Dedicated to

My Beloved Parents for their Inspiration & Patience and to My Teachers from Whom I Continue to Learn.....

CDRI

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CERTIFICATE

This is to certify that the thesis work entitled **"Expression proteomics and genomic fingerprinting studies in sodium antimony gluconate (SAG) sensitive and resistant clinical isolates of *Leishmania donovani*"** is a bonafide piece of research work. It has been carried out by **Mr. Awanish Kumar** (M.Sc. Biotechnology) under my guidance and supervision at Central Drug Research Institute (CDRI), Lucknow (India) for the degree of **Doctor of Philosophy (Ph.D.) in Life Sciences** from Jawaharlal Nehru University (JNU), New Delhi (India).

This is to further certify that this Ph.D. thesis embodies the original research work carried out by the candidate himself and has not been submitted elsewhere for any other degree.

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DECLARATION

I hereby declare that thesis entitled **"Expression proteomics and genomic fingerprinting studies in sodium antimony gluconate (SAG) sensitive and resistant clinical isolates of *Leishmania donovani*"** is my own research work carried out under the supervision of **Dr. Anuradha Dube**, Deputy Director and Scientist F, Division of Parasitology, **Central Drug Research Institute (CDRI), Lucknow (India).** I have submitted this thesis to **Jawaharlal Nehru University (JNU), New Delhi (India)**, for the award of the degree, **Doctor of Philosophy (Ph.D.) in Life Sciences** and all the resources quoted in the thesis have been indicated and acknowledged by complete references.

Date: 08/04/2009

Place: Lucknow (India)

Awanish Kumar

(Awanish Kumar)

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"The Self is an ocean without a shore"

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I would like to dedicate this thesis to my parents, for their love, patience, and understanding. Love you all!

Great thanks to all of you once again.

AWANISH KUMAR

ABBREVIATIONS

2-DGE	:	Two-dimensional gel electrophoresis
ABC	:	ATP binding cassette
AFLP	:	Amplified fragment length polymorphism
bp	:	Base pair
BSA	:	Bovine serum albumin
CBB	:	Coomassie brilliant blue
CHAPS	:	3-[(3-cholamido propyl)-dimethyl ammonio]-1-propane sulfonte
CL	:	Cutaneous leishmaniasis
CMI	:	Cell mediated immunity
cRPMI	:	RPMI-1640 with 10% HiFBS
cM199	:	Medium 199 with 10% HiFBS
cDMEM	:	DMEM with 20% HiFBS
Da	:	Dalton
°C	:	Degree centigrade
DT	:	Drug target
DTT		Dithiothreitol
EDTA	:	Ethylene diamine tetraacetic acid
ELISA	:	Enzyme Linked Immunosorbent assay
FACS	:	Fluorescence Activated Cell Sorting
HiFBS	:	Heat-inactivated fetal bovine serum
HIV	:	Human Immunodeficiency Virus
IC_{50}	:	50% inhibitory concentration
i.c.	:	Intra cardiac
i.p.	:	Intra peritoneal
i.v.	:	Intra venous
IEF	:	Isoelectric focusing
IFN-γ	:	Interferon-gamma
IgG	:	Immunoglobulin G
IPG	:	immobilized pH gradients
IL	:	Interleukin
JAK	:	Janus kinase
kb	:	Kilo base
kHz	:	Kilo hertz
MΦ	:	Macrophage
MCL	:	Mucocutaneous leishmaniasis
MALDI	:	Matrix Assisted Laser Desorption Ionization
Mb	:	Mega-base
mg	:	Milligram
µg	:	Microgram
µl	:	Microliter
ml	:	Milliliter
µM	:	Micromolar
MEP	:	Membrane enriched protein
STAT	:	Signal transducer and activator of transcription
MHC	:	Major Histocompatibility complex
mA	:	Milli amphere
µg/ml	:	Microgram per millilitre
Sb(III)	:	Trivalent antimonial
mg/ml	:	Milligram per millilitre
min	:	Minute
MDR	:	Multi drug resistance
MS	:	Mass spectrometry
MS/MS	:	Tandem mass spectrometry
M_W	:	Molecular mass
m/z	:	Mass-to-charge
nm	:	Nanometer
NO	:	Nitric oxide
OD	:	Optical density
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PAGE	:	Polyacrylamide gel electrophoresis
PgpA	:	P-glycoprotein A
pI	:	Isoelectric point
p.i.	:	Post infection
PMF	:	Peptide mass fingerprint
PTM	:	Post translational modification
RNI	:	Reactive nitrogen intermediate
ROI	:	Reactive oxygen intermediate
SAG	:	Sodium antimony gluconate
Sb(V)	:	Pentavalent antimonial
SDS	:	Sodium dodecyl sulphate
SP	:	Soluble protein
rpm	:	Rotation per minute
TDW	:	Triple distilled water
TGF-β	:	Transforming growth factor - beta
Th-1/2	:	T helper-1/2 cells
Tris	:	Tris (hydroxymethyl)-amino methane
TNF-α	:	Tumour necrosis factor- alpha
TOF	:	Time-of-flight
USD	:	United states dollar
VC	:	Vaccine candidate
VL	:	Visceral leishmaniasis
WCL	:	Whole cell lysate
WHO	:	World Health Organization

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CHAPTER 1

Introduction

"One of the advantages of being disorderly
is that one is constantly making exciting discoveries".

1. Introduction: *Leishmania* and Leishmaniasis

1.1 Historical background of *Leishmania* and leishmaniasis

Leishmaniasis has been considered a tropical affliction that constitutes one of the six entities on the list of most important diseases of World Health Organization/Tropical Disease Research (WHO/TDR) *viz.,* Malaria, Schistosomiasis, Filariasis, Chagas disease, African Trypanosomiasis, Leishmaniasis, Leprosy, Tuberculosis (Desjeux *et al.*, 2001). According to ranking after malaria it is a second most prevalent parasitic disease. Leishmaniasis caused by protozoan parasites *Leishmania*, is a disease of poverty as its victims are among the poorest.

Historical evidences revealed that representations of skin lesions and facial deformities have been found on pre-Inca pottery from Peru and Ecuador dating back to the first century AD. Some forms of leishmaniasis prevailed as early as this period (http://www.who.int/en). There are detailed descriptions of oriental sore by Arab physicians including Avicenna in the 10th century, who described it as Balkh sore from northern Afghanistan, and there are later records from various places in the Middle East including Baghdad and Jericho; many of the conditions were given local names by which they are still known.

In the Old World, Indian physicians applied the Sanskrit term kala-azar (meaning 'black fever') to an ancient disease later defined as visceral leishmaniasis (VL). Kala-azar was first noticed in Jessore in India in 1824, when patients suffering from fevers that were thought to be due to malaria failed to respond to quinine. By 1862 the disease had spread to Burdwan, where it reached epidemic proportions. In 1901, William Leishman identified certain organisms in smears taken from the spleen of a patient who had died from 'dum-dum fever'. Initially, these organisms were considered to be trypanosomes, but in 1903 Captain Donovan described them as being new. The link between these organisms and KA was eventually discovered by Major Ross, who named them *Leishmania donovani*. The search for a vector was a long one, and it was not until 1921 that the experimental proof of transmission to humans by sandflies belonging to the genus *Phlebotomus* was demonstrated by Edouard and Etienne. (Swaminath *et al.*, 2006) proved using human volunteers that the *Leishmania* parasite could be transmitted by *Phlebotomus* sandflies.

1.2 Risk factors and definition of the problem

In India, a country with a high leishmaniasis burden, 88% of leishmaniasis patients have a daily income of less than 2 USD and poor economic level (Murray *et al.*, 2005). The number of cases of leishmaniasis is increasing, mainly because of man-made environmental changes that increase human exposure to the sandfly vector (Desjeux *et al.*, 2001). Extracting timber, mining, building dams, widening areas under cultivation, creating new irrigation schemes, expanding road construction in primary forests such as the Amazon, continuing widespread migration from rural to urban areas, and continuing fast urbanization worldwide are among the primary causes for increased exposure to the sandfly (Desjeux, 2004b). The leishmaniasis causes considerable morbidity and mortality. It is the collective name for a number of diseases which have diverse clinical manifestations. Leishmaniasis has traditionally been classified in three major forms on the basis of clinical symptoms (Handman, 2001). The most deadly form is visceral leishmaniasis (VL), which if left untreated, leads to death. A number of other species of *Leishmania* cause cutaneous (CL) and mucocutaneous (MCL) leishmaniasis, which, if not fatal, are still responsible for considerable morbidity of a vast number of people in endemic foci of the world (Peters *et al.*, 1983; Prasad, 1999).

1.3 Types of leishmaniasis

Leishmaniasis is divided into three general clinical patterns according to the form of the disease.

1.3.1. Cutaneous Leishmaniasis (CL):

This is the most common form of Leishmaniasis, also known as 'Oriental sore' which first appears as a persistent insect bite. Simple skin lesions appear at the site of sandfly bite (figure 1.1) which self-heal within few months but leaves scars. The incubation period can last from few days to months. Gradually, the lesion enlarges, remaining red, but without noticeable heat or pain. Resolution of the lesion involves immigration of leucocytes, which isolate the infected area leading to necrosis of the infected tissues, and formation of a healing granuloma.

The disease is mostly prevalent in Mediterranean region, Central Asia and many places of Central Africa (Chatterjee and Ghosh, 1957). Man is the definitive host whereas gerbils, cats, dogs, and rodent act as the natural reservoir of CL. Sandflies of genus *Phlebotomus* serve as

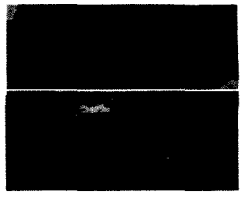


Figure 1: skin ulcer

disease. CL is usually caused by *L. major, L. tropica, L. aethiopica,* in old world and by *L. mexicana, L venezuelensis, L. amazonensis, L. braziliensis, L. panamensis, L. guyanensis* and *L. peruviana* in new world.

1.3.2. Diffuse cutaneous leishmaniasis (DCL):

This is a chronic, progressive, polyparasitic variant that develops in context of leishmanial-specific anergy and is manifested by disseminated non-ulcerative skin lesions, which can resemble lesions of lepromatous leprosy (Fig 1.2). DCL is restricted to Venezuela and Dominican Republic in the western hemisphere, and to Ethiopia and Kenya in Africa. Its main causative organisms are *L. aethiopica* (old world) *and L mexicana* species complex (new world).

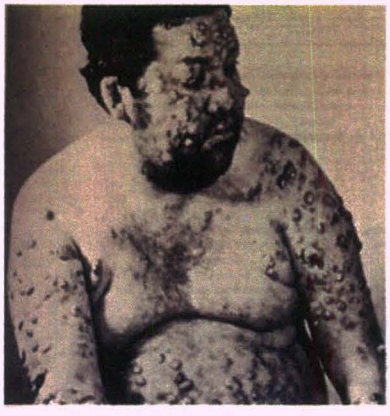


Figure 1.2: Symptoms of DCL.

1.3.3. Mucocutaneous Leishmaniasis (MCL):

This form of disease, also known as ''espundia'', causes extensive destruction of naso-oral and pharyngeal cavities with hideous disfiguring lesions, mutilation of the face (Figure1.3) and great suffering for life. MCL is occasionally reported from Sudan and other Old World foci. Classical MCL is, however, restricted to *L. braziliensis* infections in which, following the apparently complete resolution of the initial oriental sore, sometimes many years later, metastatic lesions appear on the buccal or nasal mucosa. MCL usually exists as an azoonotic infection in which lifecycle is being transmitted from rodent to rodent and mammal by the forest sandfly *Lutzomyia spp.* The reservoir hosts include rodents, opossums, anteaters, sloths and dogs etc. Human infection occurs when human invade the forest habitats.

The causative agents of MCL in old world are *L. aethiopica* (rare), and in new world are *L. braziliensis, L. guyanensis,* L. mexicana, L. amazonensis and L. *panamensis.*

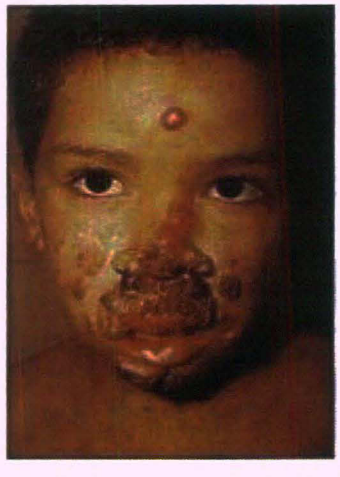


Figure 1.3: Mucocutaneous Leishmaniasis (MCL) in the patient with a perforated nasal septum and mucosal tissue destruction.

1.3.4. Visceral Leishmaniasis (VL) or Kala-azar (KA):

VL is the most dreaded and devastating amongst the various forms of leishmaniasis. VL is also known as Kala-Azar, Black Sickness, Black Fever, Burdwan fever, Dumdum fever or Sarkari Bimari etc. It is the most severe form of disease and if left untreated, is usually fatal. The parasite is responsible for a spectrum of clinical syndromes, which can, in most extreme cases, move from an asymptomatic infection to a fatal form of VL.

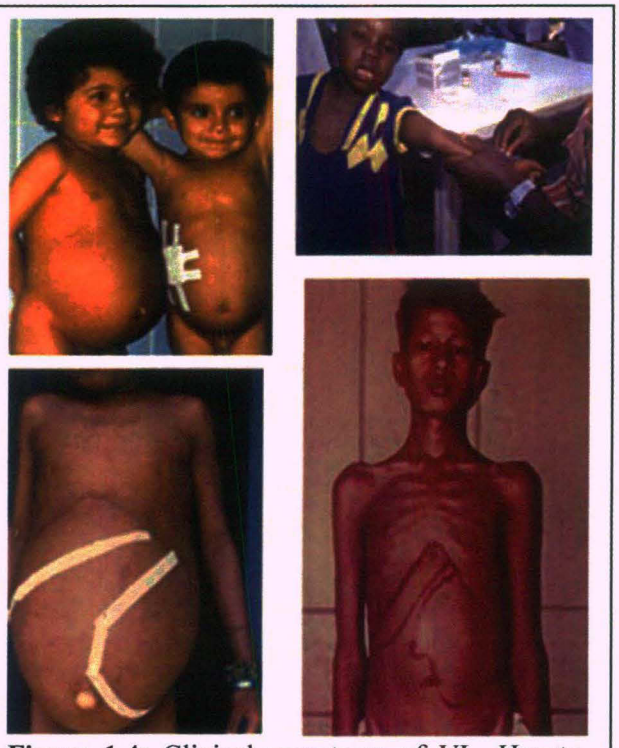


Figure 1.4: Clinical symptoms of VL. Hepato-splenomegaly and substantial weight loss are main features.

It is characterized by prolonged fever, splenomegaly, hepatomegaly, substantial weight loss, progressive anemia, pancytopenia, and hypergammaglobulinemia (mainly IgG from polyclonal B cell activation) and is complicated by secondary opportunistic infections (Figure1.4.). The parasite invades and multiplies within macrophages (free mononuclear phagocytic cells) and affects the reticuloendothelial system including spleen, liver, bone marrow, and lymphoid tissue (Aggarwal *et al.*, 1999; Boelaert *et al.*, 2000). The outcome of fully developed VL is death, usually said to be due to concomitant infection resulting from the weakened immunological state of the patient.

VL is typically caused by *L. donovani* complex, which includes three species: *L. donovani donovani, L d. infantum*, and *L. d. chagasi*. *L. donovani* is the causative in the Indian subcontinent and East Africa. *L. infantum* causes VL in the Mediterranean basin and *L. chagasi* is responsible for the disease in Central and South America (Aggarwal *et al.*, 1999; Garg and Dube, 2006; Singh, 2006). VL is emerging as an important opportunistic infection among people with HIV-1 infection (WHO, 1994; Alvar *et al.*, 1997; Desjeux and Alvar, 2003). In fact, the parasite may be a cofactor in the pathogenesis of HIV infection in human (Bernier *et al.*, 1998).

There are more than 21 morphologically indistinguishable species of *Leishmania* that infect humans. Conventionally, they are classified and named mainly according to their geographical distribution and clinical characteristics of the disease they afflict (Chang and Chang, 1985; Roberts *et al.*, 1996; Bogitsh *et al.*, 1999; Herwaldt, 1999).

The **Post kala-azar Dermal Leishmaniasis (PKDL)** is a type of non ulcerative cutaneous lesion (Figure 1.5). After recovery from *L. donovani* infection , VL patients may develop a chronic form of cutaneous leishmaniasis i.e., PKDL which is developed in about 10% of kala-azar patients generally one or two years after the completion of SAG treatment and requires a long and expensive treatment (Rees et al., 1984; Salotra and Singh, 2006). PKDL lesions develop 1-13 months post antimony treatment in Sudan, and 1-3 years post antimony treatment in India.

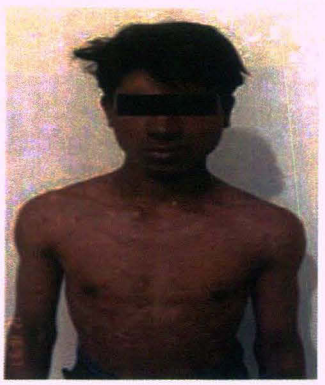


Figure 1.5: PKDL patient with papular-nodular lesions over face, chest, and arms. *[Courtesy: Murray et al., (2005). Lancet 366: 1561-77].*

1.4. Geographical distribution of leishmaniasis

Leishmaniasis occurs in 88 countries in tropical and temperate regions, of which 72 are either developing or least developed. Approximately 1,98,000 people are affected with these diseases worldwide with 5,00,000 million new cases occurring each year (WHO, 2001) but the true picture remains largely hidden since a substantial number of cases are never recorded (Herwaldt, 1999). The disability-adjusted life years (DALY) burden was 2,357,000 and total deaths were 59,000 in 2001 (WHO, 2002). It has been estimated that 90% of CL cases occur in 7 countries: Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria whereas MCL is endemic in Mexico and Central and South America (Figure 1.6 A).The annual estimate for the incidence and prevalence of kala-azar cases worldwide is 0.5 million and 2.5 million, respectively (Desjeux, 2004b; Croft *et al.*, 2006a) and of these, 90% cases occur in India, Nepal, Bangladesh and Sudan. In India VL is most prevalent in Bihar, West Bengal, Assam and Eastern Uttar Pradesh. Muzaffarpur district of Bihar is the epicenter for VL. PKDL is prevalent in India, Sudan and Kenya. A summary of clinical manifestations and geographic distribution of the *Leishmania* species is summarised in Table 1.1.

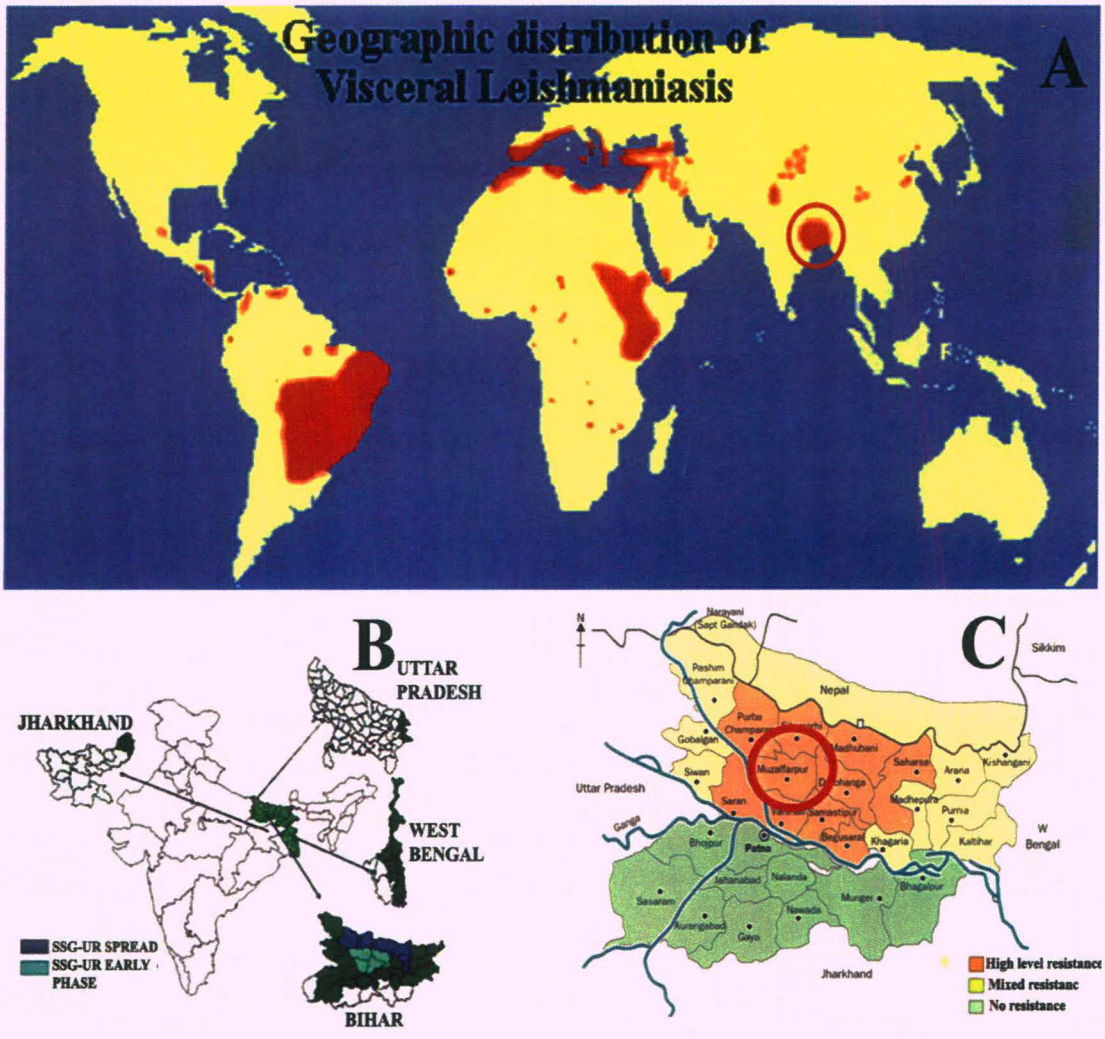


Figure 1.6: Geographical distribution of visceral leishmaniasis (VL); **(A)** Worldwide distribution of VL, **(B)** VL affected states of India and **(C)** VL affected districts of Bihar.

