies (Fig 3.2A), similar to the pattern obtained with *Ld*AG83 and *Ld*DD8. At the same time, *L. tropica* and *L. major* reference strains did not show any fluorescence with D2 antibodies (Fig 3.2B,C). Parasites of *L. major* and *L. tropica* displayed characteristic fluorescence with T-1 and T-10 antibodies while the clinical isolates did not react (data not shown). Therefore, the clinical isolates were identified as belonging to *L. donovani.*

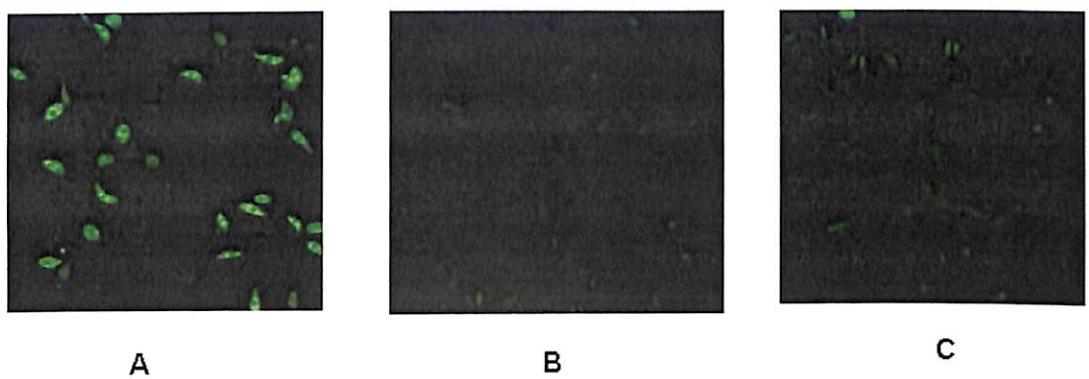
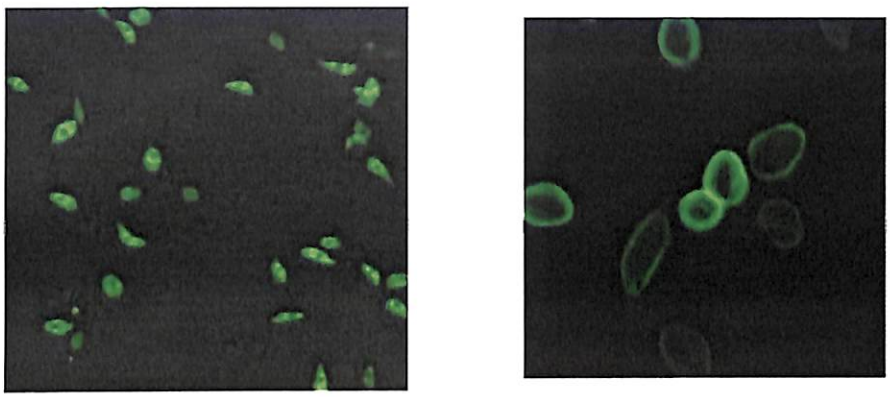


Fig 3.2 Immunofluorescence analysis of *Leishmania* parasites (A) *Leishmania* isolate (B) *L. tropica* and (C) *L. major* with D2 monoclonal antibodies.

Cytodifferentiation

The promastigotes were differentiated into amastigote stage *in vitro* for the purpose of obtaining unlimited amounts of the parasites at both the life stages without any host cell contamination as seen commonly in the case of animal spleen derived amastigotes. Both the parasite stages displayed characteristic fluorescence with D2 monoclonal antibodies that are specific for *L. donovani* (Fig 3.3).



A B

Fig 3.3 Immunofluorescence analysis of the *Leishmania* parasites in (A) promastigote stage and (B) amastigote stage with D2 monoclonal antibodies.

Isoenzyme analysis

A panel of five enzymes was analyzed for the purpose of identifying the species of the parasite isolates. Enzyme analyses of the three WHO recommended strains representing *L. donovani* (DD8), *L. donovani* (AG83), *L. tropica* (K27) and *L. major* (LV39) were also included in the study. Reliable, reproducible and distinctive profiles were obtained for 5 soluble enzymes. Fig 3.4A-E represents the isoenzyme profiles of 6 Phosphogluconic dehydrogenase, Glucose 6 phosphate dehydrogenase, malate dehydrogenase, malate esterase and nucleoside hydrolase. The isoenzyme profiles of the three leishmanial species could be differentiated by the distinct electrophoretic mobilities of the five soluble enzymes. Since all the clinical isolates had similar isoenzyme profiles as *Ld*DD8 and *Ld*AG83, they were classified as belonging to *L. donovani*.

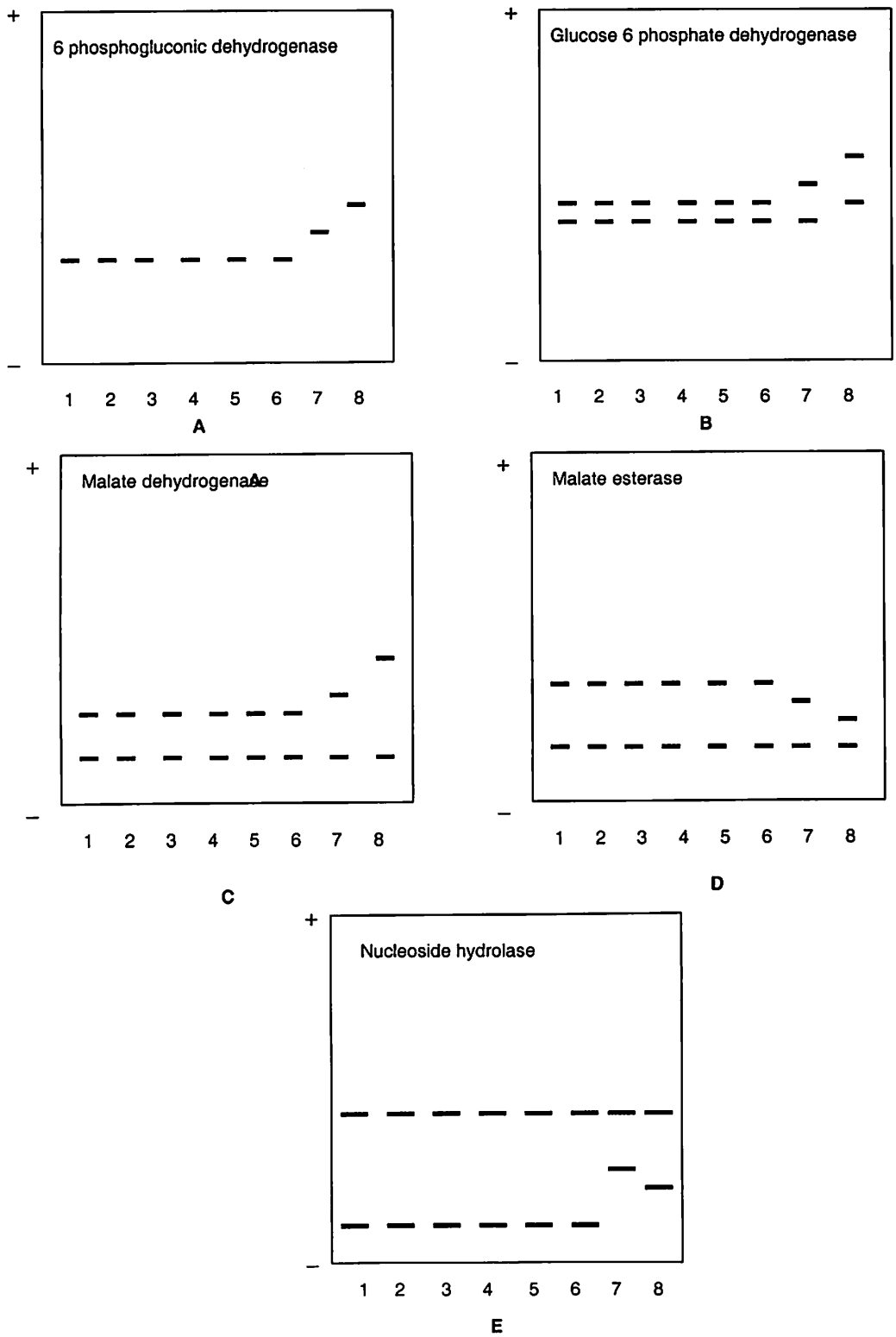


Fig 3.4. Diagrammatic representation of isoenzyme patterns of 5 enzymes prepared from *Leishmania* promastigotes. 1-4 are *Leishmania* clinical isolates, *Ld*K1-*Ld*K4; 5,*Ld*AG83, 6, *Ld*DD8; 7, *L. tropica*; 8, *L. major*. (-) and (+) denote cathode and anode respectively.

Finger printing of L. donovani isolates by AP-PCR

A subset of AP-PCR primers that were shown to yield consistent amplification products (Pogue et al, 1995a) was employed to obtain differential amplification patterns from genomic DNA of *L. donovani* isolates prepared freshly from bone marrow samples of Indian Kala-azar patients. The comparison of genomic fingerprints of 19 isolates of *L. donovani*, all from patients originating from Bihar, an area endemic for Kala-azar in India, was carried out. A large majority of isolates (17/19) displayed identical amplification profile while two isolates showed a divergent pattern with oligo AP-9 (Fig 3.5). In subsequent studies, the isolate *Ld*K1, representing the predominant pattern of amplification was taken.

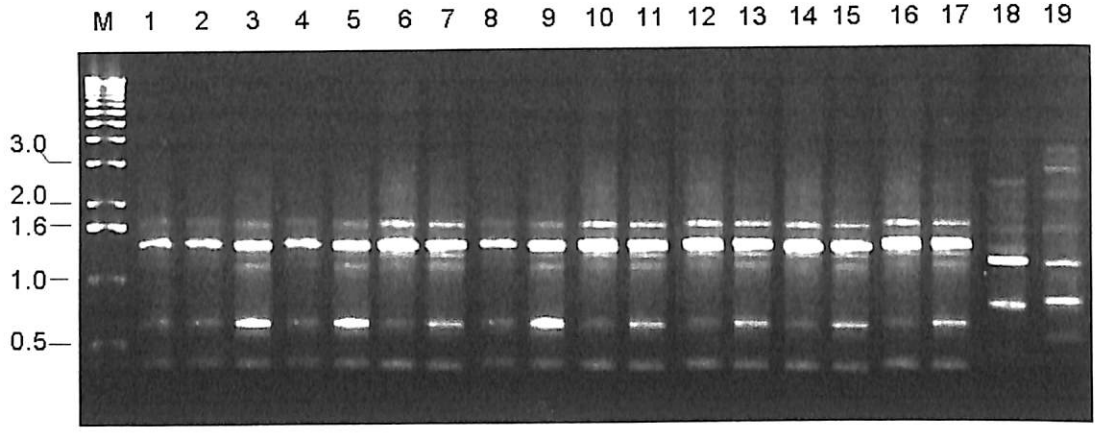


Fig 3.5 Fingerprinting of Indian isolates of *L. donovani* by AP-PCR using oligo AP-9. Genomic DNA (100ng) from parasites isolated from bone marrow samples of Kala-azar patients (K1-K19) was subjected to AP-PCR analysis. Lanes 1-19 show pattern with isolates LdK1-LdK19, M 1 kb ladder.

Comparative profile of L. donovani from distinct geographic regions

The amplification profiles from the Indian isolates were compared to *L. donovani* clonal isolates from distinct geographic locations including Sudan (*Ld*1S), Ethiopia (*Ld* WR684) and Spain (*Ld infantum*). The amplification patterns were

obtained with oligos AP-6 and AP-16A using one or a few representative isolates. With oligo AP-6 differences between the *Ld*K1 and other isolates could not be detected (Fig 3.6). Amplification with AP-16A showed different patterns between *Ld*K1, *Ld* Ethiopia and *Ld* Sudan while isolates *Ld*K1-K4 were identical to *Ld*AG83 (Fig 3.7).

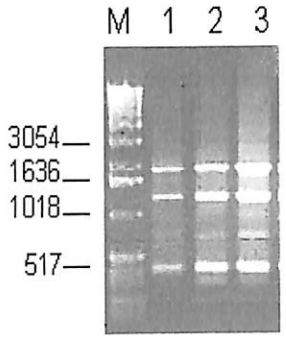


Fig 3.6 AP-PCR amplification using AP-6 primer with *L. donovani* isolates Lane 1, *Ld* Sudan; 2, *Ld* Ethiopia and 3, *Ld*K-1; M 1kb DNA ladder.

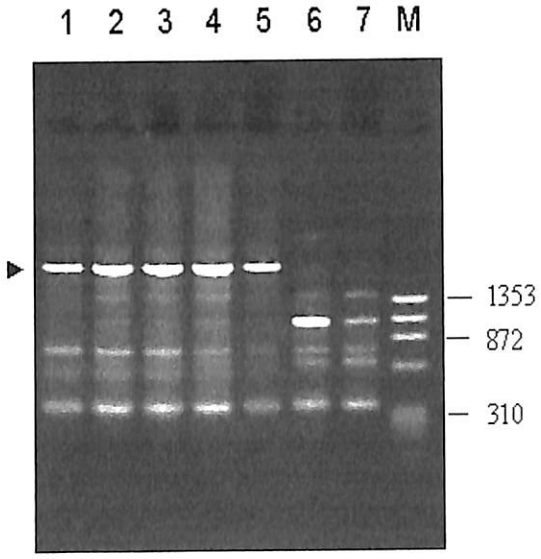


Fig 3.7 AP-PCR amplification using AP-16A primer with *L. donovani*. Lane 1,*Ld*K1; 2, *Ld*K2; 3, *Ld* K3; 4, *Ld* K4; 5, *Ld* AG83; 6, *Ld* Ethiopia; 7, *Ld* Sudan and M, ϕX 174marker

With AP-9 primer, the profile with Indian isolates was quite distinct from *L. donovani* isolates from Sudan, Ethiopia or *Ld infantum* from Spain (Fig 3.8A). Moreover, the amplification pattern with the various geographic isolates of *L. donovani* was similar to that obtained earlier (Pogue et al, 1995a,b). Differences in the AP-PCR profiles were evident between clinical isolates with minimum *in vitro* passaging and *Ld*AG83 strain that has been frequently passaged *in vitro* over last twenty years (Fig 3.8A).

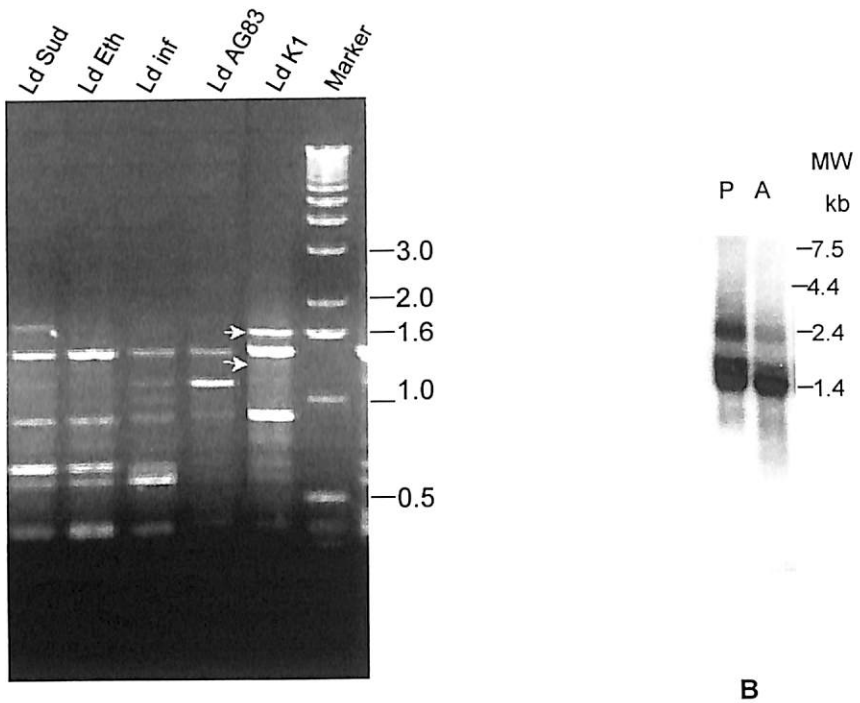


Fig 3.8 AP-PCR profile with AP-9 and identification of polymorphic fragments. Panel A- AP-PCR profile using 100ng genomic DNA as template with oligo AP-9 in *Ld* Sudan, *Ld* Ethiopia, *Ld infantum* from Spain, *Ld* AG83 from India and clinical isolate *Ld*K1. M, 1kb ladder. The arrows point to polymorphic fragments identified in *Ld*K1. Panel B. Identification of transcribed sequences by Northern hybridization Total RNA (15μg) from promastigotes (lane P) or amastigotes (lane A) was resolved in 1% formaldehyde/formamide gels, probed with K1.2 and visualized by autoradiography.

Identification of polymorphic fragments in Indian isolates

It has been shown that AP-PCR is an attractive tool in assigning specific fingerprint to *L. donovani* geographic isolates that are otherwise morphologically and biochemically indistinguishable (Pogue et al, 1995a). Using a typical isolate K1 attempt was made to detect unique DNA sequences in AP-PCR in order to identify transcribed sequences corresponding to differentially expressed genes.