Table 1.1: Summary of clinical manifestations and geographical distribution of *Leishmania* species.

Species	Clinical manifestation	Geographical Distribution
L. donovani	Visceral (kala-azar)	Old World: China, India, Bangladesh.
L. infantum	Visceral	Old World: North Central Asia, Northwest China, Uzbekistan, Middle East.
L. chagasi	Visceral	New World: South and Central America.
L. major	Cutaneous	Old World: Africa, Middle East, Northern Asia
L. mexicana	Cutaneous, Diffuse cutaneous, Mucocutaneous	New World: Southern Mexico, Belize, Northern Guatemala, Southern Texas.
L. amazonensis	Cutaneous, Mucocutaneous	New World: South and Central America.
L. braziliensis	Mucocutaneous, Cutaneous	New World: Throughout South America.

1.5. Global status of visceral leishmaniasis (Kala-azar)

VL is endemic in 62 countries, with 200 million people at risk, an estimated 500,000 new cases each year worldwide (Herwaldt, 1999; Guerin *et al.*, 2002; Thakur, 2003; Desjeux, 2004a), and 41,000 recorded deaths in the year 2000 (WHO, 2001). The disease burden associated with VL, measured in DALYs was estimated to be 1,980,000 (1,067,000 for male and 744,000 for female (Guerin *et al.*, 2002) in year 2000. VL is caused by *L. donovani* in the Indian subcontinent, Asia, Africa and by *L. infantum* in the Mediterranean region, southwest and central Asia and by *L. chagasi in* South America. In Sudan, for example, a major decade-long epidemic of VL occurred from 1984 to 1994. As this was the first epidemic in the area, populations were highly susceptible. Some studies estimate that the disease caused 100,000 deaths in a population of around 300,000 in the western upper Nile area of the country (http://www.who.int/mc/diseases/leish/diseaseinfo.htm). The health ministers of India, Nepal and Bangladesh signed a memorandum of understanding on 18 May 2005, pledging to eliminate VL from their countries. The five elements of the elimination strategy are access to early diagnosis and treatment, strengthening disease and vector surveillance, integrated vector management, social mobilization, networking and research (WHO/TDR News, 2005).

1.6. National status of VL

VL is present in India for more than 100 years. The incidence of KA in India is among the highest in the world (Desjeux, 1992). Epidemics of KA occurred in Bengal in the years 1832, 1857, 1871, 1877, and 1899. In India about 100,000 cases of VL are estimated to occur annually (TDR News No. 37, Nov, 1991). It has recently posed a serious threat in India involving 38 out of 42 districts of Bihar state, 8 districts of West Bengal and 2 districts of Eastern Uttar Pradesh (Bora, 1999) (Figure 1.6 B,C). In 1977, a sample survey in Bihar estimates the number of cases to be about 1, 00,000 with 4,500 deaths whereas in 1991 infected cases reached to 2, 50,000 with 75,000 deaths (Modabber, 1990, 1995; Thakur *et al.*, 1993). The state of Bihar now accounts for more than 90 percent of cases (Zijlstra *et al.*, 1995). Because of the rapid manner in which the disease was spreading, an alarming situation existed (Desjeux, 1992). Since, VL is more relevant in Indian context; so that research work embodied in this thesis was carried out on this form of the disease.

1.7. Morphology and Life cycle of *Leishmania donovani*

In India, VL is caused by *L. donovani*. Indian VL is anthroponotic and is transmitted chiefly through the bites of the female sandfly, *P. argentipes*. *Leishmania* exists in two forms (i) promastigotes: these are extracellular, elongated, flagellated, motile and ranges in size from 2 μm 2-20 m (Figure 1.7 A.). This form exists in sandfly and in *in vitro* cultures, (ii) amastigotes: these are intracellular, round to oval, aflagellated, non- motile and ranges in size from 2-5 m (Figure 1.7 B.). This form resides and multiplies within the phagolysosomes of macrophages of reticuloendothelial system of the vertebrate host (Handman, 1999).

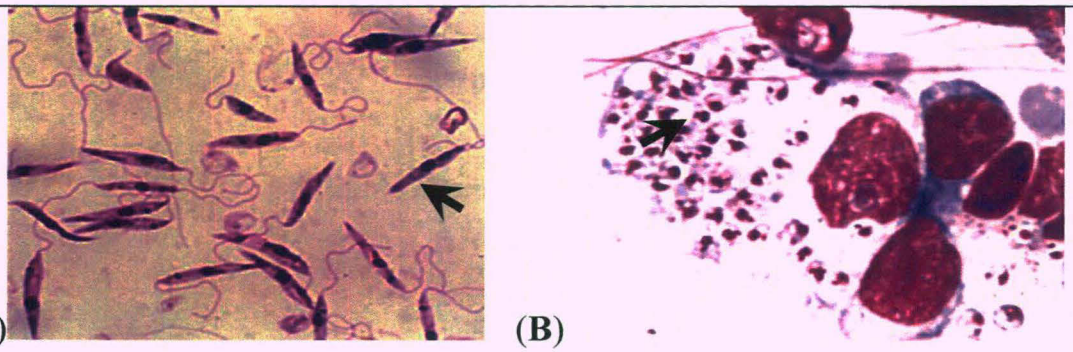


Figure 1.7: Two stages of *Leishmania* parasite; (**A**) Extracellular and motile form called promastigotes each bearing a flagellum. (**B**) Intracellular and non-motile stage called amastigotes (small dotes) as seen in Giemsa stained dab smear prepared from the spleen of *L. donovani* infected golden hamster.

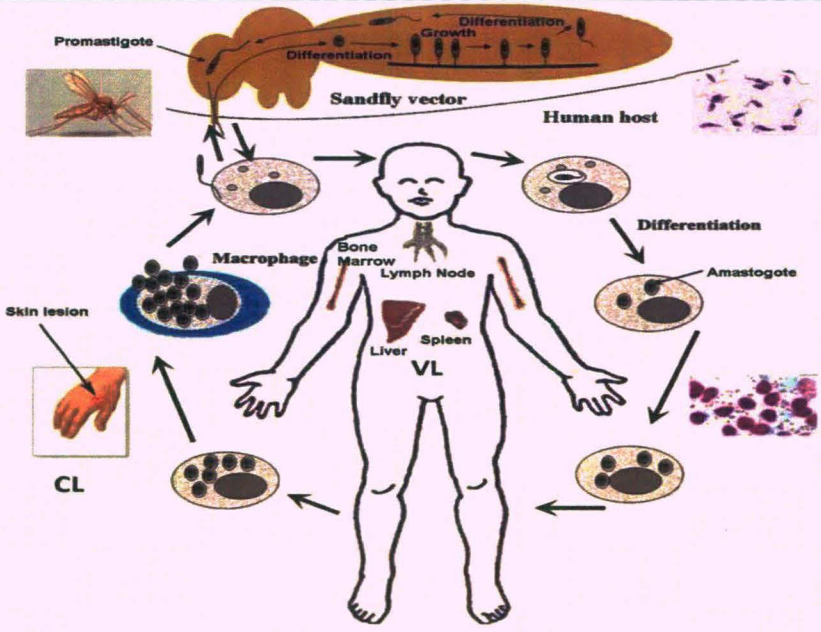


Figure 1.8: Life cycle of *Leishmania* parasite shuttles between vector sandfly and human host.

Following the sandfly bite, some of the flagellates entering the circulation are destroyed while others enter the cells of the reticuloendothelial system. Here they undergo change into amastigote form which multiplies by binary fission, with the multiplication continuing until the host cell is packed with the parasites and ruptures, liberating the amastigotes into circulation. The released amastigotes are taken up by additional macrophages and so the cycle continues (Figure 1.8). Ultimately all the organs containing macrophages and phagocytes are infected, especially the spleen, liver and bone marrow.

1.8. Vectors and transmission of the disease

Visceral leishmaniasis is transmitted by the *Phlebotomus spp.* in the old world and *Lutzomyia spp.* in the new world. *P. argentipes* is the proved vector of KA in India (Kishore *et al.*, 2006; Swaminath *et al.*, 2006). Of the 500 known phlebotomine species, only some 30 of them have been positively identified as vectors of the disease. Shadflies are very small in size (< 3.5 mm) (Figure 1.9) and may be hard to see. Sandflies are usually most active in the twilight, evening and night hours (from dusk to dawn) and less active during the hottest time of the day. Female shadfly lays its eggs in the burrows of certain rodents, in the bark of old trees, in ruined buildings, in cracks in house walls, in animal shelters and in household rubbish (http://www.who.int/en). High incidence of VL is reported during pre-monsoon season that coincides with vector abundance and increased man-vector contact due to sleeping habits of children in open space (Kishore *et al.*, 2006; Singh *et al.*, 2006).

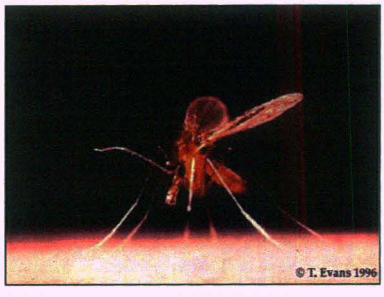


Figure 1.9: Sandfly, the vector host of *Leishmania* parasite.

1.9. Clinical symptoms of visceral leishmaniasis

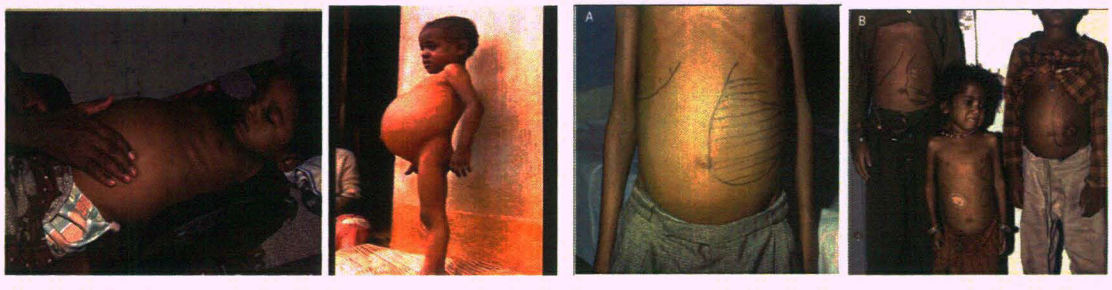


Figure 1.10: Clinical symptoms of VL; Hepato-splenomegaly and wasting are the main features. *[Reproduced from (Murray, 2005); Lancet 366: 1561–77 and TDR report, February 2004].*

VL patients are heavily infected throughout the mononuclear phagocyte system; develop life-threatening disease after an incubation period of weeks to months; and have fever, severe cachexia, hepatosplenomegaly (Figure 1.10), pancytopenia (anaemia, thrombocytopenia, and leucopenia, with neutropenia, marked eosinopenia, and a relative lymphocytosis and monocytosis), and hypergammaglobulinaemia (mainly IgG from polyclonal B-cell activation) with hypoalbuminaemia (Herwaldt, 1999). VL encompasses a broad range of manifestations of infection which remains asymptomatic or subclinical in many cases, or can follow an acute, subacute, or chronic course. Active VL may also represent relapse (recurrence 6-12 months after successful treatment) or late reactivation of subclinical or previously treated infection.

1.10. Control strategies of the disease

Efficient case management based on early diagnosis and treatment is the key to limit morbidity and prevent mortality. Effective treatment of patients is also a measure to control reservoir and transmission in anthroponotic foci, particularly for cases of PKDL, which are thought to act as a long term reservoir of the disease. In addition, vector control should be implemented wherever feasible. Spraying of houses with residual insecticides has been an important measure in the past in India but is not much used now. Insecticides used in malaria control programmes are effective on sandfly. In foci where sandflies bite at night, impregnated bednets have decreased the incidence of leishmaniasis. Indian government started Leishmaniasis Elimination Programme in 2001 with the targets of prevention of death by 2004, zero level incidence by 2007, zero level prevalence by 2010 and elimination by 2012 (Ganguly *et al.*, 2006).

1.11. Diagnosis of visceral leishmaniasis

Laboratory diagnosis of leishmaniasis can be made by light microscopic examination of the stained specimen, by *in vitro* culture, by animal inoculation, detection of parasite DNA in tissue samples, detection of parasite antigen or specific antileishmanial antibodies (Sundar and Rai, 2002). Direct visualization of amastigotes in clinical specimens is the diagnostic gold standard in regions where tissue aspiration is feasible and microscopy and technical skill are available. Diagnostic sensitivity for splenic, bone marrow, and lymph node aspirate smears is >95%, 55–97%, and 60%, respectively (Sundar, 2003). Elsewhere, serum antileishmanial immunoglobulin G in high titre is the diagnostic standard, primarily

with direct agglutination tests or other laboratory-based serological assays (Herwaldt, 1999; Abdallah *et al.*, 2004; Desjeux, 2004b). Freeze-dried antigen and rapid detection of anti-K39 antibody with fingerstick blood in an immunochromatographic strip test have advanced field serodiagnosis. In symptomatic patients, anti-K39 strip-test sensitivity is 90–100% (Sundar *et al.*, 1998; Boelaert and Dujardin, 1999; Sundar *et al.*, 2002;Veeken *et al.*, 2003; Boelaert *et al.*, 2004). This test can safely substitute for invasive diagnostic procedures in VL and is also useful for PKDL. Testing urine for leishmanial antigen or antibody is a new approach (Islam *et al.*, 2004); (Sundar *et al.*, 2005). Various immunodiagnostic tests include antibody detection, complement fixation test, immunodiffusion test, CCIEP, indirect hemagglutination, IFA test, DAT, ELISA with CSA, ELISA with fucose-mannose ligand, ELISA with rK39 antigen and rapid strip test with rK39. In addition, antigen detection test called KATEX is 68-100% sensitive. DNA detection by PCR with LDI primer using blood, bone marrow and skin are currently under use (Salotra *et al.*, 2001). Different DNA sequences in the genome of *Leishmania* have been documented in diagnosis and prognosis of VL (Schallig and Oskam, 2002; Sundar *et al.*, 2006).

1.12. Drugs against visceral leishmaniasis

The drugs currently recommended for the treatment of VL include the Pentavalent Antimonials, Amphotericin B and its lipid formulations (AmBisome®, Abelcet, Amphocil), Pentamidine, Miltefosine, Paromomycin and Sitamquine. The chemical structures, mode of action, doses, route of administration of these drugs is described below in details.

1.12.1 Pentavalent antimonials [Sb (V)]

Antimonials were first introduced in 1945 and these remain the effective drugs for all forms of leishmaniasis for more than 60 years. These are available as branded products, meglumine antimoniate (Glucantime) and sodium stiboguconate (Pentostam) (Figure 1.11) and in the generic form, Sodium Antimony Gluconate (Albert David Ltd., Kolkata). The drugs are given by i.v. and i.m. routes and their efficacies are equivalent at similar doses (Sundar and Chatterjee, 2006). The major concern for antimonials is that they exert toxic effects like arthralgia, nausea, abdominal pain, pancreatitis and cardiotoxicity. Moreover, treatment require hospitalization for a long (3-4 weeks) period. In Bihar state of India, over 60% of previously untreated patients are unresponsive to Sb (V) rendering the drug useless for routine use (Sundar *et al.*, 2000; Sundar and Rai, 2002). To date, the precise mechanism

of action of antimonial drugs remains an enigma. A general consensus is that Sb(V) acts upon several targets that include influencing the bioenergetics of *Leishmania* parasites by inhibiting parasite glycolysis (Mottram and Coombs, 1985; Berman and Gallalee, 1987), fatty acid β-oxidation (Berman *et al.*, 1989) and inhibition of ADP phosphorylation (Berman *et al.*, 1985). It has also been reported to cause non specific blocking of SH groups of amastigote proteins and cause inhibition of DNA topoisomerase I (Chakraborty and Majumder, 1988). Recent evidences have shown that both Sb(III) and Sb(V) mediate DNA fragmentation in *Leishmania* species, suggesting that antimony kills the parasite by a process reminiscent of apoptosis (Sereno *et al.*, 2001; Lee *et al.*, 2002; Sudhandiran and Shaha, 2003).

1.12.2 Pentamidine

It is an aromatic diamidine. It was initially proven to be useful in Sb(V) resistant VL cases in India in the late 1970s and early 1980s and a cure rate of 98.8% was reported without any relapse (Jha, 1983). Its leishmanicidal activity is possibly mediated via its influence on polyamine biosynthesis and the mitochondrial membrane potential (Bray *et al.*, 2003). Pentamidine has been abandoned as second-line treatment for VL because of toxicity (myalgia, nausea, headache, hypoglycaemia, irreversible insulin dependent diabetes mellitus and death) and declining efficacy.

1.12.3. Amphotericin B

Amphotericin B is an antifungal macrolide antibiotic isolated from Streptomyces nodosus in 1956. Its antileishmanial activity was first shown in the early 1960s attributed to its selective affinity for ergosterol vis-a-vis cholesterol (Ramos *et al.*, 1996). Amphotericin B, at a dose of 0.75-1.0 mg/kg for 15 to 20 infusions either daily or on alternate days, has consistently produced cure rates of about 97% and is now the drug of choice in north Bihar (Sundar and Rai, 2002). It selectively inhibits the membrane synthesis of the parasite and causes holes in the membrane, leading to parasite death (Ramos *et al.*, 1996). At present, three lipid formulations of Amphotericin B are available: liposomal Amphotericin B (AmBisome), Amphotericin B lipid complex (ABLC; Abelcet®) and Amphotericin B cholesterol dispersion (ABCD; Amphotec™). These preparations have been tested successfully for VL in India, Kenya and Brazil (Thakur *et al.*, 1996; Sundar, 2001). The lipid formulations had an upper edge as they produced distinctly lower toxicities, notably the absence of nephrotoxicity and significantly lower infusion reactions. Major limiting

factors include an almost universal occurrence of infusion based reactions like high fever with rigor and chills, thrombophlebitis and occasional serious toxicities like myocarditis, severe hypokalaemia, renal dysfunction and even death.

1.12.4. Miltefosine (Hexadecylphosphocholine)

Miltefosine, initially developed as an anticancer drug, is the first effective oral treatment of VL. Its antileishmanial activity was initially discovered in the mid-1980s and since then its efficacy has been demonstrated in several *in vitro* and *in vivo* experimental models (Croft *et al.*, 1987; Croft and Engel, 2006). These findings led to clinical trials and registration of miltefosine in India in March 2002 for oral treatment of VL (Sundar *et al.*, 2002). Its mode of action has been established to be apoptosis like cell death (Paris *et al.*, 2004; Verma *et al.*, 2007). Its adverse effects were mild to moderate gastrointestinal disturbances that include vomiting and diarrhoea in 40 and 15-20% of patients, respectively. As Miltefosine is teratogenic, it is contraindicated in pregnancy. A potential problem is prolonged half-life of miltefosine (150-200 hr) that raises concerns for emergence of drug resistance.

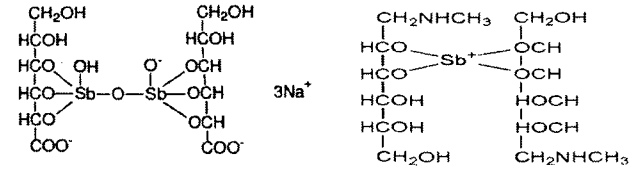
1.12.5. Paromomycin (Aminosidine)

Paromomycin (aminosidine) although developed in the 1960s as an antileishmanial agent, it remained neglected until the 1980s when topical formulations were found to be effective in CL and a parenteral formulation for VL was also developed. One World Health, the Bill and Melinda Gates Foundation, Gland Pharma Limited, IDA Solutions and WHO/TDR partnered to develop Paromomycin injection as a public health tool to be sold on a not-for-profit basis at a very low price. In phase III clinical trial, 94.6% patients treated with its injection were cured of VL. Paromomycin injection was approved on August 31, 2006 for treatment of VL in India (Sundar *et al.*, 2007). In *L. donovani,* paromomycin promoted ribosomal subunit association of both cytoplasmic and mitochondrial forms, following low Mg^{2+} concentration (Maarouf *et al.*, 1995).

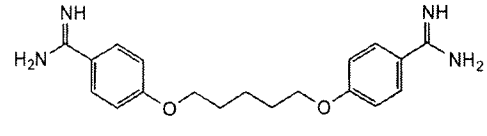
1.12.6. Sitamaquine (WR 6026)

Sitamaquine, an orally active 8- aminoquinoline analog, was originally developed as WR6026 by the Walter Reed Army Institute of Research in collaboration with GlaxoSmithKline. Its antileishmanial activity was first identified in 1970s (http://www.wrair.army.mil). Although animal studies showed very encouraging results,

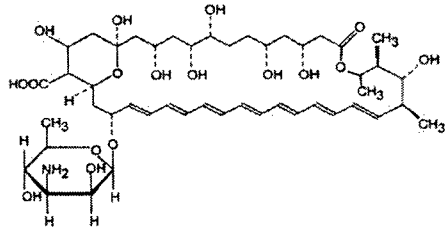
human trials done on Kenyan patients did not find it more than 50% effective (Chapman *et al.*, 1979; White *et al.*, 1989). Sitamaquine is rapidly metabolized, forming desethyl and 4-CH2OH derivatives, which might be responsible for its activity. Toxicity appears to be relatively mild as it causes mild methemglobinaemia.



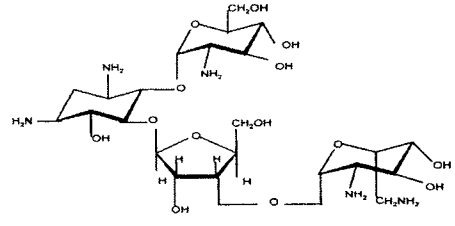
Sodium antimony gluconate **Meglumine antimonate**



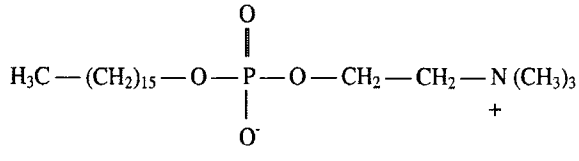
Pentamidine



Amphotericin B



Paromomycin sulphate



Miltefosine

Sitamaquine (WR6026)

Figure 1.11: Antileishmanial drugs currently available for VL treatment or under various stages of the clinical trials.

1.13. Vaccines against visceral leishmaniasis

The development of a vaccine against leishmaniasis is a long term goal in both human and veterinary medicine. In the past decade, various subunit and DNA antigens have been identified as potential vaccine candidates in experimental animals but none have so far been approved for human use. To date there is no vaccine available against VL in routine use anywhere in the world. However, there is consensus that in the long term, vaccines ought to become a major tool in the control of this group of diseases. Unfortunately, the development of vaccines has been hampered by significant antigenic diversity and the fact that the parasites have a digenetic life cycle.

Although a great number of antigens have been tested for protection against the cutaneous disease with *in vitro* cell or mouse models, no effective vaccine against human kala-azar is yet available. Though, the solid immunity observed following cure of kala-azar has suggested that the vaccination to prevent leishmaniasis is within the reach of conventional immunization methods, only few reports in literature deal with vaccines *viz.*, FML, FML-QuilA Saponin *etc.*, against canine VL (da Silva *et al.*, 2000; Santos *et al.*, 2002; Borja-Cabrera *et al.*, 2004). Immune mechanisms involved in VL and various vaccines candidates evaluated in experimental models for VL has been extensively reviewed by (Nyame *et al.*, 1994; Modabber, 1995; Handman, 1997, 2001; Brodskyn *et al.*, 2003; Scott, 2003; Goto and Lindoso, 2004; Kaye *et al.*, 2004; Ravindran and Ali, 2004; Wilson *et al.*, 2005; Garg *et al.*, 2006).

1.14. Immunology of *Leishmania*

In view of existing knowledge, lymphocytes, macrophages (MΦ) and antibody co-operation the major components of the immune system are necessary for protection against the *Leishmania* infection. The death of the parasite occurs either by stimulation of the sensitized lymphocytes through antigen load or by activating macrophages. Active VL disease is characterized by the marked elevation of humoral immune response i.e. by the production of plenty of specific as well as non-specific antibodies (Ghose *et al.*, 1980); (Ghosh *et al.*, 1995). Antibody titers, primarily IgG rise sharply during VL, but the antibodies so generated, are apparently not protective (Lainson, 1966; Bray and Manson-Bahr, 1971; Bryceson and Turk, 1971; Rezai *et al.*, 1978; Evans *et al.*, 1990). The enormous increase in serum immunoglobulin levels in active VL is due to polyclonal activation of immunoglobulin producing cells leading to increase biosynthesis of IgG and to a lesser extent of IgM. Most of the antibodies produced during infection are not parasite specific (Bunn-Moreno *et al.*, 1985; Clinton *et al.*, 1969; Evans *et al.*, 1990), but the hyper-gammaglobulinemia may have diagnostic value, IgG may reach 50gm/litre and comprise 50% of total serum proteins (Stauber, 1963).

It is held that CMI response impairment causes the pronounced immunosuppression during the active stage of the disease noted a marked decrease in circulating T-lymphocytes in kala-azar patients(Rezai *et al.*, 1978). These observations help to explain the lack of delayed type of hypersensitivity (DTH) or protective cell-mediated immunity during VL. It has not yet been ascertained whether the lack of DTH to *Leishmania* during infection is the

result of general or specific immunosuppression phenomenon. After the successful treatment, CMI response tends to be restored and the test becomes positive (Mauel, 1982) (Sacks *et al.*, 1987). Past studies explicit that in control and resolution of leishmaniasis, cell mediated immune response play a very important role in both experimental models of infection and human patients (Murray, 1982; Howard *et al.*, 1984; Stobie *et al.*, 2000). The host's immunological responses during *L. major* infection has been studied thoroughly (Locksley and Louis, 1992; Liew and O'Donnell, 1993). Resistance and susceptibility to *L. major* is mediated by Th1 and Th2 subsets of $CD4^+$ T cells, respectively. Th1 cells secrete protective cytokine IFN- , IL-2 and execute CMI responses, where as Th2 cells produce disease exacerbative cytokines IL-4, IL-10 and IL-13 (Figure 1.12) and assist in antibody production for humoral immunity (Murray *et al.*, 1983; Heinzel *et al.*, 1991; Holaday *et al.*, 1991; Lehn *et al.*, 1992; Reiner and Locksley, 1995; Suffia *et al.*, 2000). Recently, a similar Th1/Th2 dichotomy in cytokine response to *L. donovani* infection in murine and human system has also been reported (Ghalib *et al.*, 1993; Karp *et al.*, 1993; Kemp *et al.*, 1993).

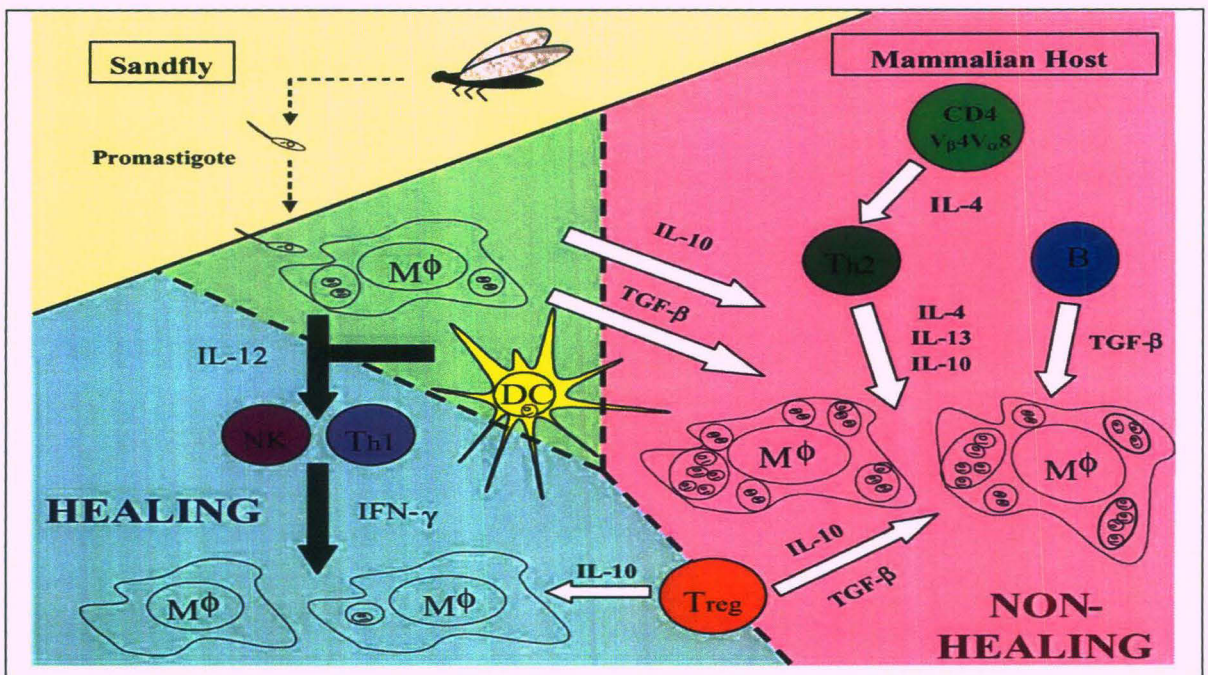
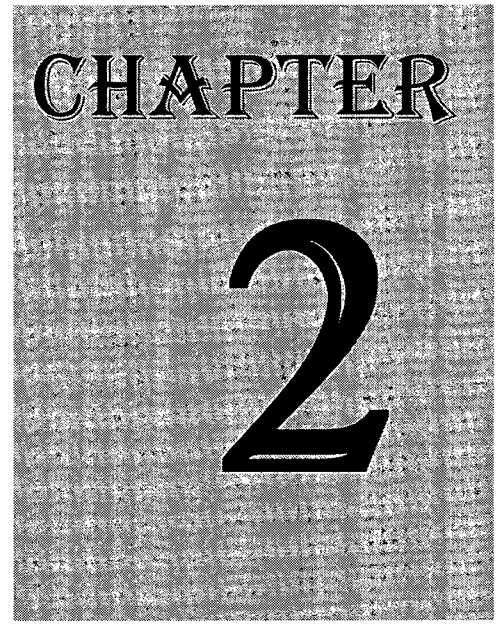


Figure 1.12: Immunological responses in *Leishmania:* During a blood meal an infected sandfly transmits metacyclic promastigotes to the vertebrate host, which convert to the amastigote form on entering macrophages and dendritic cells. IL-12 production from infected cells induces NK cell activation and $CD4^+$ T helper-1 differentiation and IFN- production. IFN- stimulates iNOS expression and NO production in the macrophage, which mediates parasite killing and therefore a healing response. Failure to produce IL-12 or alternatively IL-4/IL-13 production results in unregulated parasite replication within the infected cells facilitated by host cell IL-10 production. IL-10 production by $CD4^+$ T regulatory cells can both facilitate non-healing disease as well as maintaining latent infection and concomitant immunity.

1.15. *Leishmania*/HIV co-infections

Leishmania/HIV co-infection is emerging as an extremely serious problem. To date, it has been reported from 34 countries, with most of the cases from Southern Europe, where 25–70% of adult patients with VL are co-infected with HIV (Desjeux, 1999). The first case of leishmaniasis associated with human immunodeficiency virus (HIV) infection was published in middle of the 1980s (de la Loma *et al.*, 1985). The first case of VL/HIV co-infection in India was identified from the Bihar State of India in the year 2000 (Sinha *et al.*, 2003). Since AIDS epidemic is looming large on the horizon of new millennium in India (Sinha *et al.*, 2006), the state of Bihar needs to be looked seriously for VL/HIV co-infections. The size of $CD4^+$ cells count is $\leq 200/\mu m^3$ in 62- 90 percent of the co-infected patients. The majority of the cases of *Leishmania*/HIV co-infection have been described in adults infected by HIV-1; however, they have also been reported in patients infected by HIV-2 (Alvar *et al.*, 1997). These co-infections impose specific difficulties in terms of diagnosis and treatment (often results in frequent failure and relapses due to drug resistance). The development of the HIV/AIDS pandemic during the last 20 years has modified the spectrum of leishmaniasis in both the clinical and epidemiological fields.



Review of Literature

"It is possible to believe that all the past is but the beginning of a beginning, and that all that is and has been is but the twilight of the dawn. It is possible to believe that all the human mind has ever accomplished is but the dream before the awakening."

- H. G. Wells -

2. Review of Literature

2.1. Antimonials and Leishmaniasis

Antimonials have been known since ancient times as medicine. It is called as antimonials because antimony (Sb) - a metalloids belonging to group XV of periodic table of element present in this compound. Sb is present in trivalent Sb(III) and pentavalent Sb(V) form. In the beginning of 20th century, Tartar Emetic, an organic trivalent form of antimony was used for treatment of sleeping sickness and Gaspar Vianna introduced the drug for the treatment of MCL in 1912. The activity of Antimonials against various clinical form of leishmaniasis was soon discovered. Since, Tartar Emetic was highly toxic for human, new less toxic and more effective Antimonials were developed and evaluated. Pentavalent [Sb(V)] salts were found to be less toxic to mammal than trivalent [Sb(III)] salts. By the end of 1940s pharmaceutical research produced the current drug in use; the closely related organic pentavalent antimonial compounds Sodium antimony Gluconate (SAG) and Meglumine antimonite (Figure 1.11).

2.1.1. Leishmanicidal activity of SAG

The intrinsic susceptibility of *Leishmania* to SAG varies between different species. *In vitro* studies using macrophage-amastigote models have shown that *L. donovani* is 3-5 fold more susceptible to SAG than *L. Major, L. tropica, L. maxicana* (Allens and Neal, 1989). Likewise susceptibility for SAG varies in different life stages of *Leishmania.* Promastigote tolerate about 100 fold more concentration than intracellular amastigote. Reduced form of Sb(III) on other hand, is highly toxic for both life stages of *Leishmania.* This antimony patterns reflects the hypothetic dual mode of action of SAG discussed below:

1. Upon contact with infected macrophage (MΦ), SAG helps the MΦ to kill its intracellular guest and when reaching to the parasite, Sb (V) is reduced to Sb (III).
2. Sb (III) can further directly kill the parasite.

2.1.2. Interaction of SAG and host cell

Interaction between SAG and infected MΦ received little attention in *Leishmania* studies on Antimonials, although there always have been clear evidence pointing out the importance of MΦ for SAG. Research's observations suggested that SAG requires MΦ in functional immune system to fully unfold its activity. As we know that *L. donovani*

modulates signaling pathway of host cell extensively in favour of its own survival. Recent research now suggests that SAG modulates the signaling pathway of MΦ thereby restoring the MΦ's protective mechanism against the parasite.

2.1.3. SAG triggers RNI/ROI generation

In vitro treatment with 10mg/ml SAG (comparable to drug concentration in blood) of uninfected MΦ induces the generation of both RNI and ROI. ROI production is immediate and already breaks at 3 hrs after treatment and fading out 3 hrs latter (Mookerjee *et al.,* 2006). This effect has also been observed in the whole blood of animal after SAG treatment (Rais *et al.*, 2000).

2.1.4. SAG activates interleukins (ILs)

SAG treatments of infected or uninfected MΦ also induce expression of IL-12 (Mookerjee *et al.,* 2006). IL-12 plays central role in development of specific T–cell response crucial for control of *Leishmania* infection. However in active VL, T-cell response is futile due to suppressive action of omnipresent IL-10. SAG treatment probably helps reversing this suppressive state by inducing IL-12 expression.

Specific T-cell response acts mainly through IFN-γ which activates the infected MΦ but *L. donovani* paralyzes IFN-γ responsiveness of host cell. Thus SAG could restore IFN-γ responsiveness and thereby switching on antileishmanicidal activity of MΦ. *In vitro* study already demonstrates increased JAK/STAT phosphorylation (signaling downstream IFN-γ) upon SAG treatment (Pathak and Yi, 2001). In addition, the expression of IFN-γ receptor is also up regulated in response to SAG treatment which could further contribute to IFN-γ responsiveness of the MΦ (Dasgupta *et al.,* 2003).

2.1.5. Interaction of Sb(V)/ Sb(III) with Leishmania

Sb(V) is relatively inert and exerts minimal direct toxic activity against *Leishmania.* However Sb(V) is metabolically converted *in vivo* or *in vitro* to Sb(III) (Goodwin, 1995) which is toxic to both MΦ and amastigote, through its oxidative properties, binding functional thiol- group through its proteins and peptides. Sb(V) and Sb(III) both taken up by Promastigote and amastigote form of *Leishmania*. The uptake route of Sb(V) has not been defined clearly. Possibly, the uptake of Sb(V) is mediated by a transporter of *Leishmania* recognizing a sugar like structure resembling the gluconate like portion of SAG. Sb(III)

enters inside cell through aquaglyceroporins (AQP) (Gourbal *et al.*, 2004). AQP belongs to a major intrinsic membrane protein family that transports water and other uncharged polar solute such as glycerol and urea bidirectional. Several genes encoding AQP have been identified in the sequenced genome of kinetoplastids (Beitz, 2005). One aquaglyceroporins (AQP1) has been partially characterized in *L. major* and *L. tarantole.*

2.1.6. Mechanism of action of SAG

After 60 years of use, the antileishmanial mechanism of action of pentavalent antimonials [Sb(V)] is only now nearly understood. Nonetheless, it is now generally accepted that all pentavalent antimonials are prodrugs that require biological reduction to the trivalent form [Sb(III)] for antileishmanial activity. The site (amastigote or MΦ) and mechanism of reduction (enzymatic or non-enzymatic) remain controversial. Further studies are required to resolve this issue. Although stage-specific reduction has been demonstrated recently (Shaked-Mishan *et al.,* 2001), the mechanism by which amastigotes reduce Sb(V) is not clear. Both glutathione and trypanothione can nonenzymatically reduce Sb(V) to Sb(III), particularly under acidic conditions (Ouellette and Papadopoulou, 1993; Frezard *et al.,* 2001; Ferreira Cdos *et al.,* 2003). However, the physiological relevance of these observations is open to question since the rates of reduction are rather slow. Moreover, promastigotes contain higher intracellular concentrations of trypanothione and glutathione than amastigotes (Ariyanayagam *et al.,* 2001; Wyllie *et al.,* 2004) and both stages maintain intracellular pH values close to neutral, independent of external pH (Glaser *et al.,* 1988). Thus, it is difficult to account for the selective action of Sb(V) against the amastigote stage by a nonenzymatic mechanism. As both stages can take up Sb(III) and Sb(V) the insensitivity of promastigotes to Sb(V) cannot be attributed to drug exclusion (Brochu *et al.,* 2003). Two possible candidates for the enzymatic reduction of Sb(V) to Sb(III) in amastigotes have recently been identified. The first is a thiol-dependent reductase related to glutathione S-transferases that is more highly expressed in amastigotes (Denton *et al.,* 2004). The second is a homologue of a glutaredoxin-dependent yeast arsenate reductase (Zhou *et al.,* 2004).

The levels of expression of this protein in promastigotes and amastigotes were not reported and the low specific activity of the recombinant enzyme with glutaredoxin raises questions as to the physiological nature of the electron donor in *Leishmania spp.* The importance of these candidate proteins in conferring sensitivity to Sb(V) in amastigotes

needs to be addressed. There have been comparatively few studies on the mode of action of these drugs. Initial studies suggested that sodium antimony gluconate (SAG) inhibits macromolecular biosynthesis in amastigotes, possibly via perturbation of energy metabolism due to inhibition of glycolysis and fatty acid β-oxidation (Berman *et al.*, 1987). However, the specific targets in these pathways have not been identified. More recent studies have reported apoptosis in Sb(III)-treated amastigotes involving DNA fragmentation and externalization of phosphatidylserine on the outer surface of the plasma membrane (Sereno *et al.*, 2001; Sudhandiran *et al.*, 2003). However, these effects do not involve the classical caspase-mediated pathway (Sereno *et al.*, 2001) and do not meet the more recent stringent definition of apoptosis (Jiang *et al.*, 2004).

The mode of action of antimony in drug-sensitive *L. donovani* involves several effects on glutathione and trypanothione metabolism (Figure 2.1). Exposure to Sb(III) causes a rapid disappearance of trypanothione and glutathione from isolated amastigotes and promastigotes *in vitro*. A significant portion of these thiols are effluxed from cells in approximately equimolar amounts with the remainder being converted intracellularly to their respective disulfides (trypanothione and glutathione). The formation of the latter was ascribed to continuing oxidative metabolism in the face of inhibition of trypanothione reductase. Sb(III), but not Sb(V), has previously been shown to be a time-dependent reversible inhibitor of trypanothione reductase *in vitro* (Cunningham *et al.*, 1995). Since, Sb(III) also inhibits recovery of intracellular thiols following oxidation with diamide, this is consistent with inhibition of trypanothione reductase in intact cells (Wyllie *et al.*, 2004). The profound loss of these thiols (>90% in 4 hrs) coupled with the accumulation of disulfide (up to 50% of the residual within 4 hrs) causes a marked decrease in cellular thiol redox potential. Similar effects on thiol levels and thiol redox potential were observed when amastigotes were exposed to Sb(V), intrinsically linking the effects of the biologically active Sb(III) with the clinically prescribed Sb(V).

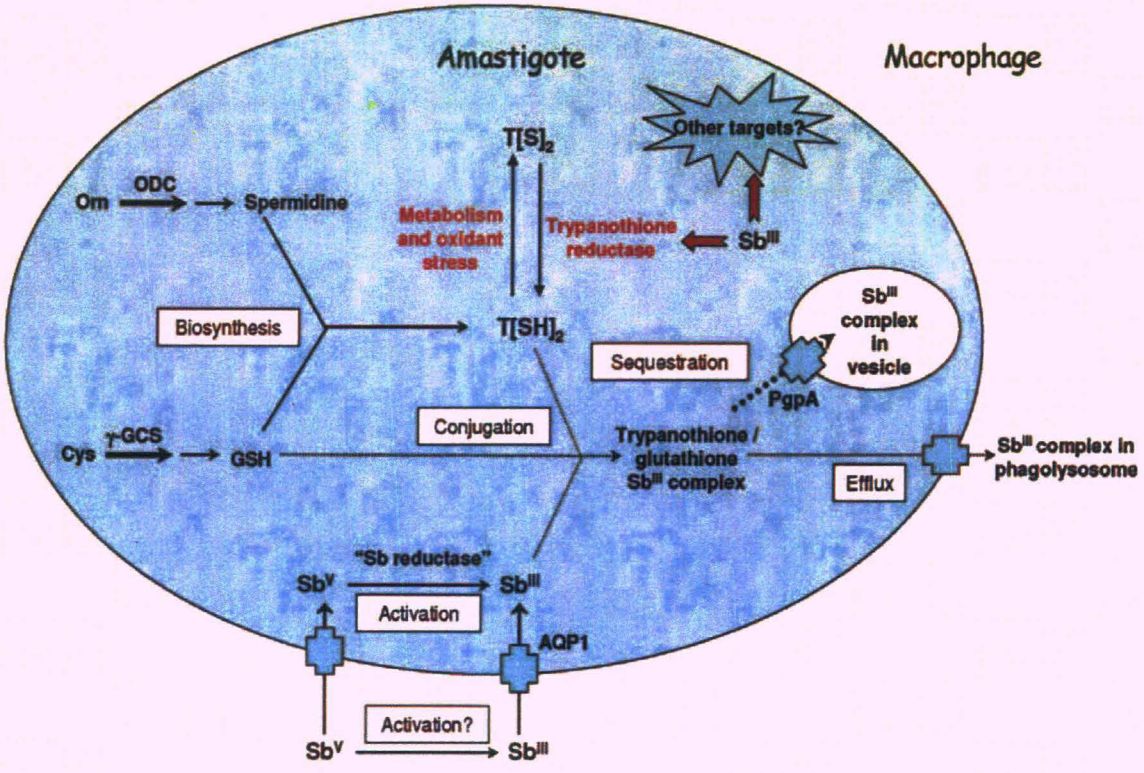


Figure 2.1: Proposed mechanisms of antimony action and resistance in *Leishmania spp.* Levels of ornithine decarboxylase (ODC), -glutamylcysteine synthetase (GCS) and an intracellular P-glycoprotein (PgpA) are elevated in some laboratory-derived resistant lines (thick lines), whereas decreased Sb reductase is observed in others. Dotted lines indicate nonenzymatic steps implicated in resistance. The red arrow indicates inhibition of trypanothione reductase and other targets. Uptake of Sb(III) is mediated via an aquaglycoporin (AQP1).