The oligo AP-6 did not yield any polymorphic fragment with clinical isolates even after repeated trials (Fig 3.6). The oligo AP-16A produced a ~2.5kb polymorphic fragment that was also apparent in *Ld*AG83 (Fig 3.7). Several polymorphic fragments were identified in *Ld*K1 in comparison with *Ld*AG83 with oligo AP-9 by altering the template DNA concentration in AP-PCR mixture since the amplification patterns in AP-PCR are sensitive to the primer and template DNA concentration. The oligo AP-9 gave two polymorphic fragments with 100ng template DNA. Comparison of the AP-PCR fingerprints of Indian isolates with geographic isolates revealed unique polymorphic DNA fragments of sizes 1.2 and 1.6 kb (termed K1.2 and K1.6 respectively, corresponding to lower and upper arrows in Fig 3.8A) in Indian isolates following amplification reaction with oligo AP-9 using 100ng of DNA. Varying the template DNA concentration to 50ng, an additional polymorphic fragment of 0.45kb was obtained (termed K0.45, corresponding to arrow in Fig 3.9A). In AP-PCR with 150ng DNA, another polymorphic fragment of size 1.4kb was identified (termed K1.4, corresponding to arrow in Fig 3.10A).

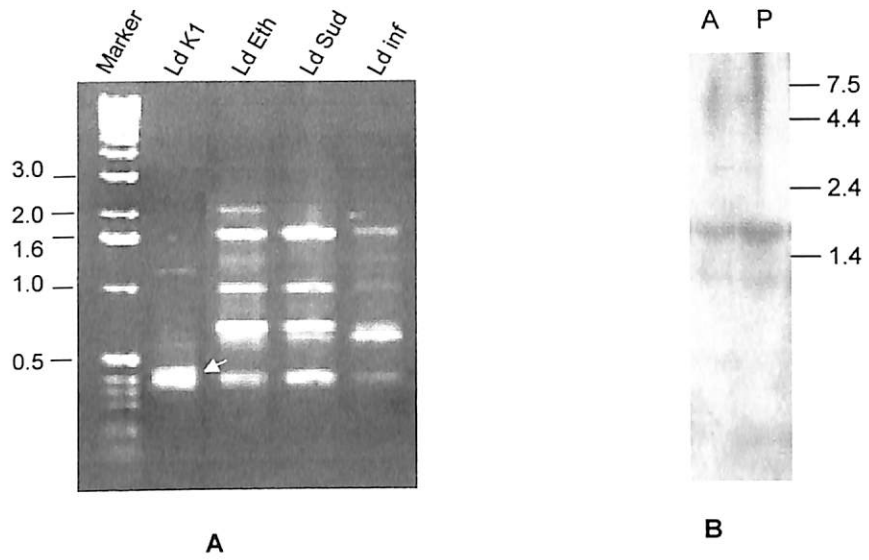


Fig 3.9 Identification of polymorphic amplified DNA fragments followed by Northern analysis. Panel A- AP-PCR profile using oligo AP-9 and 50ng genomic DNA from *Ld*K1, *Ld* Ethiopia, *Ld* Sudan, and *Ld infantum*. The arrow points to polymorphic fragment identified in *Ld*K1. Panel B. Northern hybridization with total RNA isolated from pro and axenic amastigotes using K0.45 as probe.

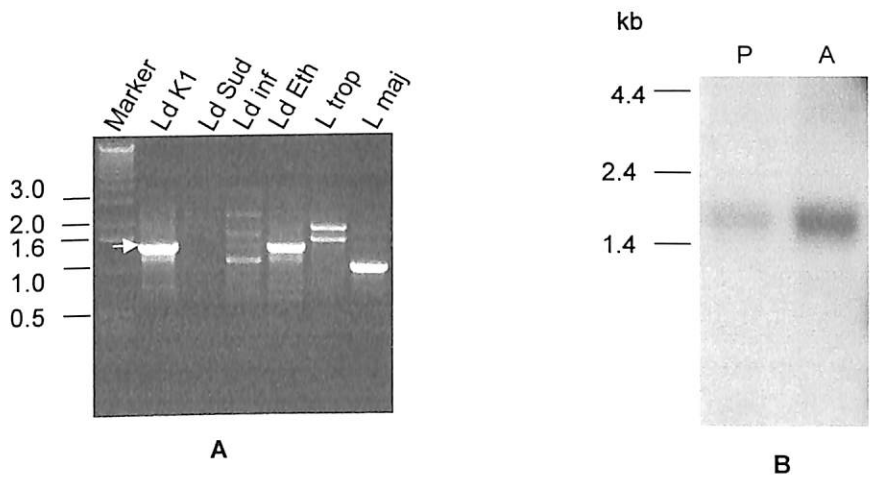


Fig 3.10 Identification of polymorphic amplified DNA fragments followed by Northern analysis. Panel A- AP-PCR profile with oligo AP-9 using 150 ng genomic DNA as template from *Ld*K1, *Ld* Sudan, *Ld infantum* from Spain, *Ld* Ethiopia, *L. tropica* and *L. major*. The arrow points to polymorphic fragment identified in *Ld*K1. Panel B. Northern hybridization with total RNA isolated from pro and amastigotes using K1.4 as probe.

Northern hybridization with cloned unique DNA fragments

To investigate if the polymorphic fragments that were obtained by AP-PCR encompass transcribed sequences, considering the low frequency of introns and high gene density in Trypanasomatid parasites, Northern analysis was performed. The four distinctive DNA fragments obtained from Indian isolates of *L. donovani* were cloned into pCRII-TOPO and used as probes in Northern blot analysis of total RNA derived from promastigotes and axenically grown amastigotes of isolate *Ld*K1. The probe containing the K1.2 fragment hybridized to a ~1.7kb RNA that showed higher expression in promastigote RNA and a major ~2.1kb RNA that showed similar expression in both the stages of the parasite (Fig 3.8B). The K1.6 DNA fragment did not yield any positive hybridization on Northern analysis, indicating that it did not represent a transcribed sequence. The K0.45 probe hybridized with ~1.8kb RNA showing 1.5 fold higher expression in promastigote stage (Lane 'P' Fig 3.9B). The K1.4 probe hybridized with a ~1.7kb RNA that displayed 2.4 fold higher abundance in amastigotes compared to promastigotes (Lane 'A' Fig 3.10B). The quantitation of the Northern data was done by AlphaEase software. The differential expression on Northern was repeated at least three times to confirm its authenticity. Further, it was validated by taking RNA from two separate parasite preparations of *L donovani* to preclude biological variation due to clonal nature of gene expression.

Sequence analysis of the differentially expressed polymorphic fragments

The three fragments that showed differential expression were taken for sequence analysis. On the basis of nucleotide sequence analysis and differential expression, two of the three fragments (K1.2 and K1.4) representing transcribed sequences in Northern analysis were selected for cloning of full length genes.

The nucleotide sequence analysis of K0.45 clone revealed that it spans the 5' end of a partial ORF (data not shown). BLAST analysis did not reveal any significant homologues. The nucleotide sequence of the K1.4 clone revealed that it contained 3' portion of a novel ORF. Cloning, sequencing and characterization of this gene was undertaken (Chapter 4). The K1.2 fragment that hybridized to ~2.1kb and ~1.7kb RNAs in Northern lead to identification of Centrin gene in *Leishmania*. Cloning, sequencing and functional analysis of this gene was carried out (Chapter 5).

Discussion

Parasites belonging to the genus *Leishmania* have wide geographic distribution and cause different clinical manifestations including cutaneous, mucocutaneous and visceral pathology. Different *Leishmania* species may be encountered within the same geographic area. Precise identification of these species is therefore important for clinical and epidemiological reasons. *Leishmania* parasites have similar morphology and cause dissimilar clinical manifestations. Hence, differentiation among species requires biochemical techniques such as isoenzyme electrophoresis, molecular techniques such as DNA probe hybridization and PCR based techniques as well as immunological techniques like immunofluorescence.

The gold standard technique for the characterization of *Leishmania* species is isoenzyme electrophoresis that involves examination of a panel of enzymatic loci (Rioux et al, 1988). This technique is also useful for intraspecies characterization and in *L infantum* has revealed the existence of a wide variety of zymodemes with a heterogeneous geographical distribution throughout Mediterranean basin. In Spain, enzymatic polymorphism studies of strains of the parasite isolated in man, dog and

sandfly have revealed the existence of 20 of the 39 zymodemes that currently constitute the *L. infantum* complex (Toledo et al, 2002).

On the basis of isoenzyme analysis, polymorphism in *L brasiliensis* has been reported previously. Evans et al (1984) detected 19 zymodemes in a sample of 22 strains isolated in Belize. Thomaz-Soccol et al (1993) found 24 zymodemes within 46 strains and Cupolillo et al (1994) distinguished 27 zymodemes within the 5 taxa of the *Viannia* subgenus, of which 9 were related to *L. brasiliensis*. Biochemical characterization of 137 *L. brasiliensis* isolates from South and Central America, and from selected endemic foci in Bolivia, Brazil and Colombia, performed by isoenzymatic electrophoresis using 10 enzymatic systems, showed a high enzymatic polymorphism (44 zymodemes obtained) based on the variation of a small number of enzymes. *L. infantum* from French and Spanish catalan focus showed 8 different zymodemes based on the variation of only the 3 enzymatic systems MDH, G6PD and NP_1 (Pratlong et al, 1995). Isoenzyme analysis revealed a clear separation of Indian and African strains of *L. donovani*. In Indian strains a single zymodemes was predominant where as in Africa 8 different zymodemes were reported (Pratlong et al, 2001). Studies on a limited number of Indian KA and PKDL isolates did not reveal any intraspecies differences and all were found to be identical to *L. donovani* (Chatterjee et al, 1995). Likewise, we obtained similar isoenzyme patterns typical of *L. donovani* in 19 isolates of Kala-azar that we tested in the present study.

Monoclonal antibodies can be used to easily and rapidly distinguish the species of *Leishmania*. Hybridoma technology has been used to produce monoclonal antibodies to *Leishmania*. The monoclonals were produced against purified membranes from *L. donovani* that have been extensively tested on a large

cross-panel of different *Leishmania* species obtained from endemic areas throughout the world (Jaffe et al, 1984). The D2 antibody produced against *L. donovani chagasi* stock used in the present study has been demonstrated to react with the membranes of both Old world and New world *L. donovani* stocks with fluorescence diffused over the entire parasite surface. In our study the D2 antibody gave a positive immunofluorescence with all the clinical isolates tested, same as the *Ld*DD8 and *Ld*AG83 strains, classifying the isolates as *L. donovani.*

Kala-azar in India is becoming increasingly difficult to treat due to several reasons. *L. tropica* was reported to cause the same clinical syndrome in the area (Sacks et al, 1995a). However, in a later study, lipophosphoglycan-specific monoclonal antibodies were used to type several clinical isolates that were predominantly drug resistant into either *L. donovani* or *L. tropica* species by agglutination of promastigotes; all were shown to be *L. donovani.* The isolates were confirmed to be *L. donovani* by isoenzyme analysis, by amplification of kinetoplast DNA, or both, in comparison with multiple reference strains; none were *L. tropica.* Therefore, rising incidence of clinical resistance to treatment is not due to a different species causing kala-azar in north Bihar (Sundar et al, 2001b).

Molecular techniques such as PCR-RFLP and RAPD have been shown to be more powerful to demonstrate the genetic variability within and between different *Leishmania* species including *L. donovani* complex (Guerbouj et al, 2001; Mauricio 2001; Schonian et al, 2000). Additionally, markers based on telomeric repeats and microsatellite markers (Jamjoom et al, 2002) for high throughput analysis have been developed, although limited studies are reported using such markers.

The genomic fingerprinting of Indian isolates of *L. donovani* obtained from Kala-azar patients originating from the endemic area, Bihar, was done by amplifying the genomic DNA with single arbitrary primers. The arbitrary PCR employed low-temperature stringency to allow sampling of diverse portions of *Leishmania* genome without any apparent bias.

The PCR with random oligos is capable of producing unique genetic fingerprints from closely related organisms and this attribute has been previously utilized to differentiate several groups of parasites from trypanosomatid (Waitumbi and Murphy, 1993; Tibayrenc et al, 1993; Pogue et al, 1995a,b; Schonian et al, 1996; Guizani et al, 2002), coccidial (Procunier et al, 1993; Shirley and Bumstead, 1994) to schistosome parasites (Neto et al, 1993). Distinct and reproducible pattern of amplified DNA fragments were obtained with various arbitrary primers examined. AP-PCR profiles with each oligo displayed high degree of similarity within the Indian isolates. Further, the standard reference isolates AG83 and DD8 could be distinguished from other Indian isolates by the additional DNA fragments that were evident in AP-PCR using oligo AP-9. Mapping of genetic diversity within *L. donovani* species is likely to have an impact on a range of problems such as drug resistance, response to vaccines and transmissibility through vector.

In the present study, the Indian isolates produced categorically divergent PCR profiles compared to *L. donovani* from distinct geographic regions. The pattern of amplified fragments obtained was identical in a large majority of Indian isolates. Two of the nineteen isolates exhibited a pattern dissimilar from the rest. Our results demonstrate that unique and reproducible fingerprints can be assigned to distinct geographic isolates within *L. donovani* species by AP-PCR. The remarkable

similarity obtained within the Indian isolates testifies to the relevance of AP-PCR technique in genomic fingerprinting. Studies on Intraspecific polymorphism in 23 Sudanese isolates of *L. donovani* revealed at least three different profiles in the ITS-1 region. However PCR fingerprinting using single primers gave highly similar fragment patterns among the 23 isolates (El Tai et al, 2001). In the present study AP-PCR profiles gave 2 distinct profiles in 19 isolates tested. More rigorous investigations are warranted to uncover if such differences point to phenotypic variations such as drug resistance or predisposition to PKDL.

The deviant pattern with WHO reference strains AG83 as well as DD8 in comparison with the clinical isolates that were minimally passaged indicates the advantage of using newly prepared isolates for the purpose of identifying differentially transcribed sequences based on the unique amplified DNA fragments since the *Leishmania* parasites are known to accumulate sequence polymorphism on longtime *in vitro* culture.

In the context of genetic polymorphism that is shown to exist among Indian isolates, it remains to be seen how this diversity translates into deducing relevant medical properties/phenotypic effects such as infectivity, virulence, relationship between pathology and refractoriness to chemotherapy. At present genetic markers for none of the above attributes exist. The occurrence of polymorphisms within the same geographic area is a significant step towards identifying such markers.

To understand *Leishmania* pathogenesis and to develop means of disease prevention and treatment, identification and functional analysis of genes conferring virulence is required. Sequence data obtained from the *Leishmania* genome project may provide some insight in the identification of unique genes, however, only a

limited number of chromosomes have been sequenced at the present time and this technology may not be readily accessible to many laboratories. Earlier arbitrarily primed polymerase chain reaction approach has been exploited in surveying the *Leishmania* genome for gene sequences of interest and several classes of differentially expressed genes from different isolates of *L. donovani* strain have been identified and some of them revealed unique expression (Pogue et al, 1995a,b). We have utilized that methodology successfully with parasites isolated from Indian Kala-azar patients.

The relative rarity of introns and short intergenic regions of *Leishmania* genome allowed us to ask if the polymorphic fragments could be used to isolate partial coding regions that are differentially transcribed between the parasite life stages. In the present study, four such fragments were identified and used as probes in Northern analysis and three of the four were observed to represent transcribed sequences. Moreover all three fragments hybridized to RNA species that exhibited differential expression in the two stages of the parasite, pro and amastigote. One such polymorphic fragment lead to identification of human homologue of Centrin in *Leishmania* (Chapter 5). Another unique fragment hybridized to RNA species that showed significantly higher expression in amastigote stage of the parasite. The nucleotide sequence analysis has shown that it does not have any homologues in Genbank database indicating that it is a novel ORF. Functional characterization of this gene termed as A-1 is described (Chapter 4). The third polymorphic fragment yielded a RNA band showing higher abundance in promastigote stage. Again, no sequence homologue was found on sequence analysis. Thus the AP-PCR analysis led to identification and cloning of Centrin and A-1 genes of *Leishmania*.

The differential expression of the genes suggest that their intensive study should lead to important insights into the differentiation process and hence virulence. It is of interest to see if these expression patterns are exclusive to viscerotropic species. Finally, successful genetic manipulation of these genes could lead to further evidence of their function and contribution to the *Leishmania* life cycle.

Cloning and characterization of A-1

Introduction

Leishmania parasites alternate between two distinct life stages, the promastigote and the amastigote. The extracellular, motile promastigotes are rapidly engulfed by phagocytic cells at the site of infection, and differentiate into the obligate intracellular amastigote in response to the acidic environment of the macrophage phagolysosome and the elevated temperature.

Understanding of the amastigote stage of *Leishmania* and its interaction with host macrophages is needed to better control leishmaniasis. Many house keeping genes are expressed constitutively while the morphological and biochemical differences between the two life stages should, in large part, result from the stage-specific expression of a discrete number of regulated genes (Bellatin et al, 2002). These amastigote-specific proteins are responsible for intracellular parasite survival and, furthermore, the interactions between such gene products and the host cell components likely determine *Leishmania* pathogenesis (Chang and McGwire, 2002).

Although differentiation from promastigotes to amastigotes is a central biological process required for survival during the life cycle, very little is known about this process. Surprisingly, few stage-specific molecules have been described and the functional role defined in still less number of cases. Amastigote-specific molecules are likely to play central roles in survival in the mammalian host and can therefore be considered virulence factors (Matlashewski, 2001).

A number of molecules from *Leishmania* have been described that can be considered virulence factors such as A2 family (Zhang and Matlashewski, 1997),

cysteine proteinase (Mottram et al, 1996), major surface glycoprotein gp63 (Joshi et al, 1998; Chen et al, 2000), lipophosphoglycan (Spath et al, 2000), secreted acid phosphatases (Ilg et al, 1991), GDP-mannose pyrophosphorylase (Garami and Ilg, 2001). More such virulence molecules need to be identified to fully understand the *Leishmania* pathogenesis. Using a genomic fingerprinting technique we have identified a stage regulated gene we termed as A-1, in *L. donovani.* The present study is aimed at characterizing the A-1 gene that showed abundant expression in amastigote stage.