2.2. SAG treatment, failure and resistance in *Leishmania*

Sb(V) has been highly effective in the treatment of Indian VL at a low dose (10 mg/kg) for short durations (6-10 days). But in the early 1980s, reports of its ineffectiveness emerged and the dose was eventually raised to 20 mg/kg for 30-40 days. In recent years, the proportion of patients unresponsive to Sb(V) has steadily increased. In hyperendemic districts of north Bihar, as many as 65% of previously untreated patients fail to respond promptly or relapse after therapy with antimony drugs, due to the development of drug resistance (Sundar *et al.*, 2000; Sundar, 2001a). The reason for the emergence of resistance is widespread misuse of the drug, as Sb(V) is freely available in India, and is easily accessible over the counter. In the endemic regions, unqualified doctors, irregular use of drugs and incomplete treatment were of common occurrence. These practices presumably expose the parasites to drug pressure, leading to progressive tolerance of parasite to Sb(V) (Sundar *et al.*, 2000; Croft *et al.*, 2006c).

The mechanism by which *Leishmania spp.* acquire resistance to antimonials has been the subject of intensive research for several decades, often yielding apparently

thiol complex remains uncertain, but two routes of elimination of the complex can be envisaged. The first involves sequestration in an intracellular compartment or direct efflux across the plasma membrane. Early studies noted that PgpA, a member of the ATP-binding cassette (ABC) transporters, is amplified in some resistant lines (Callahan and Beverley, 1991; Ouellette and Borst, 1991.). However, it soon became apparent that this transporter is not responsible for drug efflux across the plasma membrane. First, overexpression of PgpA was reported to decrease influx of Sb rather than increase efflux, possibly due to a dominant-negative effect through interactions with other membrane proteins (Callahan *et al.,* 1994). Second, overexpression of PgpA did not mediate increased efflux of radioactive arsenite from cells (Dey *et al.,* 1994) or transport of arsenite across plasma membrane preparations (Mukhopadhyay *et al.,* 1996). Finally, PgpA plays a relatively minor role in resistance (Perez-Victoria *et al.,* 2003) and is localized in membranes that are close to the flagellar pocket, the site of endocytosis and exocytosis in this parasite (Legare *et al.,* 2001). Thus, the identity of the efflux pump in the plasma membrane and its role in resistance to antimonials remain to be determined. However, the studies described above have identified PgpA as functioning to sequester Sb(III) in an intracellular vacuolar compartment in *Leishmania* (Figure 2.1). It is worth noting that resistance due to intracellular sequestration of Sb(III) as a thiol conjugate would show higher rather than lower intracellular levels of Sb(III). Thus, either sequestration plays a minor role in resistance or the conjugates must be rapidly exocytose from the cell. The next important step is to relate mechanisms observed in laboratory studies to clinical resistance.

2.3. Proteomic approaches for the study of *Leishmania* biology

2.3.1. Principles of Proteomics

Proteomics is a new fundamental concept that has emerged in the recent past years. Proteomics is defined as simultaneous analysis of all the proteins in a given cell at a given point in time. It has complemented genomic research. More than five years ago, considerable emphasis was given to genomics and the dream of having completely sequenced genomes is now a reality. The complete sequence of several genomes is known, however, the understanding of probably number of proteins encoded by thousands of genes is still a long way to go and the hard work is required to unravel the complexity of biological systems. The inability to identify functional protein targets by examining gene sequences has created a gap between genomics and drug discovery. This gap reflects the

fact that in most cases, gene sequence reveals little information about protein function or disease relevance. Proteomics is a science that focuses on the study of proteins, their roles, their structures, their localizations, and their interactions. Marc Wilkins and colleagues first introduced the term "proteomes" in 1994. The major goal of proteomics is to make inventory of all proteins encoded in the genome of an organism and analysis of interaction of these proteins. Proteomics is a combination of highly efficient technologies for separation and analysis of proteins in living organisms and to exhaustive information on biochemical properties of proteins in living systems. We may call the last decade as the "Decade of Genomics", and the first decade of the new millennium as "Decade of Proteomics". Proteomics is a key technology that is used to identify proteins and map their interactions in a cellular context. With the sequencing of genome of so many organisms, the scope of proteomics has shifted from identification and characterization of protein to structure, function and protein-protein interactions. Proteomics is a multidisciplinary research field generally combining various protein separation techniques, mass spectrometric methods and bioinformatics. Proteomic methods can be used as a toolbox of different techniques to separate, profile and identify proteins, both qualitatively and quantitatively. To study complex protein mixtures such as biological fluids or cell lysates a combination of separation methods is required to reduce the complexity of the sample. If the protein expressions of cells are studied, sub-cellular fractionation is frequently used, where the proteins generally are separated into cytosolic, nuclear and membrane protein fractions using different solubilization and centrifugation steps.

2.3.2. Classification of Proteomics

Area of proteomics study is very broad so it can be mainly divided into 5 classes:

2.3.2.1. Expression Proteomics: The expression proteomics deals with the profiling of proteins expressed in cells or tissues. In order to discover and monitor the relevance of a protein to a disease-related process, it is important to determine that up to what extent a protein is expressed. Several approaches are being used to study expression proteomics but the most popular approach is mass spectrometry based study of proteins in combination with two dimensional gel electrophoresis (2-DGE).

2.3.2.2. Functional proteomics: Genome sequencing contributed number of the protein components of cells and organisms, however, functional significance of most of these proteins is not known. Determining what these components do is the task of functional

proteomics. Proteome-scale screens for functional activities (e.g., protease and phosphatase) should be implemented in order to link new proteins with known activities.

2.3.2.3. Chemical Proteomics: It is an alternative term for chemical genomics when proteomic approaches are used to study interaction of small molecules with cells. Although it is impossible to screen for chemical reactions that are unknown, in theory, identifying small molecules that bind to the new proteins may elucidate clues to new activities. These ligands might be found by screening the new proteins against diverse chemical libraries, using existing methods such as NMR spectroscopy, microcalorimetry, or microarrays, may result in the identification of small molecule ligands. Ascribing function to new proteins by discovering small molecule ligands might be referred to as chemical proteomics.

2.3.2.4. Structural Proteomics: The aim of structural proteomics is to provide three-dimensional information for all proteins. The primary sequence of a protein determines its three-dimensional structure, which in turn determines its function. Often, proteins of similar function share structural homology in the complete absence of sequence homology. As a result, many of the newly sequenced proteins share unrecognized structural and functional homology with known proteins. Access to structural information on a proteome wide scale is of importance at several levels. Structural information can be used to ascribe function, thereby revealing new potential drug targets, validate targets based on homology to other known proteins.

2.3.2.5. Interactive Proteomics: It refers to the localization of proteins and protein-protein interactions. The protein-protein interactions are the part of most cellular processes, including cell-cycle regulation, signal transduction, certain metabolic processes as well as cellular architecture. A complete understanding of cellular function depends on a full characterization of the complex network of cellular protein-protein interactions. Therefore, elucidating these interactions will be an important step toward revealing new units of biological function and new targets for drug development. The most commonly used technology to study protein-protein interactions is yeast two-hybrid system and many new protein interactions have been discovered using this system.

2.3.3. Methods for expression proteomic study

Systems for protein separation and identification are especially important, and these have been developed at a rapid rate in the last few years. Mass spectroscopy, which is a technique

for identifying proteins, is probably the most important technique in proteomics. As such, it occupies the largest share of the market for proteomics-related supplies. The other essential technique is 2-D gel electrophoresis, a method for separating proteins prior to identifying them with mass spectroscopy. There are three main steps in expression proteomics research:

1. Separation of individual proteins through two-dimensional electrophoresis.
2. Identification and characterization of proteins through mass spectrometry.
3. Bioinformatics for analysis and comparison of the data.

2.3.4. Two-dimensional Gel Electrophoresis (2-DGE)

2-D gel electrophoresis is generally used for the separation and isolation of proteins for further characterization by mass spectroscopy. This technique can be used for two main purposes, (i) for the large scale identification of all proteins in a sample. This is undertaken for investigating the global protein expression of organisms whose genomes have been fully sequenced. (ii) For differential protein expression study that is to compare the protein expression of two or more samples. The 2-DGE technique is a high resolution separation technique in which protein samples were separated by isoelectric focusing (IEF) in one dimension and then on an SDS-polyacrylamide gel (SDS-PAGE) for size-determined separation in the second dimension. It can resolve hundreds of components on a single gel. In the classical 2-DE technique, ampholytes were used in the first dimension. The replacement of the ampholytes by immobilized pH gradients (IPG) resulted in significant improvements in the technique. The complete solubilisation and denaturation of sample proteins is a key factor in successful 2-DGE electrophoresis. The preparation of sample generally requires the use of Solubilisation-Denaturation (SD) buffer containing chaotrophe, detergent and reducing agents. Standard procedures of protein staining were studies for visualization of protein spots after separation by 2-DGE. Staining with silver, coomassie colloid solution and special fluorescent dye are the most popular and widely used methods of staining.

2.3.5. Mass Spectrometry (MS)

Mass spectrometry (MS) is workhouse in proteomics study for identification and detection of proteins and their fragments. It provides the ability to accurately measure the mass of almost any molecule that can be ionized to the gas phase. A mass spectrometer consists of three essential components an ion source, a mass analyzer and a detector. An ion

source converts molecules into gas-phase ions. Once these ions are created, they are separated in the mass analyzer by their mass (m) to charge (z) ratio and detected by an electron multiplier. MS data are recorded as 'spectra' which display ion intensities versus their m/z value.

MS allows ionization of macromolecules without destroying their chemical entity. There are two main modifications of ionization of native proteins and peptides *viz.* matrix-assisted laser desorption-ionization (MALDI, Karas *et al.,* 1988) and electrospray ionization (ESI, Fenn *et al.,* 1988). Time of flight (TOF) mass-spectrometers is mainly used for determining the primary spectra of peptides. MALDI-TOF-mass-spectrometers are used for protein identification by means of peptide fingerprinting (by treatment of the sample with trypsin or any other proteases). Tandem mass spectrometers are equipped with ion trap, quadrupole chamber for fragmentation or quadrupole-time of flight (Q-TOF) apparatus. They are used for protein sequencing during mass spectrometry of products of fragmentation of primary molecular ions of peptides or proteins within the format of chromatography-tandem mass-spectrometry (LC-MS/MS using the principle of ESI). There are a number of different types of mass spectrometers employed in proteomic research and the ones used in this thesis (MALDI-TOF) are further described below.

2.3.5.1. Matrix assisted laser desorption/ionization time-of-flight mass spectrometry: The MALDI-TOF MS instrument combines a MALDI ion source and a TOF analyzer. MALDI is a soft ionization technique, initially described in 1988, which results in most intact peptides/proteins in gas phase with little fragmentation (Karas *et al.,* 1988; Tanaka *et al.,* 1988). The sample subjected to MALDI analysis is placed on a sample plate. The sample plate is then inserted into the ion source of the instrument through a vacuum interlock, as the MALDI ion source operates under conditions of high vacuum. The technique involves co-crystallization of the sample on the sample plate with a large molar excess of a matrix compound, which strongly absorbs energy at the wavelength of the ultraviolet (UV) laser. Alpha-cyano-4-hydroxycinnamic acid (CHCA) is frequently the matrix of choice for peptides. CHCA is particularly good for generating ions above 700 Daltons (Da) since lower masses can be masked by the relatively high matrix background. The 2, 5-dihydroxybenzoic acid (DHB) matrix produces less interference than other matrices in the low m/z range and induces less fragmentation than CHCA. Therefore, DHB is often used for analyses of glycopeptides and phosphopeptides, while 3, 5-dimethoxy-4-

hydroxycinnamic acid (sinapinic acid) is suitable for intact protein analysis. The exact desorption/ionization mechanism for MALDI is not known. However, it is generally thought that the absorbed energy after laser irradiation of the analyte-matrix mixture results in vaporization and ionization of the matrix, carrying the analyte into the gas phase. The resulting ionization of the analyte probably occurs through proton transfer during the desorption process (Hillenkamp *et al.,* 1991). Predominately, MALDI results in formation of peptide/protein ions carrying a single positive charge, although ions having two or three charges can be formed. The TOF mass analyzer is well suited for pulsed ion sources, such as the MALDI technique. The analyte ions are accelerated by an electrical field between the sample plate and an extraction element prior to entering the field-free drift region. Thus, the analyte velocities become a function of their m/z ratio. As a result the analyte ions arrive at the detector at different times. For ions with the same charge, the ones with lower mass acquire higher velocity and reach the detector faster. However, during the desorption/ionization process ions of the same m/z often acquire different initial kinetic energy, and thus hit the detector at slightly different times, causing peak broadening and a lowering of the resolution.

The introduction of an electrostatic ion reflector often referred to as an ion mirror, consisting of a series of grids or ring electrodes, not only lengthens the flight path but also compensates for the difference in initial kinetic energy so that ions with the same m/z hit the detector at almost the same time (Mamyrin *et al.,* 1973). Ions with a higher initial kinetic energy will penetrate deeper into the ion mirror before reversing. Consequently, the more energetic, faster ions will have a longer flight path to the detector than the less energetic, slower ions (figure 2.2). Furthermore, the introduction of delayed extraction or time-lag focusing has provided remarkable improvement in both resolution and mass accuracy (Wiley *et al.,* 1955) and has become a standard feature of MALDI-TOF mass spectrometers. In delayed extraction, ionization occurs with no electrical field applied between the sample plate and the first extraction element. After a short time delay the electric field is switched on and the ions are accelerated towards ground potential. Ions with lower initial velocity will have travelled a shorter distance from the sample plate and will suddenly be at higher potential than the initially faster ions.

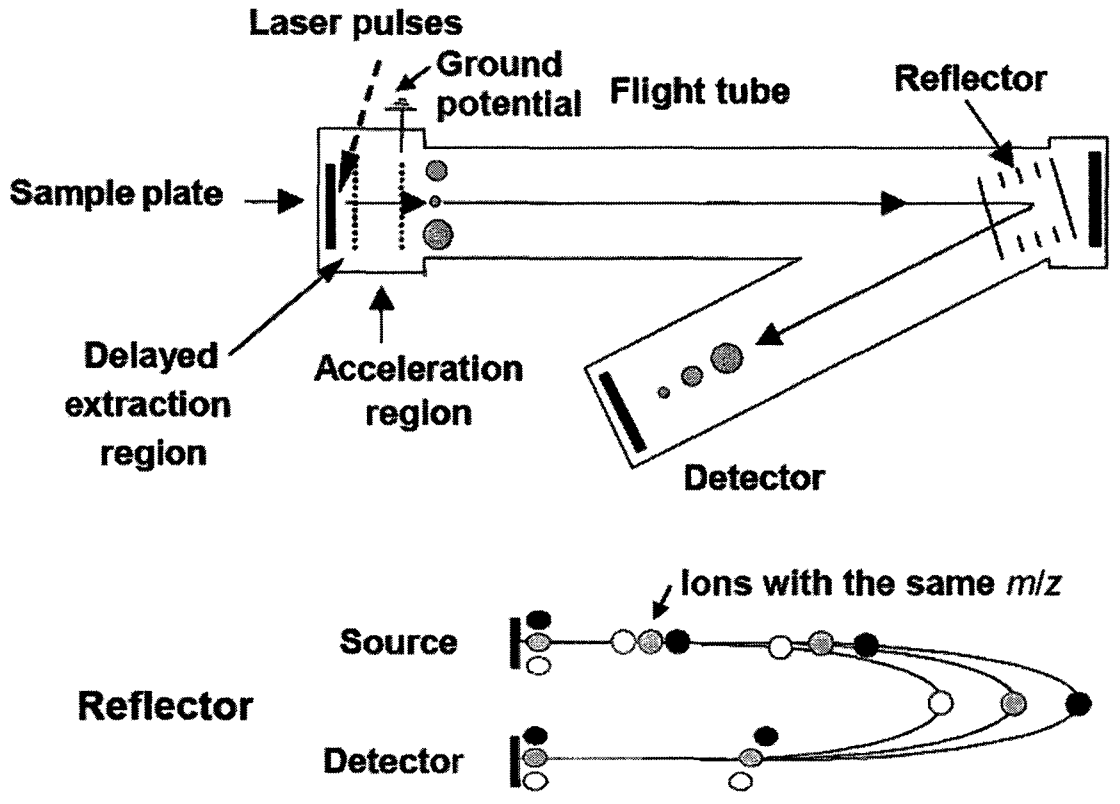


Figure 2.2: Schematic illustration of a MALDI-TOF mass spectrometer. Ionised analytes with different m/z are separated in flight tube and arrive at the detector at different times. The reflector compensates for different initial velocities of ions with the same m/z and improves the resolution. Ions with higher initial kinetic energy will penetrate deeper into reflector and travel a greater distance than less energetic ones. Thus, the ions will reach the detector at the same time.

Consequently, the originally slower ions will instead be the faster ones when they exit the acceleration region, and with properly set delay time and source voltages they will reach the detector simultaneously with the initially more energetic ions. The average of several hundreds of laser shots produces the final mass spectrum, increasing the signal. Performance in modern reflector MALDI mass spectrometers is typically in the range of a few parts per million in mass accuracy and only about a femtomole of peptide material needs to be deposited on the MALDI target to produce a signal. More recently, MALDI ion sources have also been coupled to Q-TOF and to two TOF analysers (TOF/TOF instruments, Medzihradszky *et al.,* 2000) allowing fragmentation of MALDI-generated precursor ions and subsequently providing information about the amino acid sequence for more reliable protein identification.

2.3.5.2. Surface enhanced laser desorption/ionization time of flight mass spectrometry: SELDI-TOF is an affinity-based MS method, initially described by Hutchens and Yip in 1993 (Hutchens *et al.,* 1993) and further developed by Ciphergen Biosystems into a protein Chip MS technology platform (Weinberger *et al.,* 2002). Intact native proteins are selectively adsorbed to chemically modified array surfaces followed by the addition of an energy-absorbing matrix solution. The MS part of the SELDI technique is based on the principles of MALDI-TOF MS, but modified so that the chromatographic capture step takes place on the same sample support that is subsequently used for laser desorption MS, thereby simplifying the experimental procedure, increasing reproducibility and facilitating automated analysis. The ability of the selective array surfaces to retain subsets of the proteome allows the analysis of complex biological specimens, such as serum, CSF and cell lysates. By combining different chromatographic arrays [e.g. anion exchange (Q10), cation exchange (CM10), metal affinity (IMAC) or reverse phase (H50)] and matrix molecules, a broad range of the proteome can be analyzed. The system is favorable for proteins and peptides with a MW lower than 20 kDa and is therefore a good complement to 1D/2D electrophoresis. Furthermore, small total protein quantities (~1.5 μg), and quick laboratory procedure favor SELDI-TOF compared with 2-DGE followed by MS. However, protein identities are not revealed in the process since the analytes cannot be subjected to any digestion or tandem mass spectrometry in the process. Thus, SELDI-TOF MS can be regarded as a profiling technique complementary to 2-DGE procedure, requiring downstream isolation, digestion & identification of analytes after quantification step.

2.3.5.3. Electronspray ionization (ESI) quadruple time-of-flight mass spectrometry: ESI first described in 1984 (Aleksandrov *et al.,* 1984), produces gaseous ionised molecules directly from a liquid solution of the analytes at atmospheric pressure. The sample solution is sprayed from the tip of a thin capillary and a strong electric field is applied between the capillary and a counter electrode. The ionization process is not yet fully understood. However, a fine spray of charged droplets is produced and dry gas or heat facilitates evaporation of solvent, reducing the size of the droplet. This results in increasing charge-density at the surface of the droplet and when the electrostatic repulsion between like charges exceeds the surface tension in the droplet it disintegrates. Repeated disintegrations will occur and ultimately solvent-free gas-phase ions are produced. A characteristic feature of ESI is the multiple charging of analytes, which increases proportionally with molecular

mass. This multiple charging allows for mass determination of proteins within the limited m/z range of quadrupole analysers. Low-flow electrospray, nano ESI, initially described by Wilm *et al.,* (1996), is generally used, where the spray needle is extremely thin and positioned close to the entrance of the mass analyser. These adjustments give very small droplets and hence a reduction in the amount of sample needed, enabling longer measurement times and more accurate and sensitive mass measurements. In addition, the electrospray process itself creates the sample flow through the capillary and thus no external pump is needed (Chernushevich *et al.,* 1999). The combination of ionization at atmospheric pressure and the continuous flow of solvent used in ESI allows for direct coupling with separation techniques, such as nano LC and capillary electrophoresis. In contrast to MALDI, ESI produces a continuous beam of ions and is most compatible with mass spectrometers that operate in a similar continuous fashion, such as quadrupole mass filters, while the TOF analyser requires a pulsed operation. Thus, orthogonal voltage pulsing of ion-packages into the TOF analyser was invented (Chernushevich *et al.,* 1999).