Materials

Reagents and Chemicals

Acrylamide, Ampicillin, Bovine Serum Albumin, Coomassie brilliant blue R-250, Calcium chloride, Glycine, Glutamine, Glycerol, PMSF, SDS, Imidazole, Tris, Tween-20, Isopropyl-thio-β-D-galactopyranoside and other fine chemicals were purchased from Sigma Chemicals, USA. Nitrocellulose membrane was from Schleicher and Schuell, USA. Ni-NTA agarose was purchased from Qiagen, Germany. ECL kit was purchased from Amersham, USA. The anti-6xHis-tag antibodies were from Clontech, USA. Anti-mouse IgG-HRP conjugate was from National Institute of Immunology, New Delhi.

The enzymes and chemicals used for DNA manipulation were purchased from Invitrogen, USA; Roche, Germany and New England Biolabs, USA. The oligonucleotides were obtained from CBER, FDA, USA or Microsynth, Switzerland. The vectors pCRII-TOPO and pCRT7/CTTOPO, reverse transcriptase enzyme, Superscript II and *Taq* polymerase were obtained from Invitrogen, USA.

Bacterial strains and cell lines

E. coli strains DH5α and BL21 (DE3)pLysS were kind gifts from Dr. Raj K Bhatnagar, ICGEB, New Delhi and Dr Hira L Nakhasi, CBER, FDA, USA.

Experimental methods

AP-PCR reactions

AP-PCR was performed with the genomic DNA isolated from the *Leishmania* isolate *Ld*K1 in order to isolate polymorphic DNA fragments. The pattern of AP-PCR amplification fragments is sensitive to the amount of genomic DNA template and the amount of AP oligo. Amplification reactions were carried out with 150ng DNA concentration using oligo AP-9, to obtain unique DNA fragments. The polymorphic DNA fragment was sliced from the agarose gels and purified with QiaQuick gel extraction kit (Qiagen) following the protocol supplied.

Nucleotide sequence analysis

The polymorphic AP-PCR DNA fragment that yielded differentially transcribed RNA species in pro- and amastigote stages on Northern hybridization was cloned into pCRII-TOPO vector system and sequenced on automated sequencer (Perkin Elmer). Similarly, the other products generated for sequencing purpose were cloned into pCRII-TOPO and the nucleotide sequence determined using the Prism Dye terminator sequencing kit (Perkin Elmer).

Restriction digestion

The genomic DNA was digested with different restriction enzymes at 37°C overnight in an appropriate buffer. After inactivation at the suggested inactivation temperature, the restriction products were resolved on 1 % agarose gel at 80 Volts

for 3 hrs. After electrophoresis, the gel was viewed under UV and positions of the marker noted.

Southern blot

The agarose gel was first rinsed with double distilled water for 15minutes and then with alkaline transfer buffer (0.4N NaOH, 1M NaCl) two times for 15minutes each at room temperature. Nylon$^+$ membrane of appropriate size was floated and allowed to soak in double distilled water ten minutes before capillary transfer was set up. Nylon$^+$ membrane was placed on the gel after brief treatment in the transfer buffer and capillary transfer was set up using transfer buffer. Transfer was allowed to continue for 16hrs and the membrane carrying DNA was irradiated in a UV-cross linker for 5minutes (to a total energy of 1.5J/cm^2 damp membranes). The membrane was soaked in 20XSSC for one minute and stored at -70°C till further use.

Hybridization protocol

The DNA probe was labeled to a specific activity of >10^8 dpm/μg. The membrane carrying DNA membrane was soaked in 5XSSC and placed DNA side up in the hybridization bottle and approximately 1ml of formamide prehybridization solution was added per 10cm^2 of membrane. The pre-hybridization was carried out by incubation with rotation in a hybridization oven for 3hr at 42°C. The labeled probe (1x10^7cpm) was denatured by heating at 100°C for 10minutes in a dry bath and quickly transferred to ice bath. The denatured probe was added to the pre-hybridization solution and incubation was allowed to continue for 16hours at 42°C. After hybridization, the probe was collected into a 15ml tube and membrane was washed.

Washing and autoradiography

After hybridization, the membrane was washed and subjected to autoradiography essentially as described under Northern hybridization (Chapter-3).

RT-PCR

Bone marrow or splenic aspirate samples from the human subjects were collected in Trizol reagent and immediately shipped to lab on ice and stored in -20°C until processed. Total RNA from axenic amastigote, promastigote parasites, bone marrow or splenic aspirates was isolated using Trizol reagent as described earlier. Total RNA was used in a reverse transcription reaction to synthesize first strand cDNA using oligo dT (Invitrogen).

Reverse transcription

Total RNA (5-10μg)	-	2μl
Oligo dT (0.5μg/μl)	-	2μl
DEPC treated water	-	to 12 μl

RNA was denatured by incubating at 70°C for 10 min and quickly chilled on ice for 5 min.

The reverse transcription reaction was assembled as follows.

5X first strand buffer	-	4μl
0.1M DTT	-	2μl
10mM dNTP mix	-	1μl

The reaction was mixed and incubated at 42°C for 2min. 1μl (200U) of Superscript II reverse transcriptase was added and reaction incubated at 42°C for 50 minutes followed by inactivation at 70°C for 10min.

This was followed by the PCR with specific primers from the coding region of the gene of interest involving cycles performed at 94°C for 30sec, 50°C for 30sec and 72°C for 1min.

PCR was set up as follows.

Single stranded cDNA	-	2µl
Forward primer (10µM)	-	1µl
Reverse primer (10µM)	-	1µl
dNTP mix (200µM)	-	4µl
10X PCR buffer	-	5 µl
$MgCl_2$ (50mM)	-	1.5µl
Taq DNA Polymerase (5U/µl)	-	0.25µl
Sterile H_2O to	-	50µl

Primers for amplifying A-1 ORF

A-1 Forward primer - 5' ATG GAC GCC GCC AGG 3'

A-1 Reverse primer - 5' CGA GAA AAA GAC ATG C 3'

Primers for SL-PCR

Forward 5' ACT AAC GCT ATA TAA GTA TC 3'

Reverse 5' ACG CGA ACG TAT CAC G 3'

Expression of A-1 gene from *L donovani* in *E. coli* BL21 (DE3)pLysS Cells

The vector

The vector pCRT7/CT-TOPO is a T7 based expression plasmid. The T7 expression system uses the T7 promoter and T7 RNA polymerase for high-level transcription of the gene of interest. High level expression is achieved due to the

higher processivity of T7 RNA polymerase compared to *E. coli* RNA polymerase. Expression of the target gene is induced by providing a source of T7 RNA polymerase in the host cell. This is accomplished by using BL21 *E. coli* host, which contains a chromosomal copy of the T7 RNA polymerase gene. This gene is under the control of *lacUV5* promoter that can be induced by IPTG.

Cloning into pCRT7/CT-TOPO takes advantage of the religating activity of topoisomerase I enzyme and enables faster ligation reactions with yields ≥95% recombinants. The ligation of the linearized vector, that has single 3'-T overhangs and the PCR products that possess 3'-A overhangs at each end because of the terminal transferase activity of *Taq* polymerase, is achieved in a very short interval of time.

The vector pCRT7/CT-TOPO contains all the elements essential for protein expression from the cloned gene of interest and subsequent purification, that include a strong T7 promoter, a 6X-histidine tag, gene coding for ampicillin resistance, and a synthetic ribosome binding site (RBS) along with translational stop codon and transcriptional terminator. Additionally, it also has V5 epitope region as a fusion tag for detection of recombinant polypeptides. This plasmid can be transformed into BL21 (DE3)pLysS *E. coli* host strain for efficient expression. Schematic diagram with the multicloning site and the location of the affinity tag is shown (Fig 4.1).

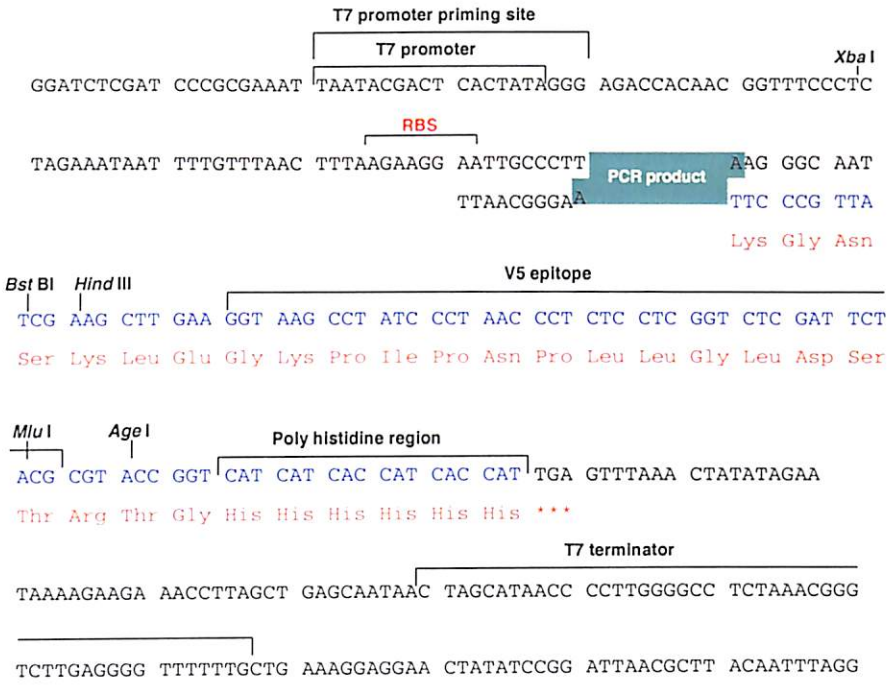


Fig 4.1 Schematic diagram of pCRT7/CT-TOPO showing multicloning site with ribosome binding site, T7 promoter, V5 epitope, Polyhistidine region and T7 terminator. Restriction sites are shown. Asterisk indicates stop codon.

Polyhistidine tracts bind tightly to a number of transition metals and transition metal chelate complexes such that a protein carrying an exposed His-6 region will bind to a resin charged with divalent nickel ions. Few natural proteins bind with significant affinities to such matrices, His-6 labeled proteins generated by recombinant methodologies can be purified substantially in a single step by affinity chromatography. Moreover, His-6 tag is much smaller than most other affinity tags and is uncharged at physiological pH. It rarely alters or contributes to protein immunogenicity, making it widely used affinity tag. In addition, His-tag allows for easy detection of the recombinant protein on Western blot.

Scheme for recombinant A-1 expression

The A-1 gene was expressed as a fusion protein with 6x Histidine affinity tag using the vector pCRT7/CT-TOPO. The A-1 gene was amplified by PCR and the amplified products were separated on 1% agarose gel. The amplified fragment of appropriate size was excised and the DNA was eluted using the Gel extraction kit (Qiagen) as described below. The PCR product and the vector were ligated overnight at 14°C and transformed into *E. coli* BL21(DE3)pLysS competent cells. Preparation and transformation of competent *E. coli* bacteria were performed according to procedures described by Sambrook et al (1989). The transformation mixture was plated on Luria agar plates containing 100µg/ml of ampicillin. The plates were incubated for 16hr at 37°C. Colonies from the plate were screened for the recombinant plasmid by mini-preparations of plasmid DNA (Sambrook et al, 1989). The desired recombinant plasmid was confirmed by restriction digestion with *Xba*I. The recombinant A-1 was expressed by induction with IPTG and the protein was purified using Nickel agarose affinity chromatography.

The experimental details are discussed below.

PCR amplification

PCR was performed in 50µl reaction volume using DNA thermal cycler in 0.5ml PCR tubes. The reaction mix consisted of 50ng of genomic DNA from *Ld*K1, 0.2mM of each dNTP, 0.2nM of oligonucleotide, 5µl of PCR buffer (10X) and 1 unit of Taq DNA polymerase. PCR amplification of A-1 gene fragment involved 94°C for 5 minute followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 5 min for final extension. PCR products were stored at -

20°C until further use. The nucleotide sequence of the amplified product was confirmed by cloning into pCRII-TOPO before bacterial expression was attempted.

Sequences of the Primers were:

A1F 5' ATG GAC GCC GCC AGG AAA C 3'

A1R 5' ACG CGA ACG TAT CAC G 3'

Extraction of DNA from agarose gel

The PCR reaction mix was fractionated by agarose gel electrophoresis. DNA fragments of right size were excised from agarose gel and were eluted from the gel slice using gel extraction kit (Qiagen) as previously described.

Ligation

The amplified DNA fragment resolved on agarose gel and extracted from gel was cloned in pCRT7/CT-TOPO. Ligation was set up as follows.

pCRT7/CT-TOPO vector DNA (50ng)	1.0 µl
A-1 DNA	3.0 µl
Ligation buffer (2 X)	5.0 µl
T4 DNA ligase (1U)	1.0 µl
Total	10.0 µl

The ligation reaction mixture was incubated at 14°C for 16 hrs.

Competent cell preparation

E.coli BL21 (DE3)pLysS competent cells were prepared as follows. The procedure used for the preparation of competent cells was a slight modification of

the one described by Cohen et al (1972). This protocol was frequently used to prepare batches of competent bacteria that yielded $5x10^6$ to $2x10^7$ transformed colonies per microgram of supercoiled plasmid. The host cell culture was streaked on a LB plate from the frozen glycerol stock. A single colony was inoculated into 5ml LB tube. 500µl of the overnight grown culture was further inoculated into 50ml (1:100) LB and allowed to grow for 2-3 hr until A_{600} reached 0.4-0.5. The culture was chilled on ice, transferred to ice cold 50ml polypropylene tubes and centrifuged at 4000 rpm for 10 min. The supernatant was decanted and the pellet was resuspended gently in 20ml of ice cold sterile 0.1M $CaCl_2$ and incubated on ice for 30min. The cells were then centrifuged at 4000 rpm for 10min. The pellet was resuspended in 1ml of ice cold 0.1M $CaCl_2$. Chilled glycerol was added to the cells to a final concentration of 20%. About 200µl aliquots were taken for checking the viability, contamination and efficiency of transformation. The rest of the suspension was kept at 4°C for 12-24 hrs to enhance the competency of the cells and then stored in aliquots of 200µl at -70°C.

Transformation of competent cells

Transformation of the competent *E. coli* cells was performed according to procedures described by Sambrook et al (1989). Briefly a 150µl aliquot of competent cells was thawed on ice and 10µl of ligation mix was added to it, mixed by tapping and incubated on ice for 30 min. The cells were subjected to heat shock at 42°C for 60 seconds in a water bath and were immediately chilled on ice for 1-2 min. 850µl of LB was added to the cells and the cells were incubated at 37°C for 1hr in a shaker incubator with constant shaking. The cells were pelleted and resuspended in 100µl

of LB and plated on LB agar plates containing 100μg/ml of ampicillin and incubated at 37°C for 16hrs.

Screening of the transformants

The presence of the ligated plasmid product was confirmed by mini-preparations of plasmid DNA. The DNA of the construct thus obtained was restriction enzyme digested with *Xba*I as described above and checked on agarose gel for the right size of insert of A-1 gene.

Expression of recombinant A-1

The expression vector pCRT7/CT-TOPO has a 6x-histidine coding sequence added downstream of the multiple cloning sites. *E. coli* BL21 (DE3)pLysS cells were transformed with the recombinant plasmid. To establish the expression of the recombinant protein, cells were grown and induced by addition of IPTG (2mM final concentration). The expressed protein was loaded on to nickel agarose column and eluted by 20mM Imidazole in wash buffer (20mM Sodium phosphate, 500mM Sodium chloride pH7.0). SDS-PAGE of the purified recombinant protein followed by the Western blotting analysis determined the presence of recombinant protein.

Polyacrylamide gel electrophoresis

A 12% separating gel was used for the electrophoretic analysis of proteins. For stacking of proteins 5% gel was used. Protein samples for SDS-PAGE were prepared by the addition of the 6X loading buffer to the final concentration of 1X, boiled for 5 minutes followed by centrifugation at 12,000g for 5 minutes at room temperature. Proteins were analysed by SDS-PAGE according to method of Laemmli (1970) at a constant voltage of 100V in a Bio-Rad mini gel apparatus. The

resolved proteins were visualized by staining the gels with Coomassie Brilliant Blue R-250 for 30 minutes followed by destaining the gel to remove excess stain.

Western blot analysis

Protein samples resolved on SDS-polyacrylamide gels were transferred to nitrocellulose membrane at a constant voltage of 30V overnight or 100V for 1hr. Blocking was done by using 10% BSA in TTBS for 3hrs. For probing the blot with antibody, the membrane was incubated for 1hr at room temperature with anti-6XHis antibodies, diluted (1:100) in Tris buffer (pH7.6) containing 0.5% BSA and 0.1% Tween-20. The membrane was washed and then incubated for 1hr in anti-mouse IgG-HRP-conjugated second antibody. After removing the excess second antibody detection was carried out using ECL blot developing reagents. The membrane was overlaid with 1:1 mixture of detection reagent 1 and detection reagent 2 in dark, for 1 min. The detection reagent mix was drained and the blot was exposed for 1-5 minutes.

Results

Identification of a differentially transcribed sequence in *L donovani*

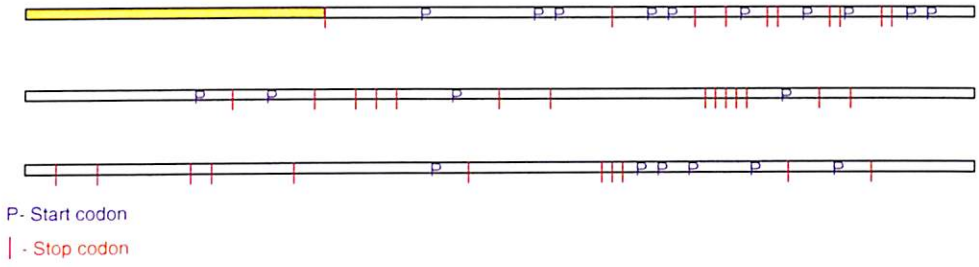
Comparison of the AP-PCR fingerprints of Indian isolates with geographic isolates revealed a polymorphic DNA fragment of size 1.4 (termed K1.4) in Indian isolates following amplification reaction with oligo AP-9 using 150ng of DNA (Fig 3.10A). The distinctive polymorphic fragment obtained by AP-PCR, K1.4, was used as probe in Northern blot analysis of total RNA derived from promastigotes and axenically grown amastigotes of isolate *Ld*K1. The K1.4 probe hybridized with a 1.7kb RNA that displayed 2.4 fold higher abundance in amastigotes compared to promastigotes (Fig 3.10B).

Cloning and sequence analysis of A-1 gene

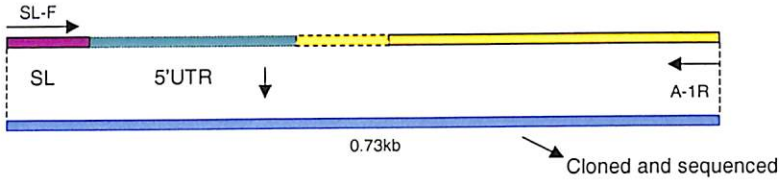
The fragment representing a transcribed sequence that exhibited 2.4 fold higher expression in amastigote stage was selected for cloning of full length gene. The nucleotide sequence of the K1.4 clone revealed that it contained 3' portion of an ORF. Accordingly, cloning of full length gene of A-1 was planned as described schematically in Fig 4.2. It was planned to obtain the 5'end of the ORF making use of the spliced leader sequence in *Leishmania*.

Synthesis of mRNA from polycistronic transcripts in trypanosomatid protozoa necessarily requires extensive post-transcriptional 5' and 3' end-processing events. A common, 39-nucleotide spliced leader sequence, also known as a mini-exon, is attached to the 5' end of all messages in a bimolecular process known as *trans*-splicing.

A Sequence analysis of K1.4 showing putative ORFs in all reading frames



B RT-PCR to rescue 5'end



C Sequence of full length A-1

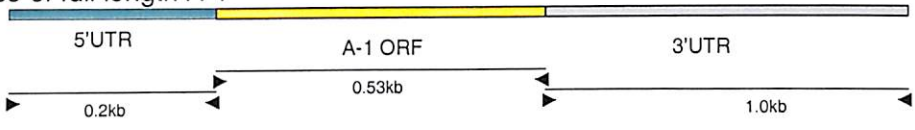


Fig 4.2 Flowchart showing the scheme of cloning of full length A-1 gene.
Panel A: Analysis of the 1.4kb AP-PCR fragment revealed a 3' portion of an ORF (shown in yellow).
Panel B: To identify the 5' end, a RT-PCR was performed with spliced leader forward and gene specific internal reverse primers. The RT-PCR product of 0.73 kb size was cloned and sequenced.
Panel C: The aggregate of the sequences resulted in the full-length nucleotide sequence of A-1gene.

To identify the 5' end of the partial ORF obtained on sequence analysis of the K1.4 kb polymorphic fragment, a reverse transcription PCR with conserved spliced leader sequence (5' ACT AAC GCT ATA TAA GTA TC 3') as forward primer and A-1 gene specific internal primer (5' ACG CGA ACG TAT CAC G 3') was performed. The reaction yielded a ~0.73kb product with pro- as well as amastigote derived total RNA (Fig 4.3). The RT-PCR product was cloned into pCRII-TOPO and its nucleic acid sequence determined. The aggregate of sequence obtained from RT-PCR product and the K1.4 fragment resulted in a 1724bp sequence (Fig 4.4). Thus, the full 5'UTR, complete ORF of A-1 and the 3'UTR sequences were obtained. Further, the A-1 ORF was PCR amplified from the *Ld*K1 genomic DNA, cloned into pCRII-TOPO and the nucleotide sequence confirmed.

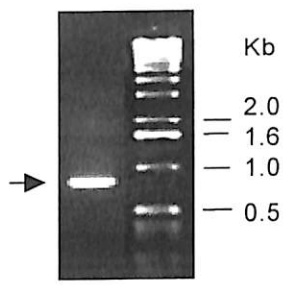


Fig.4.3 RT-PCR to obtain the 5'end of the partial ORF. Primers were designed from conserved spliced leader and the A-1 ORF sequences. The 0.73kb product was cloned and its sequence determined.

Sequence analysis indicated that the A-1 gene contained a 528bp ORF coding for a ~20kDa protein. Further, the analysis revealed the features characteristic to *Leishmania* gene organization like polypyrimidine stretch upstream of AG splice

acceptor. In addition, CA repeats were identified in the 3'UTR (Fig 4.4). A search of GenBank database with BLAST revealed that the A-1 sequence mapped to chromosome-29 clone LB00711 of *L. major* strain Friedlin (Genbank accession number: AC133777.2). This sequence referred to a 82kb clone whose functional details are not defined. Similarly, searches for homologues of A-1 in GeneDB, where the sequence information of *Leishmania* genome can be accessed, (http://www.ebi.ac.uk:parasites:leish.html) did not reveal members with significant homology. Comparison of the deduced aminoacid sequence of the A-1 by BLAST analysis in Genbank or in *Leishmania* database did not show any matches with significant homology. Based on these analyses the possible function of A-1 gene product could not be readily assessed. Therefore A-1 is likely to code for functions yet unknown. Several examples in *Leishmania* species support the notion that developmental regulation of mRNA levels is determined post-transcriptionally by sequences located in the 3'UTR that usually control mRNA stability.

Spliced leader
ACT AAC GCT ATA TAA GTA TCA GTT TCT GTA CTT TAT TGA GGT CAC TGT TAC TTG TTC GTC
ATC TGG GAT CCC CAC TGC TTC TGC AAA GGC TCA GGG TTT CAT TGA CAA GAA CCC ATT GTT
TCC CAG GAG GGG CGC ACG CTA CCC ACT GTC AGA GCA CTC GTT GCC CCT CCG CAG AAA AAA
AAG GGC CAG CGG TGC AGA

```
                ATG GAC GCC GCC AGG AAA CGT CAC AGA GGG AAC GCG TCA GAA
                 M   D   A   A   R   K   R   H   R   G   N   A   A   C    14

GCG GCA GCC AGA GGT GAT GAA GAT GAC TCG GCC GCG ATC CCC GGA CGT GAG GCA CGG CAC
 A   A   A   R   G   D   E   D   D   S   A   A   I   P   G   R   E   A   R   H    34

CAA CTG CAC AGC AGC AAA AGC GCC ATC CCT TTC ACG TCT ACT CCC GAG GAA GCG TGT GGG
 Q   V   H   S   S   K   S   A   I   P   F   T   S   T   P   E   E   A   C   G    54

GAT GAA AAC GAC TAC GCT GTC CTG GTG AAA CGG AGC ACG GCA CTG TTG GCA AAG CTC GGC
 D   E   S   D   Y   A   V   L   V   K   R   S   T   A   L   L   A   K   L   G    74

CAG CGC AAT GTA CTC GAC AAC TGC GCA GCC CAA GAA ACC AGC AGC ACG CAA AAG GAG GCA
 Q   R   N   V   L   D   N   C   A   A   Q   E   T   S   S   T   Q   K   E   A    94

ACA CAA ACC AAT GAC ACT TCC GTG AGA GTT TAC GAA TCA CAA GTT CAG CTT GTA CGG TGT
 T   Q   P   M   T   L   P   C   R   F   E   N   H   K   F   S   L   Y   G   V   114

TTC TTG CTC CGC TGG CTC TCT TGT TTT ACC CTT TGC CAC GGT GCG TGC ATA CAG CCC ACA
 S   E   S   A   G   S   L   V   L   P   F   A   T   A   A   C   I   Q   P   T   134

GAG CTG GCT GAT CGC TCC ACC AAG TGC ACA CAA ACG GAG TCA TCG ACC ACT CTA GAA GCC
 E   L   A   H   R   S   T   K   C   T   Q   T   E   S   S   T   T   L   E   A   154

CGC AAG AAG CTG CTA GCT ACG GAG TTG AGT GAG CTC TAC ACA CTG TTG CAT GTA TTT TTC
 R   K   K   L   L   A   T   E   L   S   E   L   Y   T   L   L   H   V   F   F   174

TCG TGA
 S   *
```

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TACGTTCGCG TGCTCGCGAG TTGCTTTTTT CCCCTTGCTC TTTTCTTTTC TTCACGGACA
TGCGCGCTGA TGGAAGGTTC TGCTGTACTG CGAGCACCAC TCTCTCTGCT CATTGATTTC
ACTGCTGCTG GCTTACGGCT CAGACACTGC ATCGATTCCT CGAACTCACA GTTAGCGATT
CAAGCCGTGC CTGCGTGCAT CCTCGCACGG CACCTGGCGT TGTTGTGCTC CTTCACCTCG
GGACGCTTTC TCGAGATGTC AGGAATGTTC TTTCCAGCAC ACGTTAACTG GTGGTGGCTT
TATAATAATA TATGTATATA TATATTTTGC TAGCAAGTAC CGCAGATCTT ACGCCCGCGC
CATTGGTTCT CCTTGGACCT CTGCTCATGC TTTTCCTCTC TCGTCCCTGT CTCTTTGCCT
TCTGCATCGC TGTCGTTTTT CCGCCAGGCT CTCTCTTCTG CGCGATGCCT TCGCCCTACT
CAGTAGTCGT TTGTCTTCCG CTACCCTGTC GCATTTTTTT TTCTTTCAAA GCTTACGACA
GGAATGAAAG AGGGCGGGAT GATCTAAGTA ATAAAAAAGA CGATAGGATG ATGAGGAGTG
AGTCAAGGCA CTTTCCACGA TTCCAATGAC ATGCTTCACC ACACACGTAT CTTTTCTTCA
TTTTGTTCTT CTGCTCATTT CAGATCTCGA GGAATGGAGT CATGACAAGC GTTTGCTTTT
TCCCTCCACC CGCCCTCCTC TTGATTACTC ACCATGACAG GGGGATCCTT GGGTGACGGA
AAAGGGGGAT ACGTAAGTCT ACCTATTCGT GGGTAGGGTT ACCCGATGGG GACCTGAAAA
TAACAGAATT CTCACTGATA GCTGTGCCAG CTCCAGAGGT ATCGAGGTCG CGAGCAAAGG
GATACCAAAT CGGTGTACTC GTCCACGGCA ATGGGAAACT CACCAACCAG GAAGAGTTGG
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Fig 4.4 Nucleotide sequences of A-1 ORF and 5' and 3' untranslated regions. The spliced leader sequence is underlined and is shown in green followed by the 5'UTR. The 5' untranslated region is shown in red. A-1 ORF and the deduced aminoacid sequence are shown. Asterisk indicates stop codon followed by 3' untranslated region, shown in black. Polypyrimidine tract is underlined. AG dinucleotides are indicated in red.

Recently, a 450nt region within the 'amastin' 3'UTR has been shown to confer amastigote-specific gene expression by a novel mechanism that increases mRNA translation without increase in mRNA stability. Further, this sequence was found to be conserved among a large number of *Leishmania* mRNAs in several *Leishmania* species, most of which are multicopy (Boucher et al, 2002). Therefore, the 3'UTR from the A-1 was analyzed to understand the mechanism of its regulation. The consensus 450nt sequence found to be shared by several of the differentially expressed genes in *Leishmania* was not found in the 3'UTR of A-1 indicating that its regulation follows a mechanism different from the reported scheme. Further analysis of the sequence on GCG software revealed that the sequence contains a motif shared by prokaryotic membrane lipoprotein lipid attachment site. The conserved motif is recognized by a peptidase that cuts upstream of a cysteine residue to which glyceride-fatty acid lipid is attached.

Northern hybridization of RNA isolated from different growth points

To determine if the abundant expression of A-1 as shown earlier (Fig 3.10B) was limited to a particular time in growth of parasite or if it occurred at various stages of parasite growth, A-1 expression was measured at different phases of parasite growth in both pro and amastigote stages. Northern hybridization of RNA isolated from different time points of culture 72hrs, 96hrs and 120hrs of both pro- and amastigote forms of *L. donovani* was done using A-1 coding region as probe. At all stages of growth examined, namely early, mid and late log phases of growth A-1 gene showed higher expression in the amastigotes in comparison with promastigotes. The difference in the level of expression of A-1 mRNA in the pro and

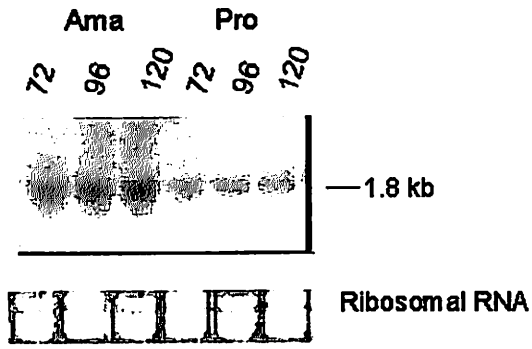

Fig 4.5 Northern hybridization of RNA (15μg) isolated from different growth points of promastigotes and amastigotes (72, 96 and 120 hours). Blots were probed with A-1ORF and visualized by autoradiography.

amastigote stages remained similar at all the growth points compared being in the range of 2.2-2.4 fold as determined by densitometric scanning (Fig 4.5). The equal loading of RNA was monitored by the comparable intensity of ribosomal RNA.

Copy number of the gene

To determine copy number of *L. donovani* A-1 in the genome, Southern hybridization of the total genomic DNA from *L donovani* promastigotes was done using A-1 as probe. Genomic DNA 5μg per lane digested individually with either *EcoR*I, or *Sph*I whose sites are not present in the coding region of the A-1 gene gave a single band of ~7.0 kb or 5.2 kb respectively. On the other hand, digestion of DNA with *Mva*I which has two sites at 11 and 183 in the *Ld*A-1 coding region resulted in three bands of 0.2, 0.75 and 2.0 kb, suggesting that A-1 is a single copy gene. Digestion with a range of restriction enzymes *Bam*HI, *Hind*III, *Pst*I, *Sal*I and *Sph*I followed by hybridization with *Ld*A-1 probe resulted in single bands of high molecular weight supporting the conclusion that A-1 is present as a single copy gene (Fig 4.6).

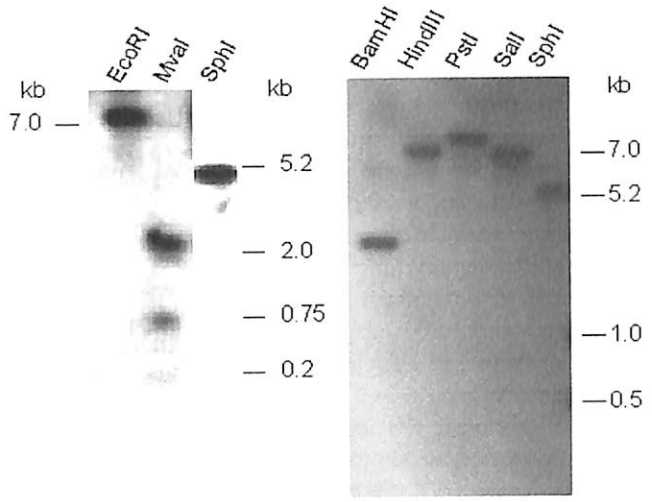

Fig 4.6 Southern blot of Leishmania A-1: *Ld*K1genomic DNA (5μg) was digested with the restriction enzymes indicated and separated on 1% agarose gels. The blots were hybridized with A-1 ORF as probe and visualized by autoradiography. Molecular weights are indicated on the side.

Identification of species-specific polymorphism in *Leishmania* using A-1 gene as a probe

It was considered appropriate to determine the genomic background of A1 in *Leishmania* parasites to explore the possibility of using the A-1 gene locus as a tool to differentiate different strains/species of *Leishmania.* To that end southern blot analysis was carried out using genomic DNA from different species of *Leishmania* such as *L. major* (LV39) and *L. tropica* (WR664). A comparative Southern blot of DNA samples probed with the coding region of A1 ORF showed that *L. donovani* displayed polymorphism with *Sac*I and *Bgl*II in comparison with *L. major* and *L. tropica* (Fig 4.7).

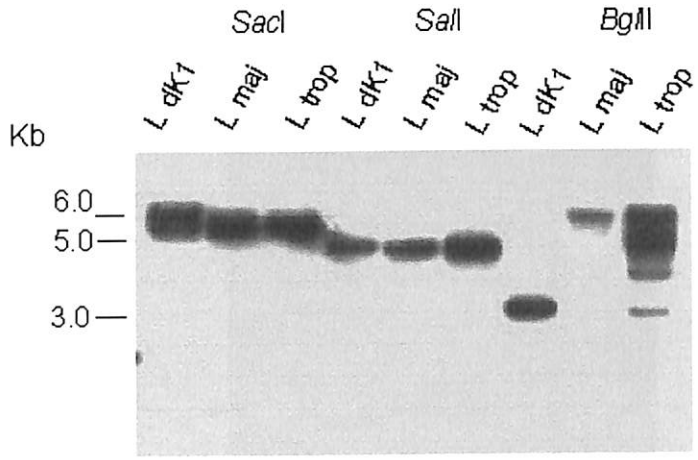

Fig 4.7 Southern blot analysis of genomic DNA (5ug) of genomic DNA from *Ld* India, *L. major* or *L. tropica* was digested with the restriction enzymes indicated, resolved on 1% agarose gel, blots probed with A-1 ORF and visualized by autoradiography.