The quadrupole (Q) TOF instrument combines the ability to obtain efficient precursor ion selection by the use of the quadrupole mass filter and dissociation in a hexapole collision cell with the high sensitivity of the TOF analyser (figure 2.3). The quadrupole mass filter in the Q-TOF separates ions according to their m/z ratio by utilising the stability of their trajectories in an oscillating electrical field. Ions that do not have a stable trajectory through the quadrupole will collide with the rods, not reaching the detector. Compared with earlier ESI-MS instruments, the advantages of the Q-TOF hybrid include better sensitivity, improved resolving power and mass measurement accuracy, attributed mainly to the narrow beam packet, pushed down into the TOF analyser which is equipped with a reflectron orthogonally to the transfer ion optics. Another advantage of the Q-TOF is the easy switching between MS and MS/MS modes and that fragmentation of a specific m/z can be carefully controlled. In MS mode, the ions drift through the quadropole mass filter, which acts as a focusing device transmitting all ions to the TOF analyser, where they are separated according to their m/z ratio. In the MS/MS mode the quadrupole mass filter is set to allow only ions within a very narrow m/z range to pass through to the collision cell for subsequent fragmentation. The precursor ion dissociates into product ions, whose ion trajectories are stabilised in the hexapole, and the m/z of the fragment ions are then measured in the TOF analyser.

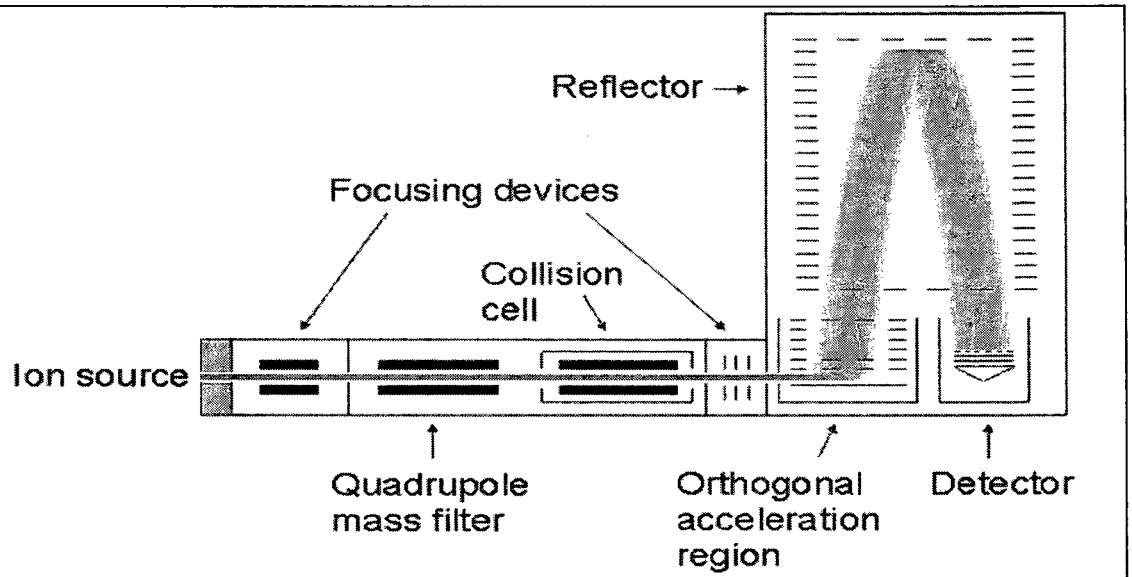


Figure 2.3: Schematic illustration of the principal components of ESI-QTOF MS.

2.3.5.4. Linear quadrupole ion trap Fourier transform ion cyclotron resonance mass spectrometry: The linear quadrupole ion trap (QIT) Fourier transform ion cyclotron resonance (FTICR) MS instrument (figure 2.4) generally also employs nano ESI for ionization of analytes. It is a hybrid instrument, consisting of a linear ion trap capable of fast, sensitive peptide sequencing combined with an ion cyclotron resonance (ICR) cell, generating extraordinary resolving power and mass accuracy (Marshall *et al.,* 1998). Ionised analytes are transmitted into the quadrupole iontrap through focusing optics e.g. quadrupoles, hexapoles or octopoles. The ion trap uses the same principles as the quadrupole filter, where different combinations of direct current and radio frequency (RF) potentials are used to select analytes of a particular *m/z* range, however in this case the ions can be trapped, forced to move back and forth in the quadrupole by applying appropriate potentials at the entrance and exit of the trap. The ability to trap ions makes it possible to select which ions should either be ejected to the detector directly, passed forward into the ICR cell or fragmented prior to detection or ICR analysis. Analytes or their fragments selected for ICR analysis are again transferred through focusing optics into the ICR cell, where ions are trapped by a strong magnetic field. The magnetic field will cause the ions to be trapped in a circular motion. For detection, coherent ion motion must be generated by applying a RF voltage to the exitation plates of the ICR cell, making the ions move closer to the detection plates. Then a small current will be induced in the plate each time an ion passes by. Since the ions with different m/z have different ion cyclotron frequencies, each

generated current frequency will correspond to a certain m/z value. The number of ions that enters the cell can be selected by the QIT. This is important because too many ions will dramatically reduce the resolution and mass accuracy of the measurements in the cell. If an appropriate number of ions are transferred to the ICR cell the high accuracy of the precursor ion, usually less than 2 ppm, enables an accurate database search for protein identification. The QIT can also be used on its own, resulting in better sensitivity but significantly reduced mass precision and resolution. Consequently, the increased sensitivity is at the expense of losses in resolution and mass accuracy.

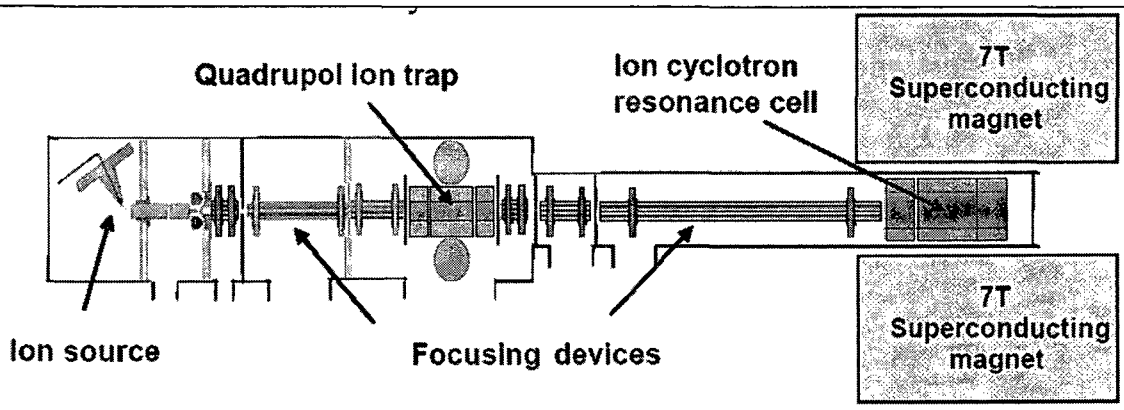


Figure 2.4: Schematic illustration of a QIT-FTICR MS instrument

2.3.6. Protein identification

A key advance in biological MS was the development of algorithms for identification of proteins by matching the mass spectrometric data to various databases containing the known protein sequences.

2.3.6.1. Peptide mass fingerprinting (PMF)

Enzymatic cleavage of proteins prior to MS analysis is usually performed by trypsin, producing a specific peptide pattern for each protein, generally referred to as a peptide mass fingerprint. Trypsin cleaves proteins C-terminally of the positively charged amino acids arginine (R) and lysine (K), giving a relatively large number of peptides available for MS analysis. Alternative endoproteases for protein digestion include Lys-C (cleaves after K), Arg-C (cleaves after R) and chymotrypsin (cleaves after F, Y, W, L and M), producing other peptide patterns. The MS-detected m/z values of the peptide pattern can then be matched to theoretically expected enzyme digests for each protein sequence present in a database. A ranked list is then produced according to the number of peptides from the sample that match

a specific protein (figure 2.5) (Mann *et al.,* 2001). The limit of PMF is normally reached when the sample contains a complex protein mixture, or if the protein yields too few digestion products. Therefore, this method is best suited for proteins separated by 2-DGE or other multi-dimensional protein separation strategies (Aebersold and Goodlett, 2001).

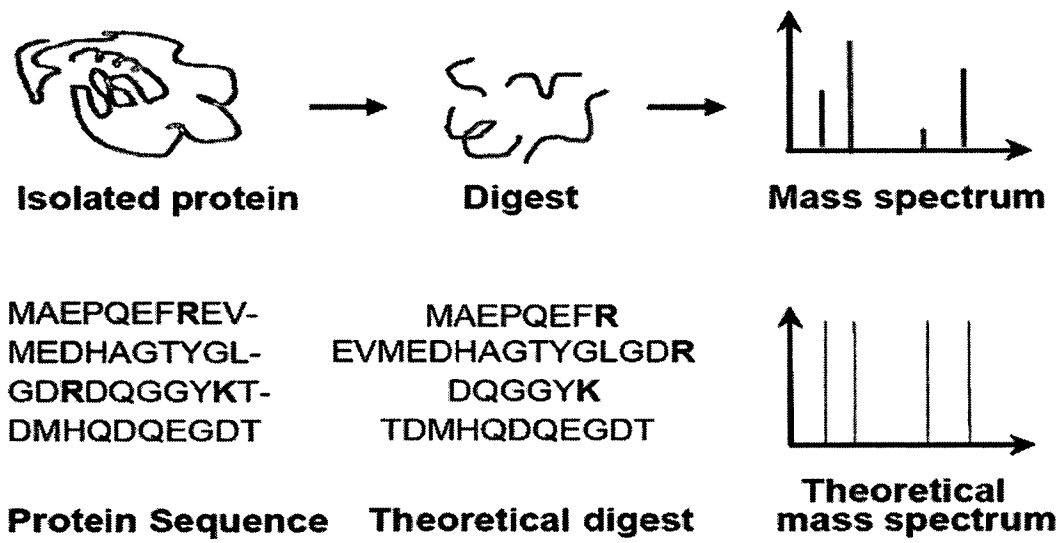


Figure 2.5: Protein identification by peptide mass fingerprinting. The isolated protein sample is enzymatically digested by trypsin. The resulting peptides are mass determined by MS. The peak list of the mass spectrum is compared with calculated masses of theoretically digested protein sequences present in databases.

2.3.6.2. Amino acid sequence analysis by tandem mass spectrometry

For protein identification using MS/MS, a single peptide precursor ion is isolated and fragmentations, mainly along the peptide bonds, are induced, resulting in a spectrum containing a pattern more or less unique for the individual peptide (Aebersold and Goodlett, 2001).The fragment ion masses are then compared with theoretical fragmentation patterns of proteins in a database search. Protein identification can be achieved by MS/MS analysis of even a single peptide (Mann *et al.,* 2001). Consequently, and in contrast to peptide mass fingerprinting, protein identification can be obtained from complex mixtures of proteins. The most common method of fragmentation is collision induced dissociation (CID). Other examples of fragmentation methods are laser-induced fragmentation (LIF), electron capture dissociation (ECD) (Zubarev *et al.,* 2000) and infra-red multi-photon dissociation (IRMPD, Little *et al.,* 1994). The CID process includes multiple low-energy collisions of the peptide precursor ion with an inert gas, usually argon, which finally leads to the dissociation of the precursor ion.

2.3.7. Role of bioinformatics in proteomics

One of the aims of genomics and proteomics is to move from an experimental to an in silico science, in which changes in cellular physiology and pharmacology can be predicted using computational methods. The integrated databases should meet the following criteria: (i) each entry in the database should be available during search by key words; (ii) the database should be connected to other databases via active links; (iii) an entry in the database should contain the main index. Programs for image analysis developed for the work with the integrated database should provide direct access of individual entries into any integrated database. SWISS-2D page is the most popular database for proteomics study. Besides images of 2D-gels with identified proteins on them and such experimental data as isoelectric point, molecular mass, this database contains information obtained during comparison of gels, microsequencing, and immunoblotting, analysis of amino acid composition and mass-spectrometry analysis of peptide fingerprints. This database also contains bibliographic references and various links on other biological servers.

Database searching using mass spectrometry data from the TOF-TOF (MS or MS/MS data) is done using the MASCOT search engine. The digested fragments were searched against the SWISS-PROT and NCBInr databases. Monoisotopic masses were used to search the database and mass tolerance was allowed less than 40 ppm and one partial cleavage. Oxidation of methionine and acrylamide modification of cysteine was considered. Where an assignment appeared ambiguous, tandem MS was conduct.

2.3.8. Proteomics approaches for studying Leishmania species/stage specific proteins

Proteomic approach has been found to be useful for analyzing the proteome of, *Leishmania spp.* including its development, evolution and pathogenicity (Figure 2.6) (Brobey *et al.*, 2006; Cuervo *et al.*, 2007; Dea-Ayuela *et al.*, 2006; Drummelsmith *et al.*, 2003; El Fakhry *et al.*, 2002; Forgber *et al.*, 2006; Foucher *et al.*, 2006; Gongora *et al.*, 2003; Leifso *et al.*, 2007; McNicoll *et al.*, 2006; Nugent *et al.*, 2004; Thiel and Bruchhaus, 2001; Walker *et al.*, 2006a; Walker *et al.*, 2006b). Proteomics is a valuable tool to extend our understanding of array of events of *Leishmania* infection (McNicoll *et al.*, 2006), about the drug resistance mechanism and to accelerate the search for novel potential clinical-associated phenotypic markers (Ouellette *et al.*, 2004). For defining the identity and location of as many proteins of an organism as possible an index map for reference mapping is carried out (Walker *et al.*, 2006b). 2-DGE reference maps have been completed for a

number of *Leishmania spp.* (Ouellette *et al.*, 2004). This seems to be a workable strategy because the availability of annotated sequenced genome (32.8-Mb genome, 8272 protein coding genes, http://www.genedb.org/leish/index.jsp) of *L. major* represents a milestone research that has opened the door for large-scale proteomic studies to dissect both protein expression/regulation and for the precise identification of *Leishmania* proteins produced by different developmental stages (de Araujo Soares *et al.*, 2003; Nugent *et al.*, 2004; Thiel and Bruchhaus, 2001) that may be involved in parasite virulence and pathogenicity (Drummelsmith *et al.*, 2003). Some of these proteins are seen as potential targets for drugs (Gradoni, 2001; Ouellette *et al.*, 2004). Despite the great deal of studies on the immunogenic properties of some leishmanial proteins and their suitability as candidates for biomarkers diagnosis and vaccination purposes against leishmaniasis (Arora *et al.*, 2005; Lemesre *et al.*, 2005; Molano *et al.*, 2003) passos (Murray *et al.*, 2005; Passos *et al.*, 2005; Santarem *et al.*, 2005), information on the functional role of relevant immunodiagnostic antigens is rather scarce (Kamoun-Essghaier *et al.*, 2005).

First proteomic study in *Leishmania* (Thiel and Bruchhaus, 2001) was carried out for comparative analysis of *L. donovani* promastigote and amastigote stages. Further progress in this developing area was made in subsequent years to generate basic proteome maps in different field isolates of New- and Old-World species including *L. amazonensis, L.(Viannia) braziliensis, L. (V.) guyanensis, L. (V.) panamensis, L. major, L. infantum,* and *L. donovani* (Bente *et al.*, 2003; Brobey *et al.*, 2006; Cuervo *et al.*, 2007; Drummelsmith *et al.*, 2003; El Fakhry *et al.*, 2002; Forgber *et al.*, 2006; Foucher *et al.*, 2006; Gongora *et al.*, 2003; Leifso *et al.*, 2007; McNicoll *et al.*, 2006; Nugent *et al.*, 2004; Thiel and Bruchhaus, 2001; Walker *et al.*, 2006a; Walker *et al.*, 2006b). Through the use of 2-D PAGE and Western blot analysis, Dea-Ayuela *et al.* (2006) showed that some antigens are mainly recognized by antibodies generated against immunizations of rodents with leishmanial proteins. This approach was found useful for identifying new immunogenic components or to provide additional functional information on already know antigens. In another study (Kamoun-Essghaier *et al.*, 2005) a combination of 2DE and LC-MS/MS and micro sequencing from gel purified bands was used for identification and characterization of proteins of a 30- to 36-kDa fraction of *L. infantum* promastigote membranes which was previously shown to be an immunodominant antigen(s) in Mediterranean VL (MVL) and a consistent and reliable serological marker of this disease.

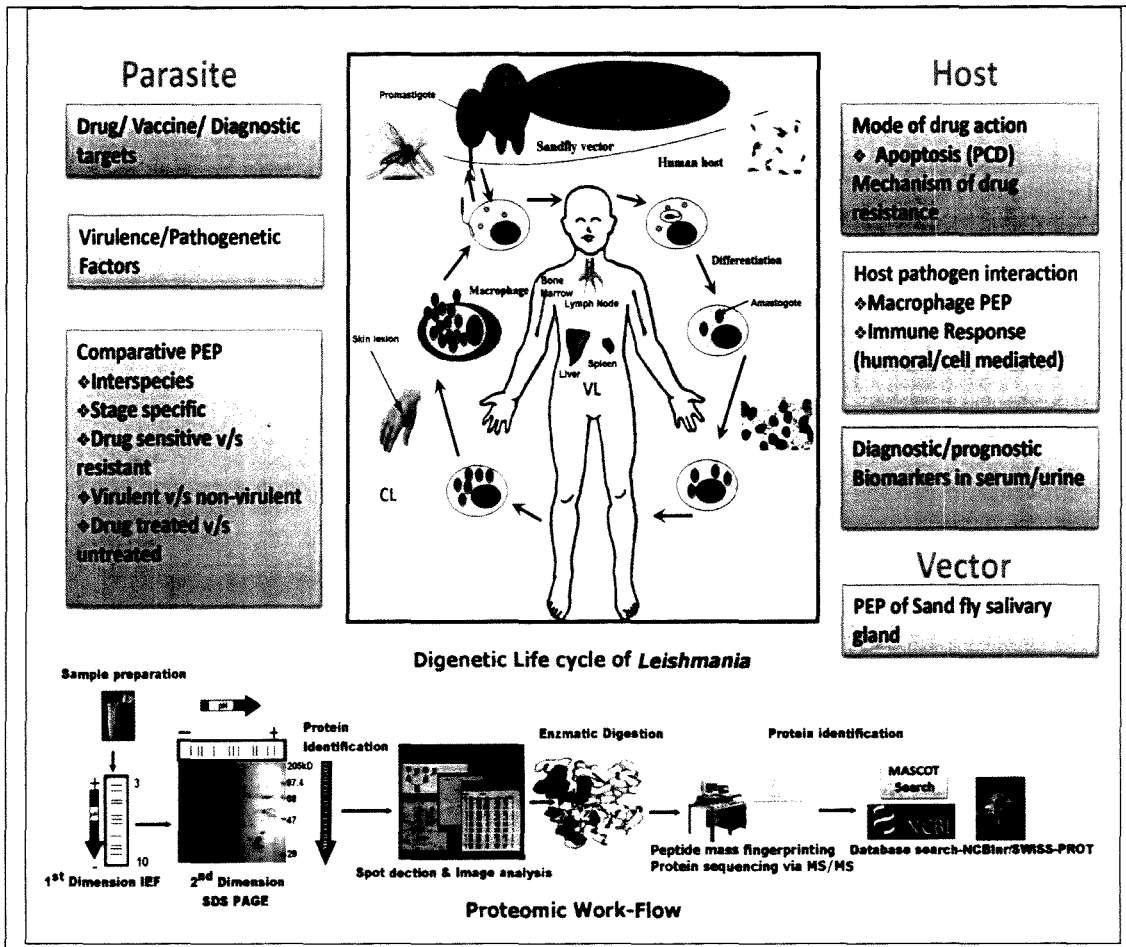


Figure 2.6: Proteomic approaches and its application in *Leishmania* parasite research.

Establishing a proteome-serological methodology, Forgber *et al.* (2006) have mapped the antigenicity of the parasites and the specificities of the immune responses in human leishmaniasis. Proteomics-based dissection of the serospecificities of leishmaniasis patients provides a comprehensive inventory of the complexity and inter-individual heterogeneity of the host-responses to and variations in the antigenicity of the leishmanial parasites. This information can be instrumental in the development of vaccines and and diagnostic devices. Further, for better understanding of the mechanisms of drug resistance in *Leishmania* parasite a complimentary strategy by using both functional genomics and proteomics approaches shall be beneficial to identify genes and proteins that are expressed in a stage-specific manner and should accelerate our understanding of key processes related to *Leishmania* biology.

Table 2.1: Proteomic approaches for various aspects study in different *Leishmania* species.