Identification of A-1 transcripts from RNA from clinical samples

To examine if the higher expression of A-1 is an anomalous phenomenon because of the *in vitro* adaptation of the *Leishmania* parasites to amastigote stage, it was sought to determine A-1 expression in conditions *in vivo*. The expression of A-1 was determined directly in the human system in different tissue samples. Total RNA was isolated from axenic amastigotes, promastigotes as well as splenic aspirate and bone marrow aspirate of a Kala-azar patient. Bone marrow aspirate of a non-KA patient was included as a negative control. RT-PCR was performed using A-1 ORF specific primers. The RNA from axenic amastigotes and promastigotes gave the expected product of size about ~530bp. Further, A-1 mRNA could be detected in the patient spleen and bone marrow samples, while the bone marrow aspirate from non-KA patient that was included as a negative control, did not show the transcript (Fig 4.8).

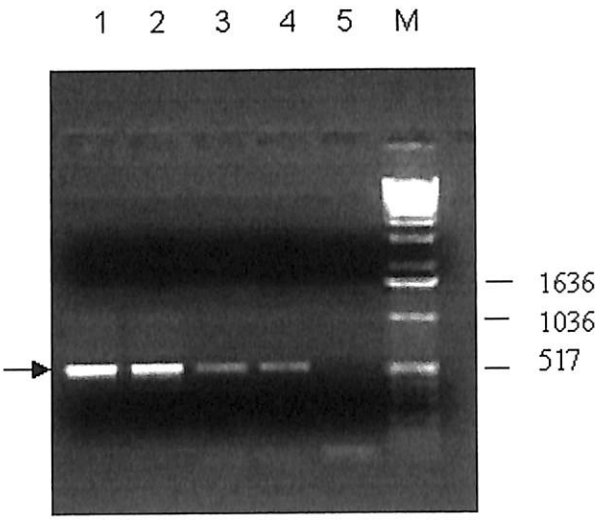

Fig 4.8 RT-PCR with total RNA isolated from clinical samples: Lanes 1: axenic amastigotes, 2: Splenic aspirate, 3: Bone marrow aspirate, 4: promastigote, 5: bone marrow from KA-negative case. M 1 kb DNA ladder.

Expression of recombinant A-1and western blotting

Towards defining the biological function of the A-1 gene product, the A-1 ORF was expressed in a bacterial system to facilitate further studies. Further, expression of the ORF was considered necessary to confirm its deduced coding capacity. The A-1 ORF was PCR amplified from genomic DNA of *Ld*K1 and was ligated into pCRT7/CT-TOPO bacterial expression vector in frame with a 3' histidine tag. The protein expression was induced by IPTG. However, the recombinant protein was expressed in low amounts. The recombinant A-1 protein was purified using nickel agarose affinity chromatography (Fig 4.9A). Western blot analysis of the recombinant A-1 protein with anti-6Xhis antibodies showed that the recombinant product was a ~23 kDa protein (Fig 4.9B).

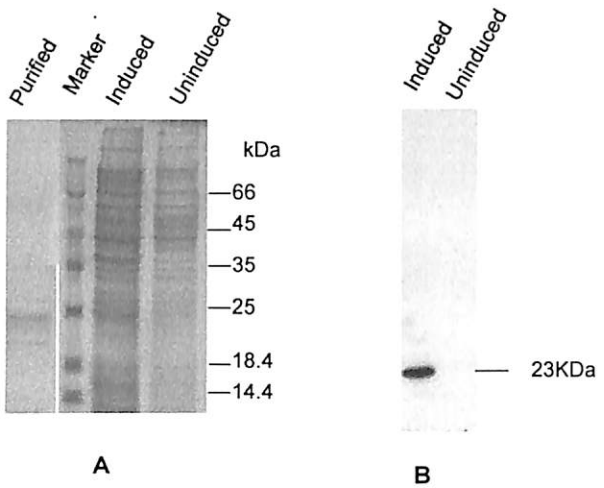

Fig 4.9 SDS-PAGE and Western blot analysis of purified rA-1 protein. The recombinant protein was purified with Nickel agarose and synthesis of the fusion protein was confirmed by SDS-PAGE (panel A) and by Western blot using anti-6XHis antibodies (panel B). Blots were reacted with anti-mouse IgG-HRP antibody and developed using ECL kit.

Discussion

The protozoan parasite *Leishmania* must undergo several metabolic and physiological changes throughout their life cycle. Understanding the mechanisms governing the developmental expression of stage-specific products essential for the adaptation in each environment will contribute to defining the molecular basis for the infectivity and the pathogenicity of the parasite. How parasites carry out the developmental transitions that are necessary for survival and transmission among their insect and mammalian hosts and the mechanisms that they use to resist, often for decades, a tremendously hostile array of defenses are of interest to biologists and clinicians who seek to control these pathogens. At present, there are no

effective strategies of vaccination against these diseases, despite considerable evidence that these are attainable goals.

We have successfully identified genes that show differential expression by genomic fingerprinting by AP-PCR technique. The A-1 gene identified for the first time, showed 2.4 fold higher abundance in amastigote stage of the parasite. The expression was monitored at several stages of the growth. These results showed that the A-1 was expressed in significantly higher abundance in amastigotes at various time points measured indicating that the higher expression in not restricted to a single stage of growth.

It is conceivable to catalog the genetic determinants of *Leishmania* into those that help the parasite invade the macrophages and those that cause persistent infection and pathology. These virulence factors help *Leishmania* successfully establish infection in macrophages by evading the humoral lytic factors, attachment of parasites to macrophages followed by entry by phagocytosis, intracellular survival, promastigote to amastigote differentiation and replication as amastigotes. However, the spreading of amastigotes to infect additional cells must be considered as crucial for the development of leishmaniasis since infection by itself does not cause the disease, though it is a prerequisite for this state. Infection must be maintained in order for the transition from asymptomatic phase to symptomatic phase, especially when host immunity becomes downregulated. Therefore, the genes that are expressed transiently at a critical point of differentiation and the products of genes that regulate differentiation into the intracellular amastigote form have great potential as targets to block the infection process. These genes may only be expressed at the time of differentiation and at very low abundance. However, because of the

difficulties in obtaining adequate amounts of viable amastigotes that are free of host cell contamination as well as the limited availability of effective and sensitive methods (Coulson and Simth, 1990; Brodin et al, 1992), molecular biology studies on the parasite particularly at the amastigote stage have been hindered greatly. Therefore, very few genes involved in the stage transitions have been characterized and the underlying mechanism of differential gene expression not fully understood (Liu et al, 2000).

The development of techniques permitting the axenic cultivation of parasites which resemble amastigotes both morphologically and with respect to a number of biochemical markers has greatly enhanced our ability to study to identify genes with stage regulated expression (Joshi et al, 1993). Successful adaptation of promastigotes obtained from clinical samples to axenic amastigote forms in our lab has allowed us to prepare RNA samples from both the stages in abundant quantities for comparison by Northern analysis.

Previously, much attention has been devoted to the infection of macrophages by promastigotes, although the manner, by which their molecules actually function in infection, remains to be elucidated. Data obtained from different host-parasite systems are also not always consistent even for the molecules more extensively studied, like gp63 and LPG. gp63 is an ecto-metalloprotease that is especially abundant in the surface of promastigotes and also released by this stage of *Leishmania* (McGwire et al, 2002). The protein is known to help promastigotes in evading lytic factors by rendering them resistant to complement-mediated cytolysis (Brittingham et al, 1995). It also appears to act, (perhaps, together with LPG) in infection of macrophages by promastigotes via receptor- mediated endocytosis

(Chang and Chang, 1986; Russel, 1986; Liu and Chang, 1992; McGwire and Chang, 1994). Both may be important for intraphagolysosomal survival (McGwire and Chang, 1994; Beverley and Turco, 1998; Chaudhuri et al, 1989). Some of the other molecules like phosphoglycans, proteophosphoglycans, cysteine proteases may be involved directly or indirectly in these and/ or the remaining events.

On the other hand, parasite molecules involved in promastigote-to-amastigote differentiation and replication of the amastigotes may have additional functions beyond infection, especially in the latter case. In the present study, we have shown that A-1 is expressed in higher quantities in amastigote stage. Further, the A-1 expression was found to be higher at several time points when its expression was monitored in both the life stages of the parasite. It is reasonable to argue that the A-1 gene identified as exhibiting consistently higher expression at several time points of amastigote growth compared to promastigote stage may be important for the replication of amastigote stage of the parasite, therefore making it an attractive target for studies involving gene disruption methods to verify if it can affect the amastigote replication. Further, pattern of A-1 expression indicated that the higher expression was not an aberration due to changes in expression patterns because of *in vitro* growth conditions.

Since *Leishmania* amastigotes are adapted to thrive in the unusual ecological niche of the phagolysosome of mammalian macrophages, it is reasonable to find several of the gene products that help the parasite adapt to the environment to be upregulated. The parasitophorous vacuole presents a strongly hydrolytic, acidic environment and the parasite apparently does not attenuate in this hostile milieu. Amastigotes maintain an intracellular pH of 7.0 even when exposed to

environmental pH as low as 4.0 (Glaser et al, 1988). Apparently proton extrusion pumps have been found to be strongly up regulated. Moreover, *Leishmania* amastigotes are covered by a densely packed glycocalyx that shields the parasite from the hostile environment. Several enzymes involved in the biosynthesis of glycocalyx components seem to be important for viability, or are important virulence factors in amastigotes but not all surface components seem to be important for virulence (Ilg et al, 2001).

The A-2 gene family was first identified as an amastigote-specific in *L. donovani* since the A2 transcripts are abundant in the amastigotes but hardly detectable in the promastigotes (Charest and Matlashewski, 1994). The A2 genes of *L. donovani* are composed predominantly of a 10-aminoacid encoding sequence that is present in multiple copies. The predominant expression of A-2 in amastigote stage, was shown to be important for the virulence when the expression of A-2 was inhibited by antisense approach (Zhang and Matlashewski, 1997).

Another class of molecules that display higher abundance in amastigote stage is the heat shock proteins. The major heat shock proteins of *Leishmania*, Hsp70 and Hsp83, are already extremely abundant under standard culture conditions (Brandau et al, 1995). In contrast to that of Hsp70 and Hsp83, the expression of the Hsp104 homolog in *Leishmania*, Hsp100, is chiefly restricted to conditions of heat stress (Hubel et al, 1995). This protein is barely detectable in unstressed insect stages of the parasite, but its intracellular level is increased by 1 order of magnitude by temperatures equivalent to those in the mammalian target tissues. Smejkal et al (1988) had shown that heat treatment increased the virulence of *Leishmania braziliensis panamensis* in the hamster model. The expression

pattern of Hsp100 (Hubel et al, 1995) suggests that this protein was the effector molecule which mediated the heat inducibility of virulence (Smejkal et al, 1988). Apart from the aforementioned classes of molecules, several genes showing higher expression in amastigote stage were identified. *L. donovani* HSP83 (Argaman et al, 1994) *L. donovani* proton motive P-type *ATPase 1b* (Meade et al, 1989) *L. mexicana CPB2.8* (Mottram et al, 1997; Souza et al, 1992) *L. mexicana* AF032464 (Liu et al, 2000) *L. donovani infantum* triose phosphate isomerase *TIM* (El Fakhry et al, 2002) *L. pifanoi CYS2* cysteine proteinase (Traub-Cseko et al, 1993) and *L. major HASPs* (McKean et al, 1997).

Studies involving *Leishmania* genomic microarray revealed that relatively a few genes showed changes in expression of more than two-fold between the stages examined. In contrast, analogous growth or developmental transitions in the yeast *Saccharomyces cerevisiae* (log to stationary phase, sporulation) were accompanied by two-fold or greater changes in expression in 18–27% of all genes (DeRisi et al, 1997; Chu et al, 1998). In another study involving Splice leader derived library based *Leishmania* microarray 147 candidates from the 1094 unique genes that are specifically upregulated in amastigotes were identified. Despite the lack of transcriptional regulation that polycistronic transcription in *Leishmania* dictates, the data provide evidence for a high level of post-transcriptional regulation of RNA abundance during the developmental cycle of promastigotes in culture and in lesion-derived amastigotes of *L. major* (Almeida et al, 2002). A proteomic approach to identify developmentally regulated proteins in *L. infantum* estimated about 250 soluble amastigote-specific proteins in *Leishmania* (El Fakhry et al, 2002).

Processing of the polycistronic RNA units into monocistronic mRNAs in *Leishmania* as in all Kinetoplasidae involves mainly a post-transcriptional control mediated by *trans*-splicing coupled to polyadenylation (Borst, 1986; Agabian, 1990; Lee and Vander-Ploeg, 1997). Several examples in *Leishmania* and *Trypanosoma* species suggest that differential expression of genes in both life stages involves mainly the 3' untranslated region of the mRNAs and implies often differential mRNA stability (Moore et al, 1996; Beetham et al, 1997; Aly et al, 1994; Nozaki and Cross, 1995; Berberof et al, 1995; Hausler and Clayton, 1996; Charest et al, 1996; Lee 1998). The implication of the 3'UTR sequences and/or intergenic regions in the stage specific regulation of *Leishmania* transcripts is further supported by the observation that differential expression of multi copy genes such as gp63, Hsp70 and β-tubulin is dependent on sequences present downstream of the different copies. Recently, amastin surface proteins were shown to contain a 450nt length conserved element in the 3'UTR that is responsible for the stage regulated expression in amastigotes (Boucher et al, 2002). The conserved 450nt was found in several of the stage regulated genes in *Leishmania*. Interestingly, this regulatory element was common to multicopy genes. The analysis of 3'UTR of A-1 did not reveal this regulatory sequence. It is possible that A-1 expression is regulated in a non-3'UTR dependent manner as are hsp83, proton motive P-type *ATPase 1b, L. mexicana CPB2.8, L. d infantum* triose phosphate isomerase *TIM, L. pifanoi CYS2* cysteine proteinase and *L. major HASPs* that are found to be single copy genes. Since A-1 was shown to be present as a single copy gene its regulation might follow a different pattern compared to the multicopy genes.

In the past, the promastigotes have been readily cultured in cell free media, while the amastigotes could only be maintained either in animals or in macrophage cell lines. The *in vitro* adaptation of the parasite into amastigote stage might result in changes in gene expression patterns. The gene expression patterns of the axenically adapted amastigotes were documented to be comparable to animal derived parasites (Saar et al, 1998; Duncan et al, 2001). Animal derived amastigotes are not well suited for the analysis of parasite gene expression because of the host cell contamination. The RT-PCR with RNA isolated from clinical samples such as bone marrow or splenic aspirates as well as *Leishmania* parasites of both the stages has yielded the A-1 ORF as its product. This indicated that the A-1 gene is abundantly expressed in amastigotes in infected tissues of KA patients. This is the first time that transcript of any gene is demonstrated in clinical samples isolated from human subjects. Though this assay was not performed to yield quantitative data, it has several favorable implications for obtaining such data. Monitoring of A-1 gene expression in clinical samples can serve as a good methodology for quantitative PCR experiments to determine active parasite burden in a range of tissues. Moreover, experimental hosts such as laboratory mice are largely used to study the immunobiology of these parasites and to screen the efficacy of newly developed drugs and vaccines (Blackwell, 1996; Handman, 2001;Sacks, 2001). Most of these studies require detection and quantitation of the *Leishmania* parasite burdens in different tissues. This is still routinely done by tissue culture methods, which have several limitations, in particular the amount of time required. A more rapid alternative is quantitative PCR, which quantifies DNA and therefore has the potential for accurate microorganism enumeration in medical or environmental samples (Jauregui

et al, 2001). Further, in microarray-based expression profiling studies an important part is validation of the microarray results by methods such as Northern blot analysis or quantitative PCR. The strategy adopted in this study is a significant advancement over the conventional methods that validate expression data with RNA isolated from cells grown *in vitro* or from infected tissues of animals. Investigating gene expression in *Leishmania* infected tissues of humans as shown in our study could serve as a good strategy for validating gene expression studies identified in microarray analyses.

Analyzing the genomic context of the A-1 locus was considered relevant since such analysis might reveal differences that can distinguish DNA from various *Leishmania* species. The A-1 locus revealed polymorphism between various geographic isolates of *Leishmania*. The polymorphism identified in A-1 locus could serve as a marker to distinguish DNA from Old World Leishmanias and can be used to identify the nature of clinical parasite isolates. Such markers are more reliable compared to monoclonal antibodies and isoenzyme analysis in distinguishing parasite species. Several such studies to map molecular differences in a range of gene loci were conducted in *Leishmania* (Banuls et al, 1999). Restriction fragment length polymorphisms within chromosomal genes and the intergenic regions in the rDNA locus as well as sequence and distribution of repetitive DNA sequences have been employed to define species-specific markers and ascertain a quantitative basis for measuring evolutionary distance between *Leishmania* species. However, multicopy gene loci have been the primary focus for such studies, making it important if low-copy gene loci also are under similar selective pressure and diverge by like mechanisms. Therefore, characterization of the conservation of low-copy

gene loci would contribute to the understanding of the molecular mechanisms involved in the divergence of parasites with widely different tissue tropisms and associated pathologies.

The A-1 ORF was successfully expressed in a bacterial expression system confirming its coding capacity. The expression was at low level for reasons that could not be readily explained even after induction with 2mM IPTG. The protein band could be readily detected on SDS-polyacrylamide gels after purification with Nickel agarose. Successful expression of A-1 should pave way for subsequent studies such as producing polyclonal antibodies to immunolocalize the A-1 gene product in *Leishmania* and detecting humoral immune responses in VL patients against A-1 protein.

With the advent of gene transfection technology with *Leishmania*, a number of innovative approaches have been used in attempts to generate vaccines. These include the inactivation of genes encoding enzymes thought to be important for parasite intracellular survival, including the dihydrofolate reductase gene (Titus et al, 1995), the cysteine proteinase genes (Alexander et al, 1998), the HSP100 heat shock protein (Hubel et al, 1997), and the trypanothione reductase (Dumas et al, 1997). Other approaches relying on the expression of antisense RNA (Zhang and Matlashewski, 1997) or of cytotoxic genes (Muyombwe et al, 1998) were also used. Gene replacement strategies by homologous recombination were readily achieved when the target gene was single copy. A-1 was shown here to be a single copy gene, making it a suitable candidate for gene replacement by homologous recombination to understand its function.

Most of the work to generate attenuated parasite strains was done with *Leishmania* species giving rise to cutaneous lesions, and with the exception of antisense RNA, recombinant attenuated *L. donovani* organisms produced by gene inactivation was reported for only *L. donovani* biopterin transporter in order to generate attenuated *L. donovani* organisms for vaccination purposes (Papadopoulou et al, 2002).

The pathoantigenic determinants may be suitable targets for considering molecular attenuation of virulence by gene deletions or modifications, thereby producing infective, but non-pathogenic mutants for vaccination. Further studies are warranted to determine if the A-1 could be a suitable target for considering molecular attenuation of virulence by gene deletions or modifications, thereby producing infective, but non-pathogenic mutants for vaccination.

Cloning and characterization of Centrin

Introduction

Leishmania donovani has a digenic life cycle. The flagellated form, known as promastigote, resides extracellularly in the gut of a dipteran sand fly insect. The non-flagellated, intracellular form, amastigote, is found in the macrophages of the infected human host. The growth and cytodifferentiation of *Leishmania* trigger differential expression of a variety of genes (Zilberstein and Shapira, 1994). Previous studies have identified genes that may have a role in growth and differentiation of the parasite (Joshi et al, 1993; Pogue et al, 1995a,b). In the present study, one such gene, centrin, was identified and its functional analysis with respect to growth of the parasite was carried out.

Centrin, also named caltractin, is particularly enriched in centrosomes. Centrin is an ancient protein of the calcium-binding, EF-hand protein, or calmodulin fold superfamily thought to have arisen within the ancestor of eukaryotes through gene duplication (Nakayama et al, 1992; Bhattacharya et al, 1993). It was first discovered in the flagellar apparatus of unicellular green algae where it is responsible for the contraction of calcium sensitive fibers that connect basal bodies to one another and to the nucleus (Salisbury et al, 1984). These fibers are involved in basal body localization, orientation, and segregation. Another set of calcium contractile, centrin-containing fibers present in the transition zone between basal body and axoneme is involved in flagellum excision (Sanders and Salisbury, 1994). Cloning of the centrin gene from *Chlamydomonas reinhardtii* revealed similarity with CDC31 gene of *Saccharomyces cerevisiae*, a gene that was discovered in a genetic screen designed to isolate cell division cycle mutants (Schild et al, 1981, Byers, 1981), and which is essential for spindle pole body duplication (Baum et al, 1986).

Centrins are cytoskeletal, calcium-binding proteins that are localized in the microtubule-organizing center of eukaryotes (Baron et al, 1991). Centrins are one of the several regulatory proteins essential for duplication or segregation of the centrosome in higher eukaryotes and basal bodies in lower eukaryotes (Wiech et al, 1996). In many organisms, more than one centrin isotype has been described e.g. three centrins in humans and mice. Though three centrin forms have been recognized in the unicellular algae *Chlamydomonas*, only one has so far been characterized (Taillon et al, 1992). One subfamily of centrins, which includes human centrin 1 (*HsCEN1*), human centrin 2 (*HsCEN2*), and *Chlamydomonas reinhardtii* centrin (*CrCEN1*), is involved in centrosome segregation (Laoukili et al, 2000). The other subfamily, which includes human centrin 3 (*HsCEN3*) and yeast centrin (*CDC31*), is involved in centrosome duplication (Middendorp et al, 1997; Khalfan et al, 2000). Results from diverse experimental systems, mostly in yeast, suggest that different types of proteins like PKic1p, protein kinase (Khalfan et al, 2000; Ivanovska and Rose, 2001), and Kar1p, a component of the half-bridge of the spindle pole body, (Durussel et al, 2000; Geier et al, 1996) bind to centrins. Though centrin has been characterized in a variety of eukaryotes, it has not been reported in the order Kinetoplastida.

The parasites of the Kinetoplastida are responsible for a wide variety of diseases affecting humans, animals, and plants (Bastin et al, 2000). Members of this group have been considered to be one of the earliest eukaryotes, developing conventional organelles, but sometimes with extreme features rarely seen in other organisms (Bastin et al, 2000). The role of such unique structural and functional features of these organelles, like the cytoskeleton and the flagellar apparatus, in

infectivity is still obscure. Hence, the identification of genes that enable *Leishmania* to grow and differentiate within the harsh and diverse environments (sand fly gut and human macrophages) continues to be an important objective (Joshi et al, 1993; Nakhasi et al, 1998). The nature and the role of either the microtubule-organizing center or the basal body apparatus in the primitive eukaryote *Leishmania* are still not known. None of the genes (e.g. centrin, calmodulin, and γ-tubulin) that are associated with these organelles in higher eukaryotes has been characterized so far in this important human parasite. As a first step toward that, cloning, sequencing, and characterization of a centrin gene from *L. donovani* (*Ld*cen) was undertaken.

Materials

Reagents and Chemicals

Acrylamide, Ampicillin, Bovine Serum Albumin, Coomassie brilliant blue R-250, Calcium chloride, Glycine, Glutamine, Glycerol, SDS, Imidazole, Tris, Tween-20, IPTG and other fine chemicals were purchased from Sigma Chemicals, USA. Nitrocellulose membrane was from Schleicher and Schuell, USA. Ni-NTA agarose was purchased from Qiagen, Germany.

DNA modifying enzymes and vectors

The enzymes and chemicals used for DNA manipulation were purchased from Invitrogen, USA; Roche, Germany and New England Biolabs, USA. The oligonucleotides were obtained from CBER, FDA, USA or Microsynth, Switzerland. The vector pCRII-TOPO was from Invitrogen, USA and pQE-70 was from Qiagen.

Bacterial strains and cell lines

E. coli strain M15 was a kind gift from Dr. Raj K Bhatnagar, ICGEB, New Delhi.

Experimental methods

***In vitro* culture of parasites**

Promastigotes and the axenic amastigotes were grown and harvested as described previously.

AP-PCR reactions

AP-PCR was performed with the genomic DNA isolated from the *Leishmania* isolate *Ld*K1 in order to isolate polymorphic DNA fragments. Amplification reactions with oligo AP-9 were carried out with 100ng DNA to obtain unique DNA fragments. The polymorphic DNA fragment was sliced from the agarose gels and purified with QiaQuick gel extraction kit (Qiagen) following the protocol described earlier.

Cloning of centrin and sequence analysis

The polymorphic arbitrarily primed polymerase chain reaction fragment K1.2, amplified specifically from *Ld*K1 DNA using AP-9 primer, hybridized to a ~2.1kb RNA that was found to be expressed significantly more in promastigotes than in axenic amastigotes and to a ~1.7kb RNA that was expressed in similar amounts in both the stages of the parasites (Fig 3.8B). Based on such differential expression, it was planned to isolate the full length gene encoded by the K1.2 fragment and an appropriate cloning strategy was designed. The K1.2 probe was used as a probe to screen a genomic cosmid library of *L. donovani* to isolate positive clones. This screening identified a positive cosmid clone bearing the K1.2 fragment. Restriction digestion followed by Southern hybridization with K1.2 yielded the positive clone of suitable size. Nucleotide sequence of the positive cosmid clone was determined.

BLAST analysis of the nucleotide sequence in Genbank database identified that the 3'end of the sequence overlapped with 5'end of untranslated region from an ORF. The deduced aminoacid sequence from this ORF was used to find homologues using BLAST program. This resulted in the identification of the ORF as a homologue of centrin gene. The centrin gene was coned from genomic DNA of *Ld*K1.

Sequence analysis

Multiple sequence alignment of centrins from various organisms was conducted in MacVector 7.0 program and used to determine cluster relationships among the sequences and to construct dendrograms representing cluster relationships. The encoded protein of 149 amino acids identified through BLAST search as a homolog of the centrin gene was used for analysis.

Northern hybridization analysis

Total RNA was isolated from promastigote and axenic amastigote cultures of *L. donovani* using Trizol as described previously. Total RNA from pro- and amastigotes was resolved on 1% formaldehyde gels and Northern hybridization analysis was performed as described earlier. The Northern blots were hybridized with a ^{32}P-labeled centrin coding region as probe. The membranes were exposed to Biomax films (Kodak) and the autoradiograms were scanned on the AlphaImager system (Alpha Imager 1220). The intensity of the hybridized bands was quantitated using AlphaEase software.

Expression of *Ldcen* from *L. donovani* in *E. coli* M15 Cells

The vector

The pQE-70 expression vector belongs to the family of pDS plasmids and were derived from plasmids pDS56/RBSII and pDS781/RBSII-DFHRS. These contain an optimized regulable promoter/operator element consisting of *E coli* phage T5 promoter and two *lac* operator sequences. It also has a synthetic ribosome binding site, RBSII designed for optimal mRNA recognition and binding and an optimized 6XHis affinity tag coding sequence, a multicloning site, translation stop codons in all reading frames, a transcriptional terminator from phage lambda and replication region and the gene for β-lactamase of plasmid pBR322. This plasmid can be expressed in *E coli* M15 strain.

Strategy for recombinant expression of Ldcen

The *Ldcen* gene was amplified by PCR and the amplified PCR products were separated on 1% agarose gel. The band of appropriate size was excised and the DNA eluted using the gel extraction kit (Qiagen) as described earlier. After digestion with appropriate restriction enzymes that generated ends to facilitate in-frame cloning, the PCR product and the vector were ligated overnight at 14°C and transformed into *E. coli* M15 competent cells. The detailed procedures are described below.

PCR amplification and sequence confirmation

PCR was performed in 50µl reaction volume using DNA thermal cycler in 0.5ml PCR tubes. The reaction mix consisted of 50ng of genomic DNA from *Ld*K1, 0.2mM of each dNTP (Invitrogen), 0.2nmol of oligonucleotide, 5µl of PCR buffer (10X) and 1 unit of *Taq* DNA polymerase. PCR amplification of *Ld*cen ORF involved 94°C for 5 minute followed by 30 cycles of 94°C for 1 min, 50°C for 1min, 72°C for 1min, and a final extension at 72°C for 5min for final extension. After the amplification reaction, samples were stored at -20°C until further use. The nucleotide sequence of the amplified product was confirmed by cloning into pCRII-TOPO before bacterial expression was attempted. Sequences of the primers used are as indicated. The restriction sites of *Sph*I and *Bgl*II are underlined.

Lcen F 5' CGC ATG CCC ATG GCT GCG CTG ACG GAT 3'

Lcen R 5' GAG ATC TCT TTC CAC GCA TG 3'

Extraction of DNA from agarose gel

The PCR products and the plasmid vector were digested with *Sph*I, *Bgl*II and *Sph*I, *Bam*HI respectively and were fractionated by agarose gel electrophoresis. DNA fragments of appropriate size were excised from agarose gel and eluted as described earlier.

Ligation

Ligation of the PCR amplified *Ld*cen fragment and the vector was set up as described.

pQE-70 vector DNA (50ng)	1.0 µl
Ldcen DNA	3.0 µl
Ligation buffer (10 X)	1.0 µl
T4 DNA ligase (1U)	1.0 µl
Sterile H_2O	4.0 µl
Total	10.0 µl

The ligation reaction mixture was incubated at 14°C for 16 hrs.

Competent cell preparation and transformation

E. coli M15 competent cells were prepared essentially as described in Chapter-4. Transformation of competent *E. coli* cells with the recombinant plasmid was performed according to the procedures described earlier.

Screening of the transformants

The presence of the ligated plasmid product was confirmed by mini-preparations of plasmid DNA. The DNA of the construct thus obtained was restriction digested with *Eco*RI, *Hind*III and checked on agarose gel for the right size of insert of *Ld*cen ORF.

Expression of recombinant Lcen

The expression vector pQE-70 has a 6x-histidine coding sequence added downstream of the multiple cloning site. *E. coli* M15 cells were transformed with the

recombinant plasmid. To initiate the expression of the recombinant protein, *E coli* cells were grown and induced by addition of IPTG to a final concentration of 2mM. The expressed recombinant protein was loaded on to nickel agarose column and eluted by 20mM Imidazole in wash buffer (20mM Sodium phosphate, 500mM Sodium chloride pH7.0). SDS-PAGE of the purified recombinant protein determined the presence of recombinant protein.

Polyacrylamide gel electrophoresis

A 12% separating gel was used for the electrophoretic analysis of proteins. For stacking of proteins 5% gel was used. Protein samples were resolved exactly as described earlier.

Western blot analysis

Protein samples resolved on SDS-polyacrylamide gels were transferred to nitrocellulose membrane at a constant voltage of 30V overnight or 100V for 1hr. Blocking was done by using 10% BSA in TTBS for 3hrs. For probing the blot with antibody, the membrane was incubated for 1hr at room temperature with anti-6XHis antibodies, diluted (1:100) in Tris buffer (pH7.6) containing 0.5% BSA and 0.1% Tween-20. The membrane was washed and then incubated for 1hr in anti-mouse IgG-HRP-conjugated second antibody. After removing the excess second antibody, detection was carried out using ECL blot developing reagents. The membrane was overlaid with 1:1 mixture of detection reagent 1 and detection reagent 2 in dark, for 1min. The detection reagent mix was drained and the blot was exposed for 1-5 minutes.

Immunofluorescence Analysis

L. donovani promastigotes were fixed in suspension in 4% (w/v) paraformaldehyde in PBS (50 mM Na_2HPO_4, 150mM NaCl, pH 7.4) for 20min at room temperature, washed three times in PBS, and allowed to attach to poly-L-lysine coated glass slides. After air drying, the slides were immersed in ice-cold methanol for 5 min, blocked for 2hrs in 1% (w/v) bovine serum albumin in PBS, and incubated 1hr with the anti-*Ld*Cenp serum (1:200 dilution) diluted in 1% bovine serum albumin in PBS. After three washes in cold PBS, cells were incubated for 1hr with fluorescein-conjugated anti-rabbit IgG antibody. These secondary antibodies (National Institute of Immunology, New Delhi) were diluted 1:200-fold in PBS containing 1% bovine serum albumin. Cells were subsequently washed three times with cold PBS and mounted in 10% glycerol in PBS. Cells were examined for fluorescence in a confocal laser microscope (Zeiss), with epi-fluorescence. The focal plane chosen in all the cells was in the middle of the cells.

Phylogenetic analysis of various centrins

The dendrogram of complete protein sequences of centrins was generated in the ClustalW-alignment section of MacVector 7.0 program utilizing systematic, bootstrap, and neighbor-joining options.

Results

Cloning and sequence analysis of the L. donovani centrin gene

The polymorphic arbitrarily primed polymerase chain reaction fragment K1.2, amplified specifically from *Ld*K1 DNA using AP-9 primer gave positive hybridization reaction yielding a ~2.1kb and a ~1.7kb RNA.

To isolate the full length gene of this fragment, the K1.2 was used as a probe to screen a genomic cosmid library of *L. donovani* (a kind gift from Dr Buddy Ullman, USA) to isolate clones encompassing this probe sequence. The cosmid library screening identified a positive cosmid clone of >25kb size. The positive cosmid clone was digested with a panel of restriction enzymes, resolved on agarose gel, blotted and hybridized with ^{32}P labeled K1.2. This hybridization reaction yielded a clone of ~3.0kb size. Nucleotide sequence of this positive cosmid clone was determined.

BLAST analysis of the nucleotide sequence in Genbank database identified that the 3'end of the sequence overlapped with 5'end of an untranslated region from an ORF. Homology search of the open reading frame revealed that it had significant similarity with centrin proteins from many organisms. A reverse transcription PCR was performed with forward primer designed from the 5'UTR sequence and oligo (dT) followed by sequencing of the RT-PCR product. Nucleotide sequence of this RT-PCR product revealed the presence of an ORF.

Primers spanning the full ORF (For- 5' ATG GCT GCG CTG ACG GAT GAA 3'; Rev- 5' CTT TCC ACG CAT GTG CAG 3') were synthesized. Using genomic DNA of *Ld*K1 PCR amplification was performed to obtain full length ORF (Fig 5.1). The PCR product was cloned and its nucleotide sequence determined. The deduced aminoacid sequence from the *Leishmania* centrin ORF was used to find homologues using BLAST program. The sequence exactly matched with that of centrin cloned from cosmid library of *L. donovani* from Sudan (Ld1S2D), submitted to the Genbank (Accession number: AF406767).

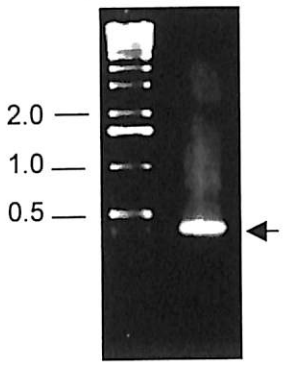

Fig 5.1PCR showing the amplification of *Ld*Cen ORF. The 0.45 kb *Ld*Cen ORF is shown by the arrow.

Leishmania centrin gene consisted of a 450bp open reading frame encoding a ~17kDa protein. The nucleotide and aminoacid sequences of the *Leishmania* centrin are shown in Fig 5.2. Analysis of the nucleotide sequence indicated the presence of at least two splice sites (AG dinucleotides) and a short polypyrimidine stretch in the 5'-untranslated region of the gene, a feature typically found in *Leishmania* genes (Fig 5.2). Further, sequence analysis revealed the presence of EF-hand (Calcium binding) domains in *Leishmania* centrin. Centrins and calmodulins, another closely related $Ca2^{+}$ binding protein, in general, have four EF-hand domains; however, the number of functional EF-hand domains vary among the centrins (Huang et al, 1988; Brugerolle et al, 2000). Sequence motif analysis of *L. donovani* centrin protein predicted only two $Ca2^{+}$ binding sites (EF-hand 1 and 4) (Fig 5.2). In addition, the *Ld*Cenp was also found to possess hydrophobic amino acids in their α-helices around both the EF-hands 1 and 4 as have been observed with other centrins.