Sp.	Parasite stage / protein form	Proteomic Tools	Proteome Map	Aspect studied	Observations/Remarks & references
Ld	Prom & Amast -WCP	2-DE of [^{35}S] methineine labeled *Leish.*	Upto 400 spots	Identification of stage specific proteins at different time, pH & temp.	Protein synthesis significantly decreased during stage differentiation from promastigote to amastigotes (Thiel and Bruchhaus, 2001)
	Prom & Amast -SP &WCP	2-DE + MALDI-TOF	~2000 spots; 31 proteins +nt only in prom ; abundance of 67 proteins during stage differentiation	Identification of stage specific proteins at different time	HSP-90 play pivotal role during stage differentiation (Bente et al., 2003)
	Prom -WCP	1D + 2D+ Immunoblotting MALDI-TOF	330 spots; 68 antigenic proteins identified	Identification of serospecific antigens	Provided new insights into the quality & identity of the antigens targeted by the immune systems of *Leishmania* patients (Forgber et al., 2006)
	Prom -SP	2-DE+ MALDI-TOF	63 spots in the range of 68-97.4 kDa; 33 proteins identified	Identification of Th1 stimulatory proteins as vaccine targets	33 proteins identified as immunogenic/ vaccine candidate & 6 hypothetical / unknown (Gupta et al., 2007)
	Prom -WCP	2-DE + MALDI-TOF	> 1600 spots	Comparative proteomic study In Sb(V) resistant vs. sensitive strains & highlighting PCD	HSP-83 & calpain-related proteins shown to be implicated in the drug-induced PCD phenotype in drug resistant isolate (Vergnes et al., 2007)
Li	Prom & Amast -SP	2-DE + LC-MS/MS	Approx. 2000 spots ; >62 proteins differentially expressed in amast	Identification of developmentally regulated proteins as vaccine/ drug targets	Provided new insight in understanding of metabolic & energy requirements for the survival of intracellular form (El Fakhry et al., 2002)
	Prom & Amast prefractionation by Amm. SO4	2-DE + LC-MS/MS+ microarray	Over 2200 spots; increased proteins by 6.1% in prom & 12.5% in amast	Identification of differentially expressed stage-specific proteins	Amm SO4 prefractionation allowed visualization of low abundant proteins; Post-transcriptional controls at translational & post-translational levels could play major roles in stage differentiation(McNicoll et al., 2006)

	Prom & Amast prefractionation by digitonin	2-DE + MALDI-TOF	1921 in WCP & 286 to 1084 spots in different fractions of digitonin.	Identification of differentially expressed proteins (cytosolic & organelle specific) in a stage specific manner	Digitonin extraction allowed identification of lower expressed proteins & provide tentative subcellular localization (Forgber et al., 2006)
	Prom -WCP	2-DE +MALDI-TOF	700 spots; 29 proteins of various functional categories identified from 72 spots	Identification of functional antigenic proteins	Identified a substantial no of antigenic proteins associated to crucial physiological & virulence functions which may be suitable targets for both vaccination & chemotherapeutic strategies (Dea-Ayuela et al., 2006)
	Prom & Amast -WCP	ICAT + MALDI-TOF+ microarray	91 differentially expressed proteins identified, 8% in amast & 20% differentially expressed in prom	Identification of differentially expressed stage specific proteins	Applicability of ICAT technology for accurate quantitative proteomic analysis of differentially expressed, stage specific proteins (Leifso et al., 2007)
Lm	Prom -WCP	2-DE + MALDI-TOF	3700 spots; identified landmark proteins	Application of proteomics to the study of potential drug targets & drug resistance mechanisms	Identified no. of landmark proteins & showed their relativity to *L. donovani*; Proven for the first time phenomena of PTM in trypanothione reductase- a potential drug target ; identified primary drug resistance mechanism in resistant strain using comparative PEP (Drummelsmith et al., 2003)
La & *Lm*	Prom -WCP	2-DE analysis	800 & 1,530 spots in *La* & *Lm* proteome respectively	Comparative proteome mapping	Inter-species profiling of *Leishmania* proteins (Brobey et al., 2006)
La	Prom -WCP	Liquid-phase IEF+2-DE	350 to 1050 spots in different fractions	Proteome mapping	Optimized a liquid-phase IEF in combination with 2-DE to reduce protein complexity & generated high resolution 2-DE maps encompassing both the acidic & basic ends of the proteome with enhanced spot representation for low-abundance proteins (Brobey et al., 2006)

Lmxi	Prom (pro & metacyclic) & Amast -WCP	2-DE &+ CapLC-QTOF	> 2000 spots in each stage; 47 proteins were stage-specific; 54 proteins changed in differentiation from pro to metacyclic, 102 in metacyclic to amast; 36 unique spots in amast	Proteome mapping & identification of differentially expressed stage specific proteins	Identified many proteins implicated in infectivity & host-parasite interactions (Nugent et al., 2004)
L(V)g	Prom -SP	2-DE analysis	Approx. 719 spots in NP-40, 322 spots in NP-40 + NH_4 acetate	Reference proteome mapping	Identified a solubilization method using NP-40 as detergent, which produced reproducible, high resolution 2-DE gels (Gongora et al., 2003)
	Prom -SP	2-DE & LC-ES-MS/MS	>800 spots	Comparative expression proteomics; identification of potential metastasis factors	Identified 2 factors viz elongation factor-1β & tryparedoxin peroxidase associated with parasite survival & persistence & thereby metastasis (Walker et al., 2006a)
L(V)g & *L(V)p*	Prom -SP	2-DE & LC-ES-MS/MS	800 & 1,500 spots in *Lg* & *Lp* proteome respectively	Comparative expression proteomics; identification of molecular determinants for virulence, vaccine targets	Characterization of the *Leishmania* proteome (the expressed protein complement of the genome) (Gongora et al., 2003)
L(V)p	Prom & Amast -SP	2-DE & LC-ES-MS/MS	> 700 spots; 75 differentially-regulated proteins , 24 uniquely expressed & 51 over-expressed proteins in amast	Proteome mapping & identification of differentially expressed stage specific proteins	Identified several novel developmentally-regulated proteins related to intracellular survival, simultaneously pinpointing therapeutic targets (Walker et al., 2006b)
L(V)b	Prom -WCP	2-DE &MALDI-TOF	101 spots; identified 75 proteins belonging to 15 functional categories	Reference proteome mapping; identification of putative virulence markers & vaccine/drug targets	Identified potential virulence factors & drug targets, including some proteins associated with the metastatic phenotype; study provided a useful tool for comparative studies of clinical isolates presenting different clinical manifestations. (Cuervo et al., 2006)

Abbreviations: La- *L. amazonensis*, Ld- *Leishmania donovani*, Li- *L. infantum*, Lmj- *L. major*, Lmx- *L. maxicana*, L(V)g- *L. (Vianna) guynanensis*, L(V)b- *L (V) braziliensis*; L(V)p- *L. (V) panamensis*, Amm. SO_4 -Ammonium sulphate, Amast- Amastigote, Prom- Promastigote, SP- Soluble proteins, WCP- Whole cell protein.

2.4. Genomic differentiations and genetic markers

Genetic differentiation refers variations of genomes between members of species, or between groups of species thriving in different parts of the world as a result of genetic mutation. It can be studied by doing genotyping or gene mapping. For many years, gene mapping was limited in most organisms by traditional genetic markers which include genes that encode easily observable morphological characteristics. The insufficient amount of these types of characteristics in several organisms limited the mapping efforts that could be done. New molecular techniques in 1980's, made it possible to examine variations in DNA, providing unlimited number of genetic markers that can be used for creating genetic maps as well as for studying linkages between diseases and genetic inheritance. Some commonly used genetic markers are

- RFLP (Restriction fragment length polymorphism)
- RAPD (Random amplification pf polymorphic DNA)
- SSR (Simple sequence repeats)
- AFLP (Amplified fragment length polymorphism)

All of these methods have expanded the availability of genetic markers for creation of genetic maps.

2.4.1. RFLP (Restriction fragment length polymorphism)

RFLP were one of the earliest molecular marker methods used which detect variations in DNA sequencing by cutting the DNA with restriction enzymes and analyzing the size of the resulting fragments by gel electrophoresis. It is the sequence that makes DNA from different sources different, and RFLP analysis is a technique that can identify some differences in sequence (when they occur in a restriction site). Though DNA sequencing techniques can characterize DNA very thoroughly, RFLP analysis was developed first and was cheap enough to see wide application. Analysis of RFLP variation was an important tool in genome mapping, localization of genetic disease genes, determination of risk for a disease, genetic fingerprinting, and paternity testing.

2.4.2. RAPD (Random amplification of polymorphic DNA)

It is a type of PCR reaction, but the segments of DNA that are amplified are random. The scientist performing RAPD creates several arbitrary, short primers (8-12 nucleotides), then proceeds with the PCR using a large template of genomic DNA. By resolving the resulting patterns, a semi-unique profile can be gleaned from a RAPD reaction.

2.4.3. SSR (Simple sequence repeats)

Also known as microsatellite repeat, consisting of short nucleotide sequences (e.g. GTA) that are repeated many times in tandem (...GTAGTAGTA...). The number of SSR tandem repeats can vary in a sequence, and many such variants (alleles) can exist in a population. Microsatellites (a SSR) consisting of two to six, but usually two or three, nucleotides that are repeated many times in tandem (e.g. GAGAGA...) and that show high variation in repeat number between individuals. By developing PCR primers for the regions flanking a microsatellite repeat, microsatellite allele variation at this site can be screened through high-resolution electrophoresis of microsatellite PCR products. Microsatellite primers developed for one species can rarely be used beyond the very closest relatives; practically, therefore, microsatellite primers need to be developed *de novo* for each new species.

2.4.4. AFLP (Amplified fragment length polymorphism)

AFLP is a PCR-based tool used in genetics research, DNA fingerprinting technique developed in the early 1990's by Vos and Zabeau in 1993. AFLP uses restriction enzymes to cut genomic DNA, followed by ligation of adaptors to the sticky ends of the restriction fragments. A subset of the restriction fragments are then amplified using primers complementary to the adaptor and part of the restriction site fragments (as described in detail below). The amplified fragments are visualized on denaturing polyacrylamide gels either through autoradiography or fluorescence methodologies. AFLP-PCR is a highly sensitive method for detecting polymorphisms in DNA.

Genomic differentiation analysis of parasites, both in the laboratory strains and in the field isolates, depends on the detection of variation between individuals. Over the past two decades, the detection of variation has depended primarily on the observation of protein variation, usually using isoenzyme gel electrophoresis (Godfrey *et al.,* 1990). More recently, a range of molecular techniques has been used, including randomly amplified polymorphic DNAs (RAPDs, Waitumbi *et al.,* 1993), microsatellites (Wang *et al.,* 1999), minisatellites and restriction fragment length polymorphism (RFLP) analysis of single and multicopy genes (Sternberg *et al.,* 1989), including repetitive DNA sequences. The molecular techniques have a number of advantages, particularly in their requiring much smaller quantities of material to work with. Amplified restriction fragment length polymorphism (AFLP) - a high throughput molecular technique analysis is able to detect genetic variation and polymorphisms without prior genome sequence knowledge using PCR (Vos *et al.,*

1995). The choice of which molecular technique to use depends on i) the application (e.g. DNA genotyping, genetic mapping, population genetics); ii) the organism under investigation (e.g. parasite, plants, animals); and iii) the resources (time and money) available. In most cases no one fingerprinting technique is ideal for all applications. However, AFLP's are quickly becoming the tool of choice for many applications and organisms. Potential applications include screening DNA markers linked to genetic traits, parentage analysis, forensic genotyping, diagnostic markers for pathogen borne diseases, and population genetics. Since the AFLP technique can be applied to a wide variety of organisms so that this technique has the potential to become a universal DNA fingerprinting tool. Due to its advancement and advantage over other technique, fingerprints are produced without prior sequence knowledge using a limited set of generic primers. AFLPs are capable of distinguishing the heterozygote from the homozygote and of converting any AFLP band of interest, without much effort, into locus-specific markers (Roos *et al.,* 1998), which can be deployed for massive locus detection and for gene isolation. Researchers in the field of molecular ecology and evolution require versatile and low-cost genetic typing methods.

2.4.4.1. General features of AFLP

AFLP fingerprints can be used to distinguish even very closely related organisms, including near isogenic lines. The differences in fragment lengths generated by this technique can be traced to base changes in the restriction/adapter site, or to insertions or deletions in DNA fragment. Dependence on sequence knowledge of the target genome is eliminated by the use of adapters of known sequence that are ligated to the restriction fragments. The PCR primers are specific for the known sequences of the adapters and restriction sites. Most importantly; AFLPs have been shown to be reproducible and reliable. This is at least partially due to the fact that limited sets of generic primers are used and these are annealed to the target under stringent hybridization conditions. The technique can be adjusted to generate consistent band patterns from DNA of any origin or complexity. Typically 50-200 bands are generated in a single lane after electrophoresis of the PCR amplified products on an analytical polyacrylamide gel. The AFLP data usually must be treated as dominant markers, since the identity of homo/heterozygote cannot be established unless pedigree studies are carried out to determine inheritance patterns of each band. However, the large number of bands gives an estimate of variation across the entire genome, thus giving a good general picture of the level of genetic variation. This type of information

is generally more applicable co genotyping, forensics, and conservation biology than detailed information on variation at one or a few loci (e.g., RFLP, microsatelliles etc).

2.4.4.2. Additional characteristics of the AFLP

i.) AFLP is relatively fast (samples is processed on automated PCR and DNA sequencers).

ii.) It assays the entire genome for polymorphic markers.

iii.) It requires relatively small amounts of genomic DNA. Typically 0.05 - 0.5 μg of DNA are required, depending upon the size of the genome.

iv.) It provides 10-100 times more markers therefore AFLP is more sensitive than other fingerprinting techniques.

v.) Unlike RAPD it is highly reproducible. Analyses performed by different workers or in different labs can be compared or reproduced. The bands (DNA fragments) can be run on an automated sequencer that resolves fragment length to single-base units.

2.4.4.3. How AFLP works

The method for generating AFLP band patterns is outlined in Figure 2.7. Double digestion of genomic DNA with two restriction enzymes (EcoRI and MseI in Figure 2.7. A) Results in three classes of fragments. Adapters are ligated to the fragment ends and PCR amplification is carried out using pre-selective (Figure 2.7. B) and selective primers (Figure 2.7. C), with a labelled primer recognizing the EcoRI adaptor. Fragments that result from digestion only with EcoRI are likely to be too big to be resolved on a standard sequencing gel because, on average, the restriction site occurs every 4096 (4^6) bp, assuming random distribution of the four bases in DNA. If these fragments were small enough to enter the gel, they would still be detected. Fragments flanked only by MseI sites will occur more frequently every 256 (4^4) bp, on average – but are unlabelled and will not be detected. The vast majority of bands detected on AFLP gels are fragments flanked by both enzyme recognition sites. A large number of possible primer combinations can be used with a single pair of restriction enzymes (Vos *et al.,* 1995). Output from AFLP analysis shows part of an autoradiograph as the spectral data (Electropherogram) for each sample in the form of peaks (Figure 2.7. D). Polymorphisms detected by AFLP will result from single nucleotide changes in the restriction sites, or adjacent to these sites in the bases complementary to the selective nucleotides, or from insertion/deletion events between the cut sites (Vos *et al.,* 1995).

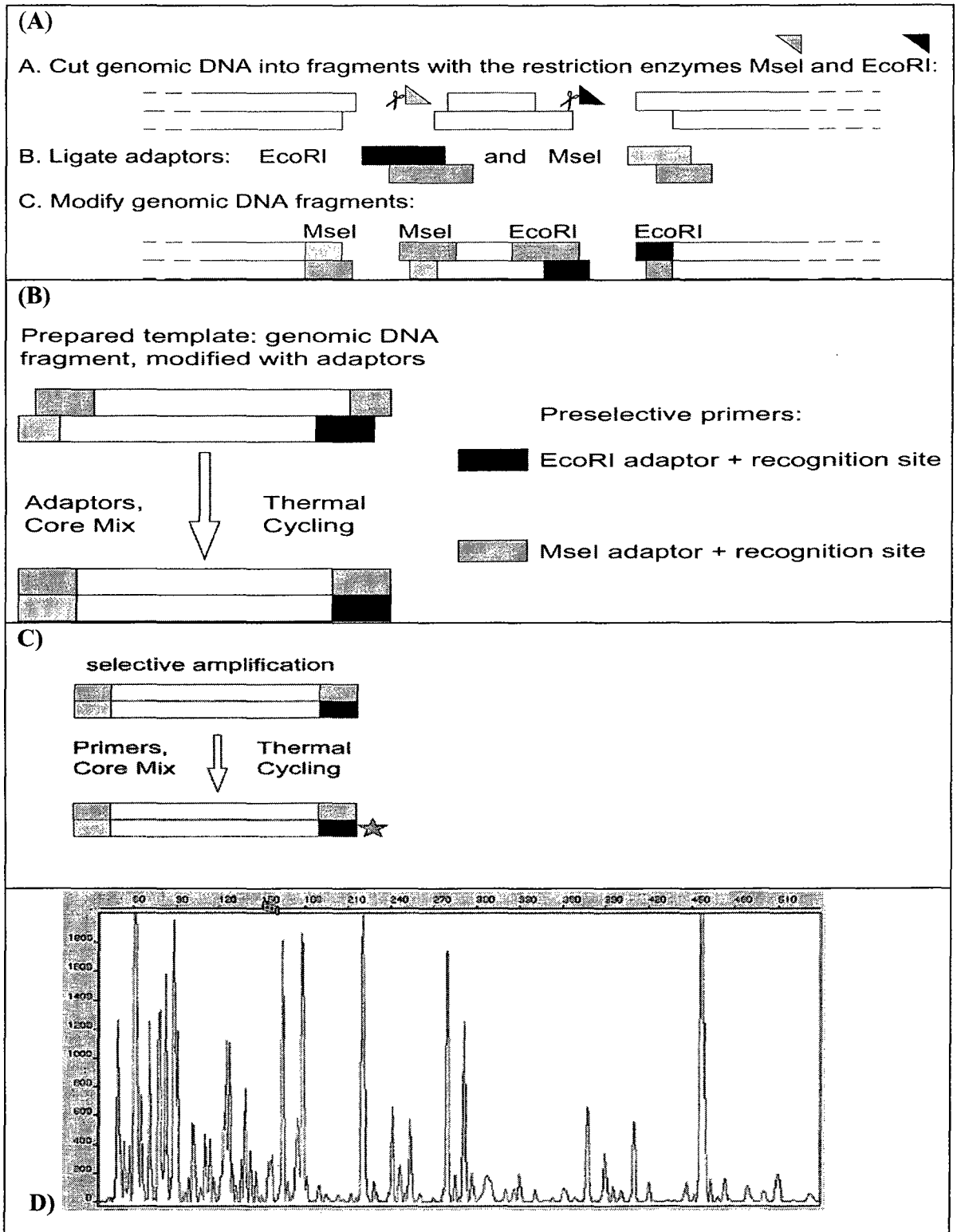


Figure 2.7: The methods for AFLP. **(A)** Template preparation & adaptor ligation, **(B)** Schematic diagram for pre-selective amplification reaction, **(C)** Selective amplification reaction with 5' fluorescent dye labeled primer (shown by star in figure). **(D)** AFLP electropherogram of sample run on DNA sequencer. Peak of different sized DNA band (Numeric value on Y-axis shows peak intensity and X-axis shows number of bands).

AFLP is likely to be superior to other more established marker methods, such as SSR, RAPD, RFLP for genetic variation study. Comparisons among some popular genetic markers generating techniques are discussed in table 2.2.

Table 2.2: Comparisons among some popular genetic markers generating techniques.

Features	AFLP	RAPD	SSR	RFLP	References
Average band informativeness	High	High	Low	Low	Archak et al., (2003)
Based on	PCR	PCR	PCR	Hybridization	Meudt et al., (2007)
Cost	Medium	Low	Medium	Medium	Naimuddin & Nishigaki (2003)
Degree of inter-specific polymorphism	High	Low	High	Low	Mueller & Wolfenbarger (1999)
Degree of intra-specific polymorphism	High	Low	Moderate	Low	Mueller & Wolfenbarger (1999)
Development time	Short	Short	Long	Long	Mueller & Wolfenbarger (1999)
Ease of use and development	Moderate	Easy	Difficult	Difficult	Mueller & Wolfenbarger (1999)
Expertise required	Good	Fair	Fair	Fair	Naimuddin & Nishigaki (2003)
Genetic diversity	High	Low	Moderate	Low	Bensch & Aesson (2005)
Genotype index	High	Low	Low	Low	Archak et al., (2003)
Highly automated	Yes	Moderate	Yes	No	Meudt et al., (2007)
Identification of hybrids and backcrosses	High	Low	Moderate	Low	Bensch & Aesson (2005)
Marker index	High	Low	Low	Low	Archak et al., (2003)
Readiness	Good	Fair	Fair	Fair	Naimuddin & Nishigaki (2003)
Reproducibility	High	Low	High	High	Naimuddin & Nishigaki (2003)
Resolution of genetic differences	High	Moderate	High	High	Naimuddin & Nishigaki (2003)
Marker nature	Dominant	Dominant	Co-dominant	Co-dominant	Mueller & Wolfenbarger (1999)
Parentage analyses	High	Low	High	Low	Bensch & Aesson (2005)
Phylogenetic reconstructions	High	Low	Moderate	Low	Bensch & Aesson (2005)
Quantity of information	High	High	High	Low	Bensch & Aesson (2005)

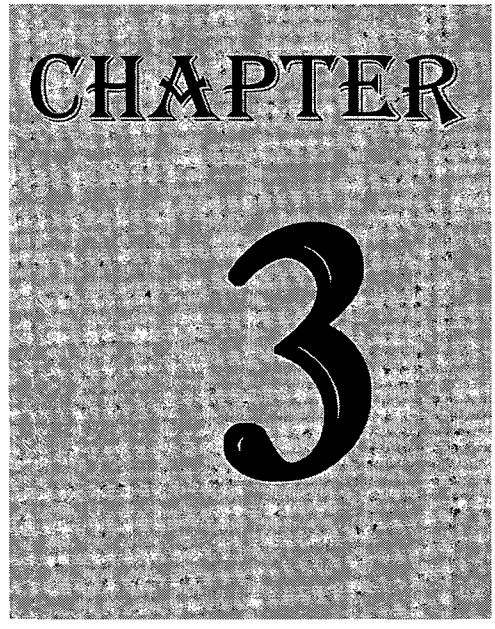
2.5. AFLP: A high throughput genomic fingerprinting technique for genomic variation study in drug sensitive and resistant isolates of protozoan parasites

AFLP technique is a relatively new and advance method for the analysis of polymorphism that has not yet been widely used in parasite study. Genetic analysis of parasites and their vectors, both in the laboratory and into the field isolates, depends on the detection of variation between the individuals. DNA profiles produced by AFLP define the clinical isolates of parasite from which they are obtained and allow differentiating among isolates. All together, there are only few studies of AFLP in parasite is notable. AFLP is not fully explores technique in parasite research.

A few recent studies in parasites (Tandon *et al.,* 2005, Troell *et al.*, 2006) have taken this notice as a starting point for finding genes that matter, or rather markers for such genes, following a strategy called 'genome scans'. The AFLP method has been used successfully in detailed genetic analysis of the some protozoan parasite like *Trypanosoma* (Masiga *et al.*, 2000; Tai *et al.*, 2002), *Plasmodium* (Rubio *et al.*, 2001; Grech *et al.*, 2002). AFLP mapping for malaria parasite susceptibility in *Aedes aegypti* was done by Zhong *et al.,* in 2006. Some RFLP/RAPD based study was done by so many workers (Gomes *et al.*, 1995; Tai *et al.*, 2001; Guizani *et al.*, 2002; Toledo *et al.*, 2002; Marfurt *et al.*, 2003; Ferroglio *et al.*, 2006) in *Leishmania* parasite. A good attempt was taken to differentiate *L. chagassi, L. amazonansis, L. braziliansis* using SSR-PCR technique by Volpini *et al.*, (2001). These few studies provide some valuable information about the genetic make-up of the parasites and further indicate to maximize genetic diversity in parasites using AFLP approach. AFLP can be used as rapid, highly discriminatory screening techniques to determine the taxonomic diversity and phylogenetic structure of parasite populations. AFLP genomic fingerprinting techniques appear to refect the genotypic, phylogenetic and taxonomic relationships of organisms (Vos *et al.*, 1995) and, therefore, we propose that this technique can be used as a rapid means of determining taxonomic diversity and phylogenic structure, especially of large collections of clinical isolates of parasite. Assessment of genetic diversity provides an efficient and effective way to estimate genetic variation and delineate phenetic relationships among isolates. AFLP markers can be routinely employed for analysis of variation in gene pool of parasite.