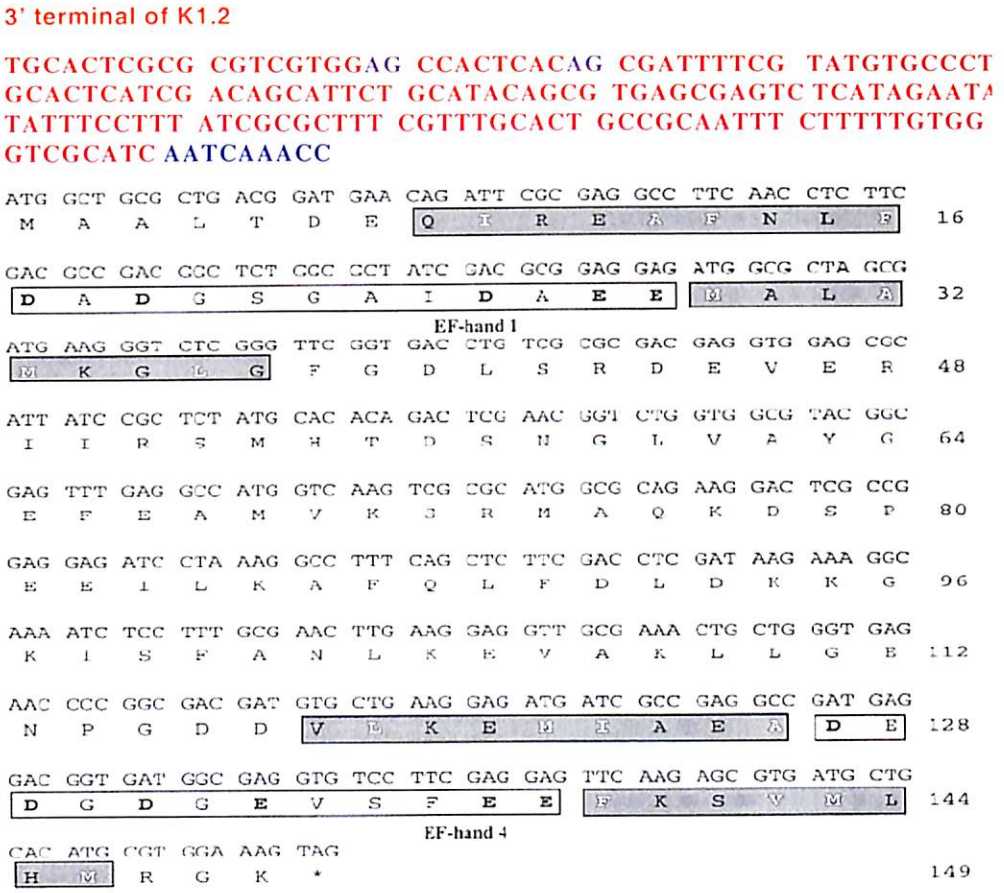

Fig 5.2 Nucleotide and amino acid sequence of *L. donovani* Centrin. The 3' end of the K1.2 fragment overlapped with 5' end of Centrin UTR. The overlapped region is shown in red. Two putative splice sites are shown in purple. The deduced amino acid sequence of *Ld*Cenp is shown in single letter code. In each EF-hand, a 12-amino acid $Ca2^{+}$ binding site (white box) is flanked on either side by a 9-amino acid α-helical stretch (gray box). Acidic amino acids in the $Ca2^{+}$ binding site are shown in bold. Hydrophobic amino acids in the α-helical regions are shown as outlined characters. Asterisk indicates the stop codon.

The amino acid sequence of *Ld*Cenp was analyzed by ClustalW alignment with centrins from different organisms. The calculated percent similarity shows that it is closer to HsCen2p (61%), HsCen1p (60%), or *Trichomonas* centrin (60%) than other centrins. Features that distinguish *L. donovani* centrin from other centrins were identified. *Leishmania* centrin had a significantly small N-terminal region compared with centrins from other species (Fig. 5.3). There was

greater sequence divergence in EF hands 2 and 3. The second EF-hand domain was clearly divergent among the four groups of sequences. The N'-terminal non-conserved domain of centrins, which is variable in length, is considered to be responsible for the functional diversity of centrins (Salisbury et al, 1995; Bhattacharya et al, 1993).

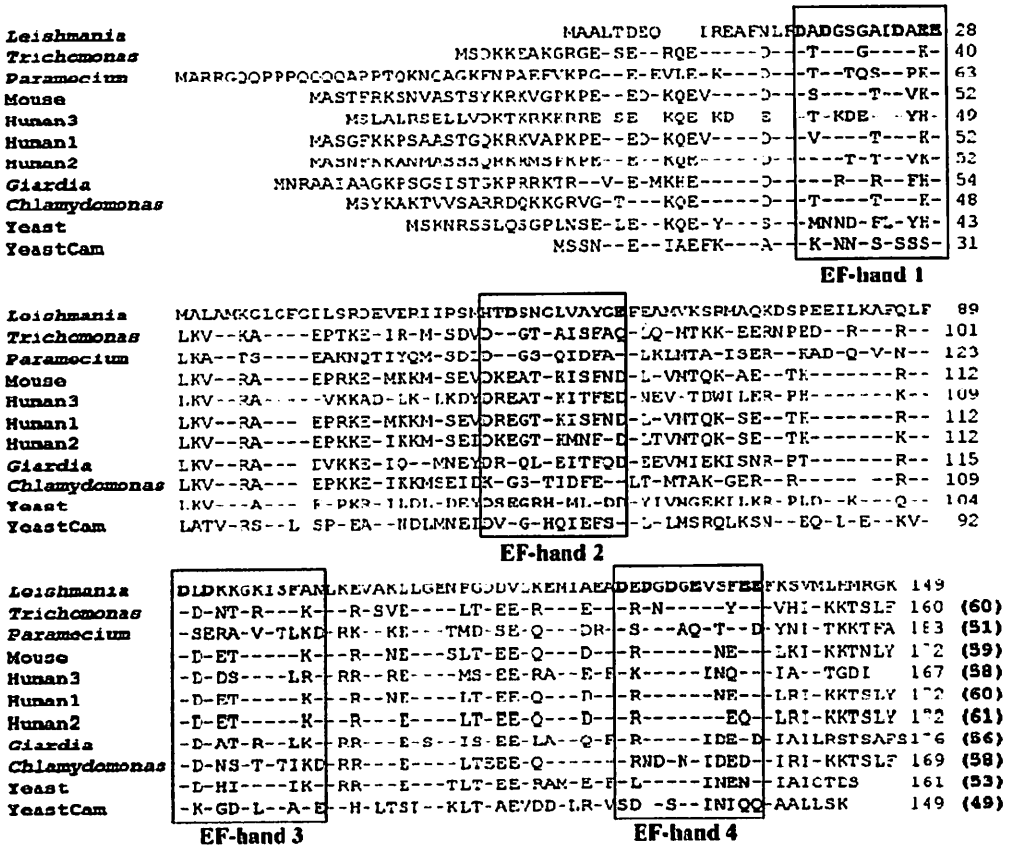

Fig 5.3 Multiple alignment of centrin sequences of various eukaryotes, *L. donovani, Trichomonas, Paramecium,* mouse, human isoforms 1-3, *Giardia, Chlamydomonas,* yeast. A calmodulin gene sequence of yeast is also included. Amino acids are listed in the standard one-letter code, and residues identical to *Leishmania* centrin are indicated by dashes. The gray boxes (EF-hands 1-4) are the putative $Ca2^{+}$ binding domains. Acidic amino acids in the boxes are printed in bold. Bold numbers in parenthesis at the end of the last lines represent the percent similarity of each to *L. donovani* centrin.

Phylogenetic analysis

Based on aminoacid sequences of centrins of various eukaryotes a neighbor-joining systematic tree was constructed, to study the phylogenetic relationship of *Ld*Cenp with centrins of other organisms. Two distinct clusters were seen in the tree (Fig. 5.4). One cluster had CrCen1p, mouse centrin, HsCen2p, and HsCen1p, and the second cluster had *Giardia* centrin, CDC31p, and HsCen3p. However, centrins of *Paramecium* and *Leishmania* branched off independently from the common ancestor of all the centrins.

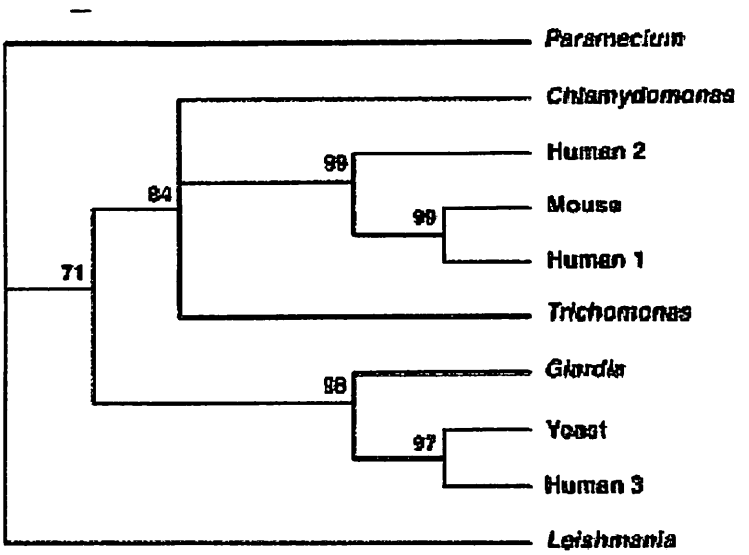

Fig 5.4 Phylogenetic analysis of centrins from various eukaryotes. The dendrogram of complete protein sequences of centrins was generated in the ClustalW-alignment section of MacVector 7.0 program utilizing systematic, bootstrap, and neighbor-joining options. The numbers on the nodes indicate the proportion of times (%) the centrins (shown on the right) grouped together in-1000 bootstrap samples in the program. The branching order rather than the actual distances on the tree, is shown.

*Parasite growth regulated expression of Ld*Cen

Centrins have been implicated to have an essential function during the cell division cycle (Schiebel and Bornens,1995). As a first step toward understanding the role of *Ld*cen in *Leishmania* growth, the correlation between the expression of *Ld*cen and the parasite growth was investigated. The level of expression of centrin mRNA was measured at different stages of promastigote and axenic amastigote growth by Northern analysis. The *Ld*cen hybridized to ~1.7kb RNA that showed comparable expression in both promastigotes and amastigotes, however, its expression varied markedly according to parasite growth stage. Quantitation of Northern data indicated that the level of *Ld*cen mRNA was maximal in the exponentially growing culture in both the promastigotes and axenic amastigotes. The levels of mRNA steadily declined as the parasites progressed from log to stationary phase, thereby indicating that the expression of centrin correlated with the growth of *L. donovani* (Fig 5.5).

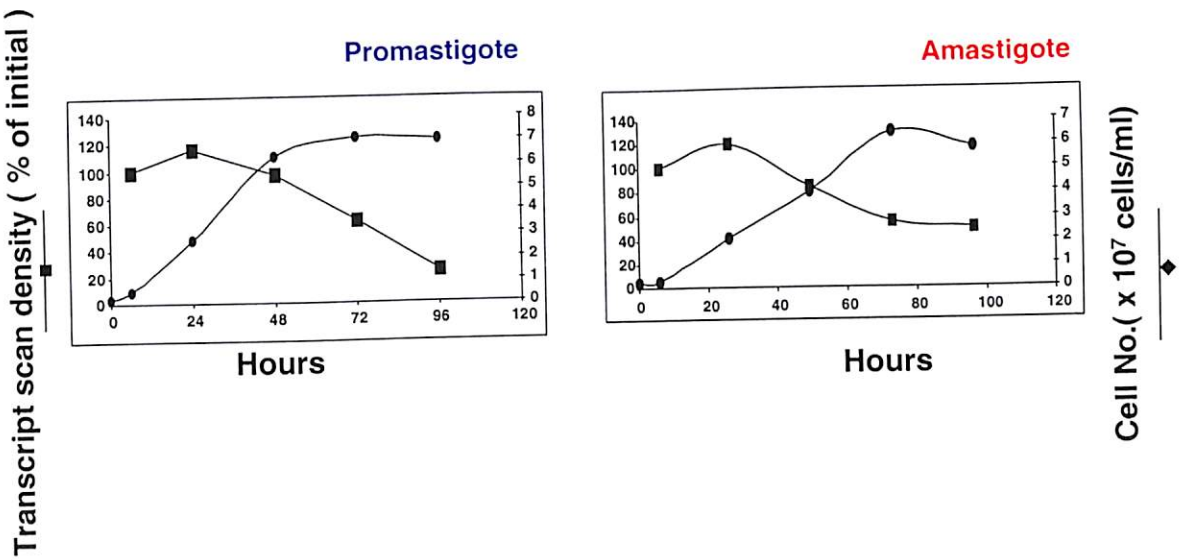

Fig 5.5 Growth regulated expression of *Ld*cen. Cells were collected at different time points during growth and stationary phase of both promastigote and axenic amastigote cultures. These cells were then processed for isolation of total RNA. The RNA samples were analyzed by Northern blot using *Ld*cen as probe. Fig shows the quantitation of Northern blots at various time points. The data were the representative of two independent experiments.

Bacterial expression of Ldcen

Recombinant *Ld*cen was expressed in *E. coli* M15 and was affinity purified to near homogeneity through Ni-NTA column. Cloning and expression of *Ld*cen is schematically described (Fig 5.6). The recombinant protein was purified by Nickel agarose affinity chromatography. The approximate molecular mass of the *Leishmania* centrin protein was determined as 17kDa on SDS-polyacrylamide gel. The recombinant *Ld*cen protein reacted with anti-6xHis antibodies confirming the synthesis of *Ld*cen in *E. coli* (Panel B, Fig 5.6). The recombinant protein was used to determine if *Ld*cenp elicited humoral antibodies in VL. The sera from VL patients did not react with *Ld*cenp when tested by Western blotting (data not shown).

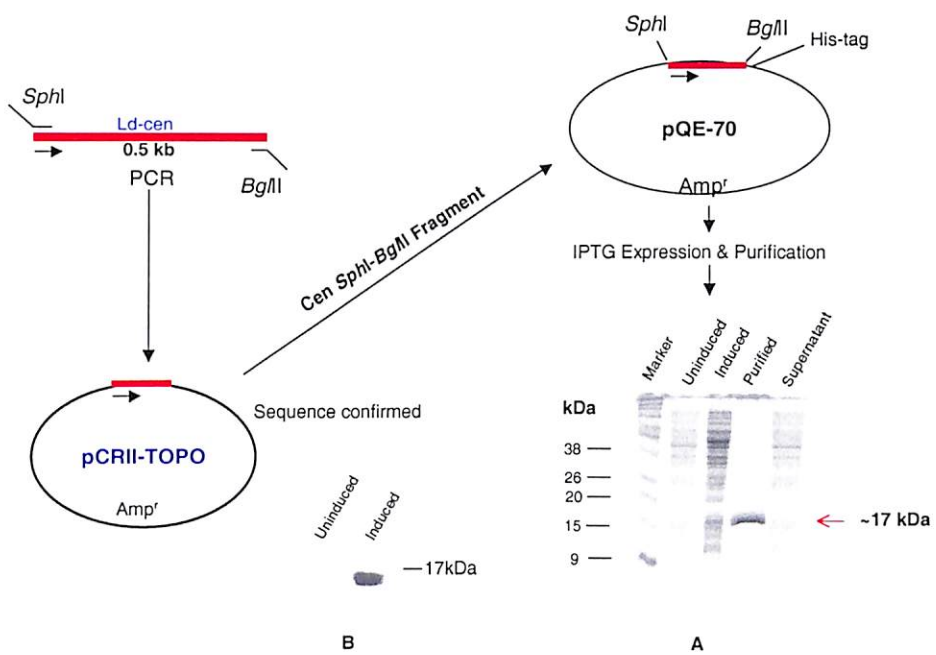

Fig 5.6 Bacterial expression and purification of *Ld*cen. Panel A. *Ld*cen ORF was expressed in pQE70 by induction with IPTG and the product purified by nickel NTA affinity chromatography. Panel B. Expression of recombinant *Ld*cen was confirmed by western blotting with anti-6Xhis antibodies and detected by anti-mouse IgG-HRP using ECL kit.