As per discussion above it can be inferred that AFLP is a powerful molecular tool to generate and identify molecular markers. Our main aim was to develop unique DNA marker for quick identification of sensitive and resistant drug strain in VL patients and also to develop the understanding of drug targets. The parasitic strain-specific markers can be used to identify drug sensitive/resistant gene. It is very promising technique for parasite strain variance study. Keeping this in view genomic differentiation study was done in SAG sensitive and resistant strains of *L. donovani*. The AFLP method can be used on organisms with no prior molecular information. The high reliability of AFLP markers could lead to the displacement of RAPD markers, and the user-friendliness of AFLP markers might cause a partial replacement of other high-resolution markers. Thus, AFLP could be the main tools for the analysis of genetic variation. Although AFLP–PCR is not a panacea for all molecular problems, but it offers many advantages over othr fingerprinting techniques and therefore it will probably replace several other techniques.

The AFLP technology has the capability to detect various polymorphisms in different genomic regions simultaneously. As a result, AFLP has become widely used for the identification of genetic variation in strains or closely related species of plants, fungi, animals, and micro-organism. The AFLP technology has been used in criminal and paternity tests, in population genetics to determine slight differences within populations. There are many advantages to AFLP when compared to other marker technologies including RAPD, RFLP and microsatellites (Weber and Helentjaris, 1989). AFLP not only has higher reproducibility, resolution, and sensitivity at the whole genome level than other techniques, but it also has the capability to amplify between 50 and 100 fragments at one time. In conclusion, the AFLP technique has been shown to be a very good molecular technique for genomic fingerprinting and variation study and will be useful in a wide range of applications.



Rationale, Aims and Objectives

"No great discovery was ever made without a bold guess".

- Isaac Newton -

3. Rationale, Aims and Objectives

3.1. Rationale of the present study

As discussed in the previous chapters following points have emerged:

1. VL caused by *L .donovani* is a complex anthroponotic disease of poverty particularly of India and Sudan and is usually fatal if not treated properly.

2. The current treatment measures available against VL are not entirely satisfactory due to severe toxicity or side effects, painful route of administration, prolonged treatment schedules that require hospitalization leading to high cost of the treatment.

3. Pentavalent antimonials Sb(V) are being used worldwide as first-line drugs for the treatment of VL and CL since 1940s. Two major pentavalent antimonials - Sodium antimony gluconate (SAG, Pentostam) and meglumine antimoniate (Glucantime) are currently used for the treatment of the infection of *L. donovani* but the resistance against this drug has been alarmingly increasing in several parts of the world Widespread primary failure (>65% unresponsiveness) to SAG has also been reported from North Bihar, India which has been attributed to poor compliance of patients to long treatment schedules (Croft *et al.*, 2006b; Lira *et al.*, 1999; Sundar *et al.*, 2000; Sundar *et al.*, 2001). However, in most parts of the world, over 95% of previously untreated patients with VL do respond to pentavalent antimonials.

4. The mechanism of resistance is now partly understood in laboratory bred isolates but it is lagging behind in respect of field or clinical isolates. Using intracellular susceptibility testing, parasites isolated from Sb (V) unresponsive patients were found to be more resistant to Sb(V) as compared to parasites isolated from Sb(V) responsive patients (Faraut-Gambarelli *et al.*, 1997; Hadighi *et al.*, 2006).

5. Recent studies with field isolates (Decuypere *et al.*, 2005; Singh *et al.*, 2003) suggested the similarities and differences with laboratory resistant isolates but it is not yet clear whether any of the highlighted mechanisms is central to the resistance phenotype of field isolates.

6. With the alarming rise of drug resistance, there is an urgent need to identify and characterized parasite targets for the development of novel drug/vaccine therapies.

7. Genomics approach for genetic variation study among clinical isolates of *L. donovani* can provide blueprint for moving forward in the field of disease marker discovery and development. To draw the genetic variation, AFLP can be done which is a reliable DNA fingerprinting technique that has provided one of the most informative approaches to ascertain about molecular variance, genomic differentiation and phylogenetic structure. The genome of the parasite includes approximately 8000 genes (Leifso *et al.*, 2007) coding for number of proteins that indicates about the complexity. Therefore the scanning of multiple loci across the whole genome for the presence of polymorphism (Tandon *et al.*, 2005) was performed by AFLP. These new high-throughput approaches definitely provide the opportunity to identify numerous genes/proteins differentially expressed during the life cycle of *L. donovani*. Some of the identified genes/proteins could be used for the development of new diagnostic and therapeutic tools in *Leishmania* research.

8. Using proteomics approach, preliminary efforts in this direction include the generation of a partial 2-D gel map of *L. donovani* and analysis of differentially expressed proteins through MALDI-TOF have been pinpointed (Faraut-Gambarelli *et al.*, 1997). Proteomics refers to the systematic analysis of protein profiles of entire cells, tissues, organisms, or species. It represents the protein counterpart to the analysis of gene function. A proteome map of the species being sequenced is required, and the work done here may provide a framework of 2-D gel and mass spectrometry (MALDI-TOF/TOF) based approaches to quantitative proteomics in *L. donovani.* Proteomic methods can be applied to study sub-cellular localization, cell function, organelle composition, changes in protein expression patterns in response to drug exposure, drug-protein binding and validation of data from genomic annotation and transcript expression studies. Proteomics offer increased hope for the discovery of promising new drug targets/ vaccine candidate by virtue of their ability characterize complex parasite biology and biochemistry.

9. Since, the genome and proteome are complimentary; the correspondence study between the genome and proteome shall be more effective for target validation in drug resistance of parasite. The correlation between genomics and proteomics can give a strong and better conclusion in target finding.

3.2. Aims of the study

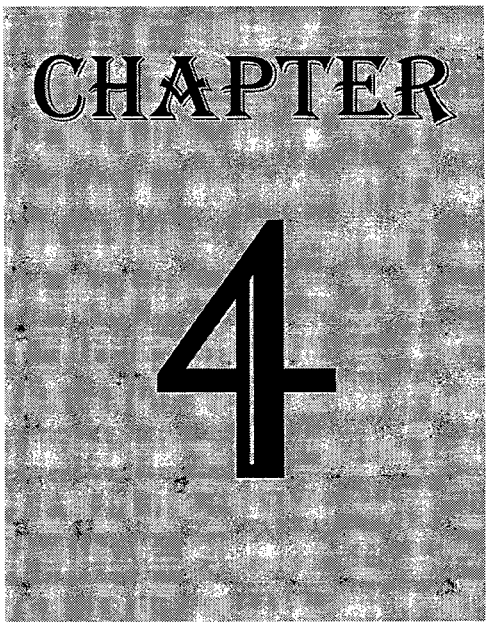
In the light of above rationales the aims of this study was to increase our understanding in Sodium antimony Gluconate (SAG) resistance mechanism and eventually to link genome and proteome of *L. donovani* to identify novel drug targets/ vaccine candidate. Further, since, *Leishmania* parasite has significant antigenic diversity due to its digenetic life cycle, the identification and characterization of the structural and functional proteins in both membrane as well as soluble content of the promastigote stage which occurs in vector and can be easily cultured *in vitro* in abundance, would provide a solid base.

In this study, through amplified fragment length polymorphism (AFLP) analysis the possible variations among the SAG sensitive/resistant isolates of *Leishmania* may lead to the identification of (i) strain-specific markers to SAG sensitive/resistant gene of *Leishmania* and (ii) molecular marker(s) that can be used to develop the understanding of drug targets. Further, using the proteomic approach, with the availability of annotated sequenced genome of *L. major* and *L. infantum,* a comprehensive proteome mapping in promastigote stage of the *Leishmania* parasite would provide a base for the screening of differential proteomic expression in order to compare a genetically related pair of SAG resistant and sensitive Indian *L. donovani* field isolates. This may provide lead towards identification of those proteins which are involved in resistance mechanism and can eventually be used as a potential drug/vaccine target(s).

3.3. Research objectives

With these aims the specific objectives of this study have been drawn as follows focusing on *L. donovani* parasite:

1. Genomic fingerprinting study of SAG sensitive and resistant clinical isolates of *L. donovani* through amplified fragment length polymorphism (AFLP).
2. Proteomic analysis of membrane as well as soluble proteins in clinical isolates *of L. donovani* by 2-Dimensional Gel Electrophoresis (2-DGE) and Matrix Assisted Laser Desorption Ionization -Time of flight (MALDI-TOF) mass spectrometry.
3. Identification of differentially expressed membrane/soluble proteins between sodium antimony Gluconate (SAG) sensitive and SAG resistant *L. donovani* isolates by 2-DGE and MALDI-TOF.



Maintainance of Leishmania

"He who risks and fails can be forgiven. He who never risks and never fails is a failure in his whole being."

- Paul Tillich -

4. Maintenance and drug sensitivity of *Leishmania donovani* clinical isolates

The causative organism of leishmaniasis exists in two forms, a flagellated motile form promastigote, found in the gut of *Phlebotomus spp.* and an aflagellated, non-motile form, called amastigote, parasitizing the vertebrate host. Regular availability of parasite is a pre-requisite for any experimentation. The promastigote stage has been widely cultured *in vitro* in a variety of biphasic and monophasic media (Bhatnagar *et al.*, 1989; Chatterjee and Ghosh, 1957) and the amastigote stage is maintained *in vivo* by regular passage in susceptible animal hosts.

4.1. Preparation of culture media

4.1.1. Rosewell Park Memorial Institute (RPMI) – 1640 Medium

The medium was prepared by adding 13.4 gm RPMI–1640 (Sigma, USA) powder supplemented with 0.3g/L L-glutamine in 500 ml of tripled distilled water (TDW) followed by the addition of 2 gm $NaHCO_3$, 2 gm glucose, 5.94 gm N-2-Hydroxy-Ethyl-Piperazine-N-2-Ethane sulphonic acid (HEPES) with constant stirring for about 45 min and the pH was adjusted to 7.2 ± 0.2. Finally, volume was adjusted up to 900 ml with TDW. This is called as incomplete RPMT. The medium was filtered through 0.2 μm (pore size) Millex-GV filter units (Millipore, MA, USA) and 40 mg Gentamycin and 10% heat inactivated fetal bovine serum (Hi-FBS) was added. Now, RPMI medium (Complete) was tested for sterility by putting a drop in thioglycollate medium (2.98 g). Thioglycollate medium was dissolved in 100 ml TDW (pH 7.2±0.2) and was sterilized by autoclaving at 15 lbs for 15 min) and incubated at 37°C for 24 hr. Since, thioglycollate supports rich bacterial growth even slight bacterial contamination grows luxuriously and makes a white ring on the top of the medium. Complete RPMI-1640 was finally stored at 4°C.

4.1.2. Leibovitz (L-15) medium

L-15 medium (14.7 g; Sigma, USA) supplemented with 0.3 g L-glutamine was dissolved in 800 ml of distilled water followed by the addition of 2 g glucose with constant stirring for 30 min. About 2.95 gm Tryptose Phosphate Broth (TPB) (HiMedia Labs, India) was dissolved in 100 ml of TDW and autoclaved at 15 lbs pressure for 15 min. In the above-mentioned L-15 medium 10% TPB and 10% FBS (Sigma, USA) were added, pH was

adjusted to 7.2 ± 0.2. Finally the volume was adjusted 1000 ml with triple distilled water. The mixture was passed through millipore filters (0.2 μm). Finally 40 mg Gentamycin was added and stored at 4°C.

4.1.3. Medium 199 (M199)

This medium was prepared by adding 13.4 g M199 (Sigma, USA) powder in 500 ml of deionized water followed by the addition of NaHCO3 (2.0 g), glucose (2.0 g), HEPES (5.94 g) with constant stirring for about 30 min and final pH was adjusted to 7.2 ± 0.2. Finally, the volume was adjusted up to 900 ml. The medium was filtered through 0.2 μm Millex-GV filter unit (Millipore, MA, USA). Gentamycin (40 mg/L) and 10% Heat inactivated FBS (HiFBS) were added and the medium finally stored at 4°C.

4.1.4. Dulbecco's modified Eagle's Medium (DMEM)

DMEM was prepared by adding 13.4 gm of powdered DMEM (Sigma, USA) in 500 ml of deionized water followed by the addition of $NaHCO_3$ (2.0 gm), glucose (2.0 gm), HEPES (5.94 gm) with constant stirring for about 30 min and final pH was adjusted to 7.2 ± 0.2. Finally the volume was adjusted up to 900 ml. The medium was filtered through 0.2 μm Millex-GV filter units (Millipore, MA, USA). Gentamycin (40 mg/L) and 10% HiFBS were added and the medium finally stored at 4°C.

4.2. Maintenance of *Leishmania* Parasite

4.2.1 In vitro:

The promastigote form of both the *Leishmania* strains was maintained in biphasic, NNN medium (Chatterjee and Ghosh, 1957) in screw cap vials using RPMI-1640 medium as an overlay (Bhatnagar *et al.,* 1989) and mass cultivation of promastigotes was accomplished in monophasic medium, L-15 (Leibovitz) with 10% heat inactivated fetal Bovine serum (FBS) and 10% tryptose phosphate broth.

(a) L. donovani WHO reference strain Dd8 (MHOM/IN/80/Dd8)

This strain was procured in 1981 from Imperial College, London through the courtesy of Dr. P.C.C. Garnham. This strain was originally isolated from a human patient from Bihar (India) in 1979. It was found to be virulent in animals and produced fatal disease. Thus, it was considered appropriate to use this as the test parasite for various experimental studies. The promastigote form of the parasite was cultured 26°C in RPMI-

1640 medium (Sigma, USA) supplemented with 10% HiFBS, penicillin (100 U/ml) and streptomycin (100 µg/ml). Parasites were sub-cultured every 3-4th day.

(b) Clinical isolates of L. donovani

A number of field isolates were procured from patients admitted to the Kala-azar Medical Research Centre of the Institute of Medical Sciences (IMS), Banaras Hindu University (BHU), Varanasi, Uttar Pradesh, India and also from its affiliated hospital at Muzaffarpur, Bihar, India (Table 4.1) and these were put into the cultures.

Table 4.1: Clinical isolates of *L. donovani* from Bihar & Uttar Pradesh (India).

Designation of clinical isolates	Year of isolation	Place of isolation	Patient gender	Clinical history of patient	Response to SAG therapy
Dd8	1979	Patna, Bihar	-	No treatment	Responded
2001	Jan. 2000	Varanasi Uttar Pradesh	Male	No treatment	Responded
2039	May 2000	Muzaffarpur, Bihar	Female	SAG (3 dose) 1 month	Not responded
2041	May 2000	-do-	Female	SAG (4 dose) 1.6 months	Not responded
2087	Dec. 2003	Varanasi Uttar Pradesh	Male	No treatment	Responded
2093	Dec. 2003	Muzaffarpur, Bihar	Male	SAG (2 dose) 1 month	Not responded

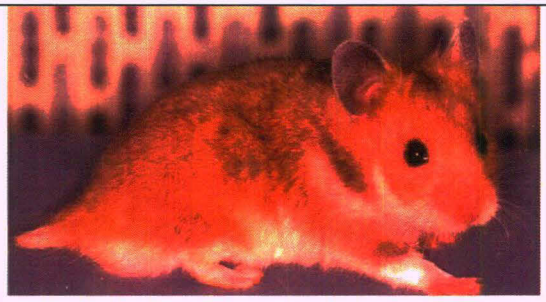
SAG sensitive isolate (2001, 2087) were obtained from kala-azar patient who responded to therapeutic course of SSG at the Institute of Medical Sciences, BHU, Varanasi. SAG resistant isolate (2039, 2041, 2093) were obtained from patients previously undergone SAG treatment and which fail to cured with regular SAG course thereafter at Kala-Azar Medical Research Center, Muzzaffarpur, Bihar. The designation of responsive patients was based on the absence of fever, clinical improvement with reduction in spleen size and the absence of parasites in the splenic aspirates. Patients who showed the presence of amastigotes were labeled as unresponsive cases. These patients were subsequently treated successfully with amphotericin B. The biopsy materials were cultivated initially at 26°C in NNN-agar tubes and subsequently promastigotes were cultured in cM199 supplemented with 20% HiFBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 26°C in 25 cm^2 tissue culture flasks (Singh *et al.*, 2002).

4.2.2 In vivo

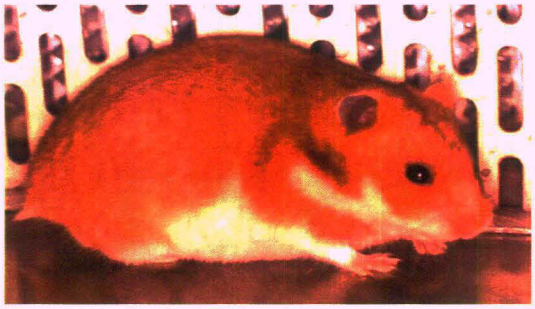
The parasites were maintained *in vivo* in hamsters through amastigote to amastigote passages or at times by inoculating the stationary phase promastigotes.

4.2.2.1. Experimental host for Leishmania

The experimental host should be small for manipulative handling, of standard breed with minimum genetic variation and should be easily available in adequate numbers. Syrian golden hamsters (*Mesocricetus auratus*) satisfy almost all the conditions mentioned above. Thus, *L. donovani*/ hamster system appeared appropriate for experimental investigations and these were employed as the experimental host for *L. donovani* infection for all the studies embodied in the present thesis. Laboratory bred male golden hamsters weighing 45-50 g (Figure 4.1 A) were housed in plastic cages in climatically controlled rooms and fed with standard rodent food pellet (Lipton Ltd., Bombay) and the water *ad libitum*. Experiments on the animals were performed according to the guidelines of Institutional Animal Ethics Committee of Central Drug Research Institute (CDRI), Lucknow (India).



(A) Naive hamster.



(B) *L. donovani* infected hamster.

(C) Spleen of normal and *L. donovani* infected hamsters.

Figure 4.1: Golden hamster as experimental host; **(A)** Naive hamster **(B)** *L. donovani* infected hamster and **(C)** Spleen of normal and *L. donovani* infected golden hamsters.

4.2.2.2. Infection to hamsters

(i) ***Preparation of infective inoculum***

(a) Promastigotes:

Promastigotes of all the strains (Table 4.1) harvested from 7-10 day old cultures were washed 2-3 times by centrifugation at 2000 rpm for 20 minutes (4°C). Pellet was suspended in phosphate buffer saline (PBS) and promastigotes number was adjusted to $1x10^8$/0.1 ml.

(b) Amastigotes:

The hamsters with well established infection (15-60 day old) were sacrificed, spleen removed aseptically in sterile Lock's solution (4 gm NaCl, 0.10 gm KCl, 0.10 gm $CaCl_2$, 0.15 gm KH_2PO_4, 1.250 gm glucose was dissolved in 500 ml distilled water, pH 7.2) and cut into small pieces. The splenic tissue was homogenized gently with the help of motor driven tissue homogenizer, consisting of glass mortar and Teflon pestle at 4^0C. Suspension was centrifuged at 500 rpm for 10 min at 4°C, allowing the tissue debris to settle down. The supernatant was centrifuged at 2000 rpm for 20 min. at 4°C. The supernatant was discarded and sediment containing amastigotes was resuspended in PBS, adjusting the count with the help of Neubaur's chamber (haemocytometer) to the concentration of 1 x 10^7 amastigotes per 0.1 ml of the cell suspension.

(ii) Inoculation to animals

Male golden hamsters weighing 40-45 gm were each inoculated intracardially with $1x10^8$ promastigotes or $1x10^7$ amastigotes (Stauber, 1966).The animals were kept in plastic cages (38x27x13 cms) with standard rodent pellets diet (Lipton India Ltd.) and water ad *libitum.*

(iii) Assessment of infection

The infection levels in the infected hamsters (Fig 4.1 B) were assessed on day 20-25 p.i. by performing splenic biopsies. Hamsters were anaesthetized by injecting 80 mg/kg of sodium pentabarbitol (Thiopentone) through i.p. route. An incision of about 1 cm over the gastro-splenic region was made aseptically and the omentum was drawn exposing the spleen. A small piece of spleen was cut and kept on a filter paper. The spleen is carefully inserted back to its position and muscles and skin is sutured using curved needle (3/4", 22-25 gauge) and braided nylon stitch thread (0 No.). Tincture benzoin is layered on the sutured

portion and neosporin powder is sprinkled on it. One day prior to operation and on 2 days post operation, neomycin sulphate (10 mg/kg) is administered orally to prevent bacterial infection. The biopsied splenic tissue was dabbed over filter paper so as to absorb excess blood and then impression smears were made on clean microscopic slides. The smears were air dried, fixed in absolute methanol for 60 sec and stained with 10% Giemsa for 45 min. The slides were washed with running tap water; air dried and examined for the number of amastigotes per 1000 macrophage nuclei under the 100 × oil-immersion objective of a light microscope.

In case of clinical isolates (2001, 2039, 2041, 2087 and 2093) the hamsters were initially infected intracardially (i.c.) with 1×10^7 stationary phase promastigotes in 0.1 ml of PBS. Splenic biopsies were performed intraperitoneal (i.p.) on day 20-21 to assess the establishment and level of infection in animal hosts. Once infection was established, further passages were carried out serially using spleen-derived amastigotes as described earlier.

4.3. Primary Culture of Promastigotes

The 45-60 days old infected hamsters were necropsied to dissect out the infected spleen (Figure 4.1 C) which was immediately given 2-3 washings in 1x PBS containing gentamycin antibiotic at a concentration of 2mg/100ml media to maintain sterile condition. Splenic tissue was then transferred to the petridish containing incomplete RPMI medium + same concentration of gentamycin as mentioned above. After 2-3 washings in the iRPMI, tissue was transferred into iRPMI (5-10 ml) + antibiotic containing flasks and kept at 26°C. Flasks were examined daily for up to 7-10 days. When the presence of moving promastigotes was noticed in the flasks, 20 µl of primary culture was inoculated to fresh flasks containing 5-10 ml complete RPMI (cRPMI) +antibiotic.

4.4. Macrophage cell line culture and maintenance

J774A.1, a mouse macrophage cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 medium, respectively supplemented with 20% hiFBS, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C in 5% CO_2 in humidified atmosphere. J774A.1 cells were sub-cultured every 2–3rd day. This cell line were then used for establishing the infection and carrying out drug susceptibility studies (Chang, 1980).

4.5. Sensitivity of clinical isolates to SAG

4.5.1 In vitro

The infectivity of strain Dd8 and clinical isolates to mouse macrophage cell line J774A.1 was investigated as per protocol described by Neal and Croft (1984). Briefly, macrophages (10^5 cells/well), laid in 16-well chamber slides (Nunc, IL, USA), were infected with promastigotes at a ratio of 10:1 (parasites/macrophage) and incubated at 37°C in 5% CO_2 for 8–12 h after which the chamber slides were washed thrice with phosphate-buffered saline (PBS, pH 7.2) and finally supplemented with complete medium. Different concentrations of SAG (100, 50 and 25 μg/ml), amphotericin B (10 and 1 μg/ml) and miltefosine (10 and 1.0 μg/ml) were added to the wells in triplicate and macrophages were examined at 24, 48 and 72 h post inoculation for the transformed intracellular amastigotes. The infected as well as control (infected and untreated) cells were fixed with methanol and stained with Giemsa. The assessment of infection was carried out microscopically. At least 100 macrophages per well were counted for calculating number of amastigotes per 100 macrophages as well as percentage infected macrophages. IC_{50} value of all the standard drugs in all the clinical isolates were assessed as shown in table 4.2. As evident in the table IC_{50} value of SAG was higher with 2039, 2041 and 2093 strains (isolated from the patients non responsive to SAG) as compared to the other isolates as well as Dd8 which were quite sensitive to SAG as well as to the other antileishmanials.

4.5.2. In vivo

Once the infection established in hamsters with the promastigote form, further passages in hamsters were carried out with splenic amastigotes. For this, animals carrying 40–60 day old infection were necropsied, their spleen removed aseptically, homogenized in Locke's solution and centrifuged at 900 rpm for 5 min at 4°C to sieve out tissue debris. Supernatant was centrifuged at 2,500 rpm for 10 min, pellet washed twice with PBS. Animals were infected intracardially with $1x10^7$ amastigotes in 0.1 ml PBS. Animals carrying 25–30 days old infection were employed for drug screening with five hamsters for each dose of different drugs. SAG was administered at the doses of 80, 40 and 20 mg/kgx5 whereas Amphotericin B, at 5 and 2.5 mg/kgx5 intraperitoneally (i.p.). Miltefosine was given orally (p.o.) at 100, 50 and 25 mg/kgx5. Five infected hamsters were kept as untreated control. Splenic biopsies were again performed as described above [section 4.2.2.2

(iii)] on day 7 post treatment (p.t.) after the administration of the last dose of each drug. Parasite burden in both treated and untreated infected animals was assessed and percentage parasite inhibition in treated animals was calculated using the formula as described below.

$$\% \text{ inhibition} = \frac{AT \times 100}{IT \times TI}$$

Where AT: the actual number of amastigotes/100 spleen cell nuclei in treated animals,
IT: the initial number of amastigotes/100 spleen cell nuclei in treated animals, and
TI: is the times increase in untreated control animals.

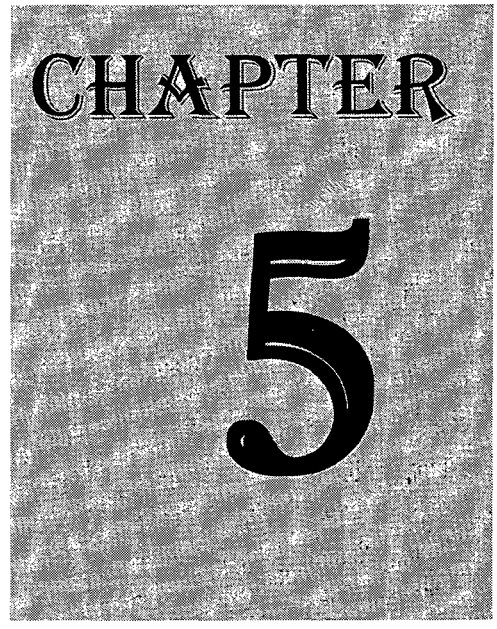
The ED_{50} value of different drugs in hamsters infected with the clinical isolates has been shown in table 4.2. As observed in *in vitro* experiments the ED_{50} values of SAG was found to be higher in hamsters infected with 2039, 2041 and 2093 parasites.

Table 4.2: Evaluation of chemo-sensitivity of clinical isolates of *L. donovani* from Bihar & Uttar Pradesh (India) to sodium antimony gluconate (SAG) in *vitro* & *in vivo*.

Designation of clinical isolates	Response to SAG therapy	IC_{50} (µg/ml) against intra-macrophage amastigotes	ED_{50} (mg/kg x 5 i.p.) in golden hamsters
Dd8	Responded	53.8 ± 7.4	12.5 ± 5.4
2001	Responded	56.3 ± 5.6	23.5 ± 7.2
2039	Not responded	>100	>60
2041	Not responded	>100	>60
2087	Responded	62.1 ± 7.4	15.6 ± 4.5
2093	Not responded	>100	>60

4.5.3. Drug sensitivity of isolates after repeated passages

To check the persistence of SAG-resistance in clinical isolates, splenic amastigotes were isolated from infected animals after 1, 6, 12 and 24 passages and allowed to differentiate into promastigotes in RPMI-1640 medium. These promastigotes were used for infecting J774A.1 macrophages and drug sensitivity assay as per the method described above. The isolates were tested for their susceptibility to SAG and other antileishmanials in hamsters after 1, 6, 12 and 24 passages. These isolates are being maintained for the last four years in the laboratory with no loss of their specific properties including SAG resistance.



Genomic fingerprinting study through amplified fragment length polymorphism (AFLP)

"Basic research is like shooting an arrow into the air and, where it lands, painting a target".

- Homer Burton Adkins -

5. Genomic fingerprinting study of SAG sensitive and resistant clinical isolates of *L. donovani* through amplified fragment length polymorphism (AFLP)

5.1. Introduction

As reviewed in earlier chapters (1 & 3), the pentavalent antimonials such as Sodium Antimony Gluconate (SAG) are the main first line drugs used for the treatment of the infection of *L. donovani* but unfortunately, the incidence of parasite becoming resistant to these drugs is increasing in several parts of the world including South America (Rojas *et al.,* 2006), Europe (Carrio *et al.,* 2001), Middle East (Hadighi *et al.*, 2006) and most notably in India (Sundar *et al.,* 2000). In India, it is most prevalent in Bihar, West Bengal, Orissa, Assam, and Eastern Uttar Pradesh. The mechanism of resistance is now partly understood in laboratory bred isolates but it is still lagging behind in respect of field or clinical isolates. Recent studies with field isolates (Singh *et al.,* 2003; Decuypere *et al.,* 2005) suggest similarities and differences with laboratory resistant isolates but it is not yet clear whether any of the highlighted mechanisms is central to the resistance phenotype of field isolates. In the previous chapter the clinical field isolates taken from the patients were subjected to SAG sensitivity since few of them *viz.,* 2039,2041 and 2093 were isolated from the patients which were non responsive to SAG treatment. It was observed that these three strains did not respond to SAG treatment in laboratory conditions also. In order to investigate whether this unresponsiveness to SAG is due to genomic variations a comparative genomic differentiation analysis of these strains was neccessary.

Amplified fragment length polymorphism (AFLP) is a high throughput DNA fingerprinting a technique used for the genomic differentiation and comparative analysis of a genetically related group of organisms. Since, prior sequence knowledge is not necessary to conduct AFLP experiments; hence, this method is particularly applicable to organisms for which no substantiative DNA sequence data are available (Masiga and Turner, 2004). AFLP has good reliability and reproducibility (Castillo *et al.*, 2007) and has been used widely to investigate organisms ranging from very simple unicellular bacteria to complex multicellular organisms (Barker *et al.*, 1999; Hayashi *et al.*, 2004; Hill *et al.*, 2004; John *et al.*, 2004; Ross *et al.*, 2003). This approach has been explored in this chapter for genomic differentiation and comparative analysis of SAG resistant and sensitive clinical isolates of *L. donovani* isolated from VL patients of Bihar and Uttar Pradesh (India) so that it could be

helpful to give the clue on SAG resistance. This study shall provide a clue to the (1) usefulness of AFLPs in differentiating resistant and sensitive strain of *Leishmania*, (2) development of unique molecular markers against resistant strain for identification and (3) determination of genetic relationships among the clinical isolates.

5.2. Materials and Methods

5.2.1. Isolation and cultivation of clinical isolates of L. donovani

A number of clinical isolates of *L. donovani* (Table 4.1) were procured from Kala-azar patients were isolated and cultivated as described previously in chapter 4.

5.2.2. Parasite culture and maintenance

L. donovani strains were cultured as described previously in chapter 4.

5.2.3. Isolation of Genomic DNA

Genomic DNA of all *L. donovani* strains was isolated as per protocol described previously (Kelly, 1993). Briefly, logarithmic phase promastigotes were disrupted in NET lysis buffer (50 mM NaCl, 50 mM EDTA, 1% SDS, 50 mM Tris–HCl, pH 8.0), and then incubated overnight with proteinase K (100 mg/ml, Sigma-Aldrich) at 37°C. DNA was purified further by phenol-chloroform extraction and ethanol precipitation.

5.2.4. Restriction enzyme digestion and adaptor ligation

AFLP was performed as described by Vos *et al.,* (1995). Briefly, 500 ng genomic DNA was digested using two restriction endonucleases, EcoRI and Tru9I (an isoschizomer of MseI) for 4 h at 37°C. Adapter ligation was performed in a 50 µL reaction mixtures containing EcoRI and MseI adapter primers, T4 DNA ligase and digested template DNA at 16°C over night. The reaction mixture was consisted of 500 ng of DNA (5.5 µl), 1 µl 10X T4 DNA ligase buffer, 1 µl 0.5 M NaCl, 0.5 µl 1mg/ml BSA, 1 µl MseI adaptors (Applied Biosystems, Foster City, CA, USA), 1 µl EcoRI adaptors (Applied Biosystems Foster City, CA, USA). The reaction was then diluted (1:20) to a total volume of 200 µl with TE (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0).

5.2.5. Pre-selective amplification

After ligation, the pre selective PCR amplification reaction was performed using template DNA, a pair of primers based on the sequences of the EcoRI and MseI adapters,

including one additional selective nucleotide at the 3'end of the MseI primer (MseI + C) and the EcoRI primer (EcoRI + A). The ligated adaptors served as primer binding sites for a low-level selection in the preselective amplification of restriction fragments. Only the genomic fragments having an adaptor on each end amplified exponentially during the PCR. The preselective amplification mix was prepared by adding 4 µl of 20-fold diluted DNA from the restriction-ligation reaction, 0.5 µl AFLP preselective primer (EcoRI, Applied Biosystems Foster City, CA, USA), 0.5 µl AFLP pre-selective primer (MseI, Applied Biosystems), and 15 µl AFLP core mix. The preselective amplification was carried out in a thermal cycler programmed as: 72°C for2 min; 20 cycles of 94°C for 20 sec, 56°C for 30 sec and 72°C for 2 min; 60°C for 30 min; and 4°C for infinity.

5.2.6. Selective Amplification

The preamplified DNA was diluted 20-fold with T10E0.1 buffer and selective amplifications were carried out using different MseI and EcoRI primer combinations (Applied Biosystems). Primer combinations were tested using 5' fluorescent-labelled EcoRI primers (EcoRI-ACA, EcoRI-ACG, EcoRI-AGC, EcoRI-ACT, EcoRI-AGG, EcoRI-AAC, EcoRI-AAG) and MseI unlabeled primers (MseI-CAA, MseI -CAC, MseI-CAT, MseI-CTC, MseI-CAG, MseI-CTA, MseI-CTG, MseI-CTT). The EcoRI primers contained fluorescent dyes, FAM (6-carboxyfluorescein) for blue, JOE (2, 7-dimethoxy-4, 5-dichloro-6-carboxyfluorescein) for green and NED (N-(1-naphthyl)-ethylene diamine) for yellow, labeled at the 5' end. The EcoRI primers contain 3 selective nucleotides with the sequence 5' [Dye-Primer-Axx]-3', while the MseI primers had the 3 selective nucleotides starting with C with the sequence 5' [Primer-Cxx]-3'. For selective amplification the reactions were set up as follows: 3 µl of 20-fold diluted preselective amplification product, 15 µl AFLP core mix, 1 µl MseI primer 5'-[Primer-Cxx]-3', 1.5 µl EcoRI primers 5'-[Dye-Primer-Axx]-3'{0.5 µl of 3 EcoRI primers each were pooled here}. Selective amplification was carried out in a thermal cycler programmed as: 94°C for 2 min; 10 cycles of 94°C for 20 sec, 66°C for 30 sec, 72°C for 2 min; 20 cycles of 94°C for 20 sec, 56°c for 30 sec, 72°C for 2 min; 60°C for 30 min; and 4°C for infinity. All the polymerase chain reactions (PCR) were performed using AFLPTM® core mix which constitutes Taq DNA polymerase, dNTP's, taq buffer (Applied Biosystems Foster City, CA, USA).

5.2.7. Electrophoresis

AFLP products were separated on a 5% denaturing polyacrylamide gel using an ABI PRISM® 377 DNA sequencer following manufacturer's protocols supplied by Applied Biosystems, USA. Briefly, 3 µl of the selective amplification reaction product was mixed with 4 µl of loading buffer [ROX-500 size standard (10%), blue dextran (10%), and deionised formamide (80%)]. Samples in loading buffer were denatured at 95°C for 3 min, 1.5 µl was loaded in each well on gel. Electrophoresis was carried-out under 3 V, 60 mA and 200 W electric field at 51°C for 2 h.

5.2.8. Data analysis

Analysis of AFLP output was performed using the ABI GENESCAN Analysis Software (Applied Biosystems, USA) which automatically detects the DNA fragments and assigns the molecular weight of the bands obtained based on the internal standard dye. Different DNA fragments amplified with each primer were treated as discrete characters and numbered sequentially. For diversity, analysis bands were scored as present (1) or absent (0) to form a raw data matrix. Matrix of similarity was then obtained using Jaccard similarity coefficient (Jaccard, 1908) by SPSS 10.0.1 software .The average similarity matrix was used to generate a tree for cluster analyses by UPGMA (Unweighted Pair Group Method with Arithmetic average) method using NTSys v2.1. Principal component analysis was carried out with help of SPSS software. The support values for the degree of confidence at the nodes of the dendrogram were analyzed by BOOTSTRAP analysis (Felsenstain, 1985) using two softwares WINBOOT (Yap *et al.,* 1995) and FREETREE (Pavlicek *et al.,* 1999). These softwares were originally designed for the analysis of the results of DNA fingerprinting methods that provide binary character data (presence/ absence of the characters). For such data, the program computes the distance matrix, constructs the phylogenetic tree by using the UPGMA linkage algorithm and also computes bootstrap values for internal branches of the tree.

5.3. Results

5.3.1. AFLP analysis

The AFLP analysis of genomic DNA (Figure 5.1) of 6 clinical isolates was carried out according to Vos *et al.,* (1995) with minor modifications. A total of 2338 informative AFLP markers were generated using ten selective primer combinations (Table 5.1.). The Representative DNA Sequencer gel image showing AFLP banding profile of *Leishmania* clinical isolates was shown in figure 5.2. It was observed that the number of unique bands varied greatly between the different strains. In most of the cases, the unique band was present in only one population. Out of 2338 AFLP bands, number of polymorphic ones identified were 1294 and that of monomorphic - 1044. On the basis of all the ten primer combinations used the percentage of polymorphism was calculated to be 55.35 and of unique ones were 9.28.

Table 5.1: Showing polymorphism on the basis of ten selective primer combinations.

Selective Primer Combinations	Number of All Bands	Number of Monomorphic Bands	Number of Polymorphic Bands	Number of Unique Bands	Percentage of Polymorphism
1) MseI CAA/ ECoRI ACA	253	174	79	7	31.22
MseI CAA/ ECoRI ACG	49	30	19	1	38.77
MseI CAA/ ECoRI AGC	119	42	77	19	64.70
2) MseI CAC/ EcoRI ACA	92	54	38	2	41.30
MseI CAC/ ECoRI ACG	45	24	21	2	46.66
MseI CAC/ ECoRI AGC	62	42	20	5	32.25
3) MseICAA/ EcoRI ACT	86	48	38	13	44.18
MseICAA/ EcoRI AGG	87	6	81	5	93.10
MseICAA/ EcoRI AAC	25	18	7	2	28.00
4) MseICAC/ EcoRI ACT	67	30	37	7	55.22
MseICAC/ EcoRI AAG	86	54	32	6	37.20
MseICAC/ EcoRI AAC	20	12	8	0	40.00
5) MseICAT/ EcoRI ACA	92	12	80	11	86.95
MseICAT/ EcoRI ACG	32	6	26	12	81.25
MseICAT/ EcoRI AGC	35	0	35	10	100
6) MseICTC/EcoRI ACA	46	6	40	6	86.95
MseICTC/ EcoRI ACG	47	12	35	5	74.46
MseICTC/ EcoRI AGC	54	12	42	12	77.77
7) MseICAG/ EcoRI ACA	80	30	50	7	62.50
MseICAG/ EcoRI ACG	43	12	31	2	72.09
MseICAG/ EcoRI AGC	89	30	59	8	66.29
8) MseICTA/ EcoRI ACA	174	42	132	11	75.86
MseICTA/ EcoRI ACG	135	66	69	9	51.11
MseICTA/ EcoRI AGC	118	42	76	11	64.40
9) MseICTG/ EcoRI ACA	82	42	40	9	48.78
MseICTG/ EcoRI ACG	95	60	35	9	36.84
MseICTG/ EcoRI AGC	59	36	23	9	38.98
10) MseICTT/ EcoRI ACT	63	42	21	5	33.33
MseICTT/ EcoRI AAG	43	18	25	7	58.13
MseICTT/ EcoRI AAC	60	42	18	5	30.00
Total No. of Bands	2338	1044	1294	217	

5.3.2. Dendrogram and Phylogeny

The phylogenetic tree was obtained by using two softwares WINBOOT (Figure 5.3.) and FREETREE (Fig 5.4). Bootstrap value and dendrogram pattern of both the softwares were found to be almost similar. The dendrogram clearly separated clinical isolates of *Leishmania donovani* into three main clusters. The first cluster from the top constitutes of Dd8 and 2001 sharing 50.8 % similarity. The second cluster was divided into two sub clusters, the first one comprising of isolate 2041 sharing 83.2% similarity with the second one having isolates -2087 and 2093. Interestingly, the later isolates too shared 56.5% similarity among themselves. The first and second main clusters have shown 55.7% similarity. The third cluster 2039 isolate was observed to be completely out grouped form others, showing high rate of polymorphism.

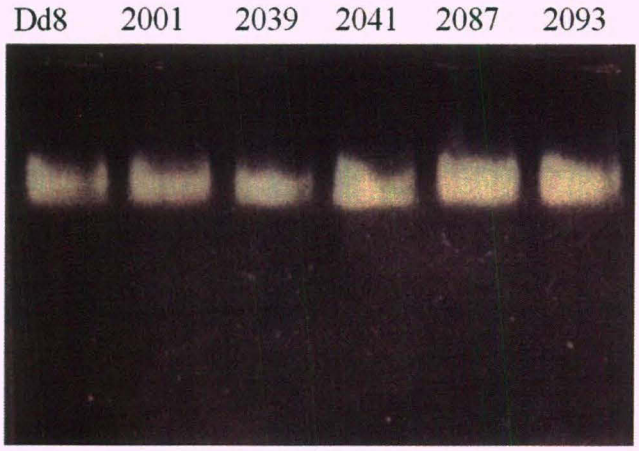


Figure 5.1: Genomic DNA of 6 clinical isolates (Dd8, 2001, 2039, 2041, 2087, 2093) of *L. donovani.*

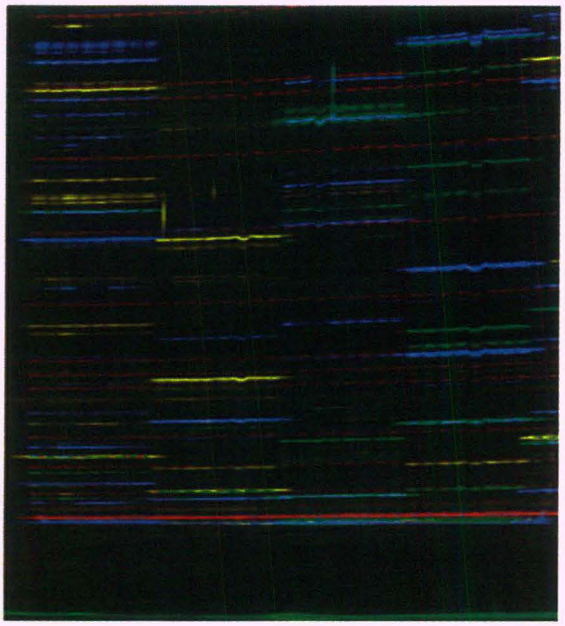


Figure 5.2: DNA sequencer gel image of an AFLP banding profile of *L. donovani* using different fluorescent primer combinations shown in blue, green and yellow coloured bands. Red coloured band is standard marker