LdCenp localizes at the basal body region of the promastigotes

To ascertain the localization of centrin in *L. donovani*, immunofluorescence analysis was carried out. Polyclonal antibodies raised in rabbit against *Ld*cen protein were obtained commercially (Spring Valley Labs, USA). Paraformaldehyde-fixed mid-log phase promastigotes were stained with anti-*Ld*Cenp antibody and examined by confocal microscopy. The intensity of fluorescence by anti-*Ld*Cenp antibody was mostly concentrated in the anterior part of the parasites. The basal body has been shown to be localized at the flagellar root that remains tightly associated to the

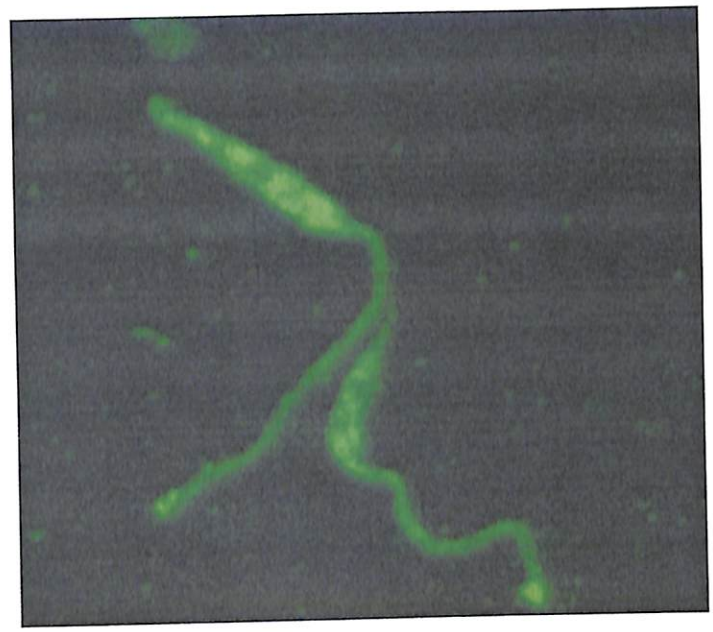

Fig 5.7 Immuno-localization of Centrin in *Ld*K1 promastigotes. Immunofluorescence analysis of promastigotes using rabbit anti-LdCenp antibody. Image was viewed under the confocal microscope.

kinetoplast in *Leishmania.* The results showed that *Ld*Cenp was predominantly localized close to the kinetoplast of growing promastigotes and may be associated with the basal body of *Leishmania* (Fig 5.7). The fluorescence intensity was high when cells in actively dividing phase were used and significantly reduced in the resting cells.

Discussion

Centrin is a calcium-binding protein involved in the contractile function of the cytoskeletal structures in eukaryotes (Salisbury, 1995; Schiebel and Bornens, 1995). In the present study, we have cloned and characterized centrin for the first time in Kinetoplastid parasites. *Ld*cen was cloned from the genomic DNA of *L. donovani* isolate *Ld*K1 and the recombinant protein was synthesized. Further, the *Ld*cen gene that we have identified showed parasite growth regulated expression when its expression was monitored at several stages of the growth. The aminoacid sequence of *Ld*cen was compared to those from other organisms to understand the function. The cloned gene *Ld*Cen, from the *L. donovani* parasite was more homologous to centrin than the other closely related $Ca2^{+}$ binding EF-hand proteins such as calmodulins. Recently, a Ca2+ and calmodulin-dependent phosphatase from *Leishmania* has been identified (Banerjee et al, 1999).

Numerous centrin proteins have been described in various species from all kingdoms. Centrin genes have been cloned in several protists, *Giardia lamblia*, a diplomonad, *Paramecium*, or *Naegleria gruberi*, an amoebo-flagellate, and in a land plant, *Atriplex nummularia*. Centrin genes have also been cloned in vertebrates, including the amphibian *Xenopus laevis* and the mammals *Mus musculus* and *Homo sapiens* (Salisbury, 1995).

Centrins from other organisms have been studied in some detail especially in green algae and in yeast. Centrin functions in animal cells remain largely unknown. Injection of heterologous centrin in two-cell stage *xenopus* embryos led to undercleavage suggesting an essential function of animal centrin during the cell

division cycle as was found in *S. cerevisiae*. However, centrosome duplication did not appear to be affected (Paoletti et al, 1996).

Sequence motif analysis of *L. donovani* centrin protein (*Ld*Cenp) predicted only two $Ca2^+$ binding sites (EF-hand 1 and 4) unlike other members of the family that have multiple $Ca2^+$ binding sites. In case of human centrin 3 only one functional $Ca2^+$ binding site was predicted. In order to understand the function of centrin it is necessary to verify its calcium binding capacity. *In vitro* calcium binding assays with three *Ld*Cenp mutants that had either the first, fourth, or both putative $Ca2^+$ binding domains deleted confirmed that the EF hands 1 and 4 were the only functional calcium binding domains in *Leishmania* (Selvapandiyan et al, 2001). Quantitative estimate of this binding revealed that the *Ld*Cenp ΔN (N terminal deleted) bound 16-fold more ^{45}Ca than the *Ld*Cenp ΔC (C terminal deleted).

Centrins are distinguished structurally from calmodulin by a longer N-terminal variable region. This longer N-terminus is thought to confer functional diversity to centrins (Wiech et al, 1996; Salisbusry, 1995). However, this region is smaller in *Ld*cen than all other centrins and calmodulins. The short N terminus may reveal its ancestral position in the evolution of centrin. Phylogenetic analysis revealed that *Leishmania* centrin is closest to human centrin2 (61%homology). This finding was substantiated by the observation that *Ld*Cenp cross-reacted with the antibody raised against human centrin protein (Selvapandiyan et al, 2001). Phylogenetic analysis revealed that *Ld*cenp branched off independently from a common centrin ancestor, while most other centrins could be grouped into two clusters. These clusters interestingly also correlate with their distinct biological functions as observed (Laoukili et al, 2000; Middendorp et al, 1997; Khalfan et al, 2000). HsCen1p,

HsCen2p, and CrCen1p of one cluster have been involved in the segregation of centrosome, whereas CDC31p and HsCen3p of the other cluster have been involved in centrosome duplication during cell division (Laoukili et al, 2000; Middendorp et al, 1997; Khalfan et al, 2000). Despite *Leishmania* centrin's early divergence, the unique binding of the anti-HsCen2p antibody suggests structural relatedness of LdCenp and Hscen2p.

The *Ld*cen ORF was expressed in a bacterial expression system. The protein band could be readily detected on SDS-polyacrylamide gels after purification with Nickel agarose chromatography. Expression of *Ld*cen allowed us to produce polyclonal antibodies and to immunolocalize the *Ld*cen gene product in *Leishmania.* In the present study, *Ld*Cenp was found to be localized at the basal body of the parasite, and its expression was high when cells were actively dividing but significantly reduced in the resting cells. This correlation of expression pattern suggested that *Leishmania* centrin may have a role in the growth of the parasite. Multiple centrins are localized at the centrosome and expressed in all the dividing cell types (Schiebel and Bornens, 1995). Specifically HsCen2p expression level increases during ciliogenesis in the tracheal epithelial cells, and its involvement in cell division as well as cellular motility has been demonstrated (LeDizet et al, 1998; Wolfrum and Salisbury, 1998).

Centrin's association with growth in several eukaryotes has been studied (Taillon et al, 1992). Injecting recombinant *Chlamydomonas* centrin or human centrin 2 in two cell stage frog embryos delayed cleavage and promoted the formation of abnormal blastomeres (Paoletti et al, 1996). Expression of both antisense and sense transcripts of centrin arrested spermiogenesis in *Marsilea vestita* (Klink and Wolniak

2001). The importance of the N-terminal sub-domain for centrin function was emphasized similarly by observing slower growth rate in the yeast cells expressing centrin (CDC31), which had the N-terminal region replaced with that of *S. dubia* centrin. Secondly, yeast centrin interacts with the cellular protein Kar1p at its C-terminal region (Wiech et al, 1996; Geier et al, 1996).

Centrins have been implicated to have an essential role during the cell division cycle. The present study demonstrated growth regulated expression of centrin in *Leishmania.* When the deletion constructs lacking either the N- or C-terminal $Ca2^{+}$ domains or both were transfected into the *L donovani* parasites to understand the role of centrin in the growth of *L. donovani*, it was found that the mutant parasites expressing N-terminal deleted centrin displayed a significant reduction in the parasite growth (Selvapandiyan et al, 2001).

In conclusion, understanding the role of centrin in *Leishmania* growth could provide ways to alter the growth of the parasite and clues for the development of attenuated *Leishmania* vaccine candidates.

The arbitrary-primed PCR (AP-PCR) technique was employed with the twin goals of identifying genetic polymorphisms within the Indian isolates of *Leishmania donovani* and to identify differentially expressed gene sequences. The parasite isolates from Indian Kala-azar patients could be differentiated from *Leishmania donovani* isolates from distinct geographic regions such as Ethiopia, Sudan and Spain. Moreover, differences within the Indian isolates could also be identified. A majority (17/19) of the Indian isolates gave identical AP-PCR pattern, while two isolates gave consistently divergent pattern. Using a typical isolates *Ld*K1, several polymorphic fragments were identified. The distinctive AP-PCR fragments obtained with Indian isolates were used as probes in Northern blot analysis to identify genes that might have a role in parasite growth or virulence. Three such fragments were found to represent transcribed sequences. These transcribed sequences led to cloning and characterization of two important genes of *Leishmania*; a novel gene termed A-1 gene that is over-expressed in amastigote stage of the parasite and the Centrin gene that showed growth regulated expression. The study demonstrated the utility of random genome sampling methods in genomic fingerprinting and in identifying differentially transcribed sequences whose gene products could potentially contribute to parasite virulence.

Cloning and characterization of a novel gene Ld A-1

A novel amastigote specific gene was cloned and its molecular characterization was undertaken. The nucleotide sequence analysis showed that the gene comprised of a 528bp ORF encoding a ~20kDa protein. The A-1 gene showed consistently upregulated expression in amastigote stage at several

points of the parasite growth indicating its importance in amastigote replication. In *L. donovani* genome A1 was found to be present as a single copy gene. Sequence analysis of the 3'UTR showed that this gene did not contain the conserved 450nt sequence shown to be regulating stage specific expression in some other characterized amastigote specific genes, majority of which are multicopy genes. Nucleotide polymorphism in the A-1 locus indicated that this could be used as a molecular tool to discriminate *Leishmania* species. For the first time, the presence of transcripts of a gene showing higher expression in amastigote stage were demonstrated in bone marrow and splenic aspirates of KA patients, confirming the expression data obtained with RNA derived from *in vitro* grown parasites, thus making A-1 gene a good candidate for quantitative PCR experiments to determine active parasite burden in a range of tissues. The recombinant A-1 was expressed in *E coli* as a ~23kDa protein. The recognition of prokaryotic membrane lipoprotein lipid attachment site on protein motif search suggested A-1 to be a membrane bound protein.

Further studies are warranted to determine if the A-1 may be a suitable target for considering molecular attenuation of virulence by gene deletions or modifications, thereby producing attenuated mutants for vaccination.

Cloning and characterization of Ld Centrin

Centrin gene was identified for the first time in kinetoplastid protozoa. Structural and functional characterization of *Leishmania* centrin was undertaken. Centrin is a calcium-binding cytoskeletal protein essential for centrosome duplication or segregation. The cloned *Ld*cen had an ORF of 450bp encoding for a 17kDa protein. The deduced amino acid sequence of LdCenp was analyzed

by ClustalW alignment with centrins from different organisms. The calculated percent similarity showed that it was closer to HsCen2p (61%), HsCen1p (60%), or *Trichomonas* centrin (60%) than other centrins. *L. donovani* centrin had a significantly small N-terminal region compared with centrins from other species. Centrins and calmodulins, another closely related $Ca2^+$ binding protein, have in general four EF-hand ($Ca2^+$ binding) domains; Sequence motif analysis of *L. donovani* centrin protein (LdCenp) predicted only two $Ca2^+$binding sites (EF-hand 1 and 4). Immunofluorescence analysis localized the protein in the basal body. The levels of centrin mRNA were high during the exponential growth of the parasite in culture and declined to a low level in the stationary phase.

The growth-regulated expression pattern indicated that Centrin is essential for parasite proliferation. It is of interest to see if a mutant lacking such an essential gene could be tested as an attenuated vaccine candidate.

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Appendix

Preparation of reagents

10X MOPS

0.2M MOPS pH 7.0, 20mM Sodium acetate, 10mM EDTA pH 8.0

Filter sterilized with 0.45μm filter.

DEPC water

0.1% diethylpyrocarbonate was added to 1ltr double distilled water in a fume hood and mixed well. After incubating it for 1hr at 37°C it was autoclaved.

100X Denhardt's solution

Ficoll400	10g
Polyvinylpyrrolidone	10g
Bovine serum albumin Fraction V	10g
ddH_2O	500ml

Filter sterilized and stored at 4°C.

20X SSC

175.3gm of NaCl, 88.2gm of Sodium citrate were dissolved in 800ml H_2O. pH was set to 7.0 with HCl. Volume was adjusted to 1ltr, aliquots prepared and the solution was sterilized by autoclaving.

Preparation of bacterial culture media

LB medium (Luria Broth)

10 gm of Tryptone, 5gm of Yeast, 5gm of Sodium chloride (Hi Media) were dissolved in 1liter of double distilled water. Media were sterilized by autoclaving for 20 minutes at 15 lb/sq.in.

LB Agar

15gm of agar powder, 10gm of Tryptone, 5gm of Yeast, 5gm of Sodium chloride, (Hi media) were dissolved in double distill water. Media were sterilized by autoclaving for 20 minutes at 15lb/sq.in. LB agar was allowed to cool to 45°C and poured (~30 ml per plate) in 90 mm disposable petri plates (Tarsons) along with appropriate antibiotics and allowed to solidify.

Antibiotics

Ampicillin

100mg/ml ampicillin stock was prepared in autoclaved double distilled water and sterilized by filtration through 0.22 μm filter. 100μl aliquots were stored by freezing at –20°C.

Stock solution of commonly used reagents

1M Tris

121.1gm of Tris base was dissolved in 800ml of double distilled water and pH set (6.8, 7.4, 8.0) with concentrated HCl. Volume was made up to 1liter and autoclaved.

0.5M EDTA

186.1gm of disodium EDTA.-$2H_2O$ was added in 800ml of double distilled water, stirred vigorously on a stirrer, pH set to 8.0 with NaOH (~20 gm of NaOH pellets) and volume made up to1liter and autoclaved.

3M sodium acetate

204.5gm of $C_2H_3O_2Na$. $3H_2O$ was dissolved in 400ml of ddH_2O, pH set to 5.3 with glacial acetic acid, volume made up to 500 ml and autoclaved.

10% SDS

10gm of electrophoresis grade SDS was dissolved in 70ml of ddH_2O, heated at 60°C to dissolve and the volume made up to 100ml.

Ethidium Bromide (10 mg/ml)

10mg of ethidium bromide was dissolved in 1ml ddH_2O, stored in a opaque bottle.

30% Acrylamide Stock

29.2gm of acrylamide and 0.8gm of bis-acrylamide were dissolved in 50ml of ddH_2O. Volume was made up to 100ml, the solution filtered through Whatman no. 1 paper, degassed and stored in an opaque bottle.

Calcium Chloride (0.1 M)

1.47gm of $CaCl_2.2H_2O$ was dissolved in 100ml of ddH_2O and sterilized by autoclaving.

IPTG (1M)

238mg of IPTG was dissolved in 1ml of ddH_2O, filter sterilized and stored at -20°C in 50µl aliquots.

Sodium Phosphate (1M)

Monobasic

138gm of $NaH_2HPO_4.H_2O$ was dissolved in 800ml of ddH_2O and volume made up to 1liter.

Dibasic

268gm of $Na_2HPO_4.7H_2O$ was dissolved in 700ml of ddH_2O and volume made up to 1liter.

Ammonium persulfate (10%)

To 1gm of ammonium persulfate, 10ml of ddH_2O was added and the solution stored for several weeks at 4°C.

10 X TAE buffer (Tris acetate, EDTA)

4.84gm of Tris base in 80ml of ddH_2O was dissolved and 1.2ml of glacial acetic acid and 2ml of 0.5 EDTA pH 8.0 were added. Final volume was made up to 100ml.

10X TBE buffers (Tris borate, EDTA)

8gm of Tris base, 55 gm of boric acid and 9.3gm $Na_2EDTA.\ H_2O$ were dissolved in 700ml ddH_2O and the final volume made up to 1 liter.

Phosphate Buffer Saline (PBS)

8gm of NaCl, 2gm of KCl, 1.44gm of Na_2HPO_4 and 0.2gm of KH_2PO_4 were dissolved in 800ml of ddH_2O. pH was set to 7.4 with HCl. Final volume was made up to 1 liter and sterilized by autoclaving at 15lb/ sq.in for 20 minutes and stored at room temperature.

SDS-PAGE electrophoresis buffer

3gm of Tris base, 14.4gm of glycine and 1gm of SDS were dissolved in 1 liter of ddH_2O.

Protein transfer buffer

5.8gm of Tris base, 2.9gm of glycine and 0.33gm of SDS were dissolved in 500ml of ddH_2O. 200 ml of ethanol was added and the final volume was made up to 1 liter.

2X SDS-PAGE sample buffer

The composition of sample buffer is as follows

Tris-Cl (pH6.8)	100mM
DTT	200mM
SDS	4%
Bromophenol blue	0.2%
Glycerol	20%
β-mercaptoethanol	10%

10X Ligation buffer

Tris.Cl pH 7.8	500mM
$MgCl_2$	100mM
DTT	100mM
ATP	10mM

10 X Amplification buffer

Tris.Cl pH 8.3	100mM
$MgCl_2$	15mM
KCl	500mM
Gelatin	0.1%

DNA loading dye (6X)

0.2gm bromophenol blue, 0.2gm of xylene cyanol and 30ml of glycerol were dissolved and volume set to 100 by autoclaved ddH_2O.

SDS-PAGE reagents

Composition of resolving gel (12%) 10 ml

30% acrlyamide solution	4.0 ml
1.5M Tris-Cl pH 8.8	2.5 ml
ddH_2O	3.3ml
10% SDS	100µl
10% APS	100µl
TEMED	10µl

Composition of stacking gel (5%) (5.0 ml)

30% acrylamide solution	0.83ml
1.0M Tris.Cl pH 6.8	0.68 ml
ddH_2O	3.4ml
10% SDS	50µl
10% APS	50µl
TEMED	5µl

Staining solution

1gm of coomassie blue was dissolved in 450ml of methanol. 100ml of glacial acetic acid was added and the volume made up to 1liter by double distilled water, filtered through Whatman no.1 filter and stored at room temperature.

Destaining solution

Methanol: water: acetic Acid were mixed in the ratio of 45:45:10 and stored at room temperature.

Publications

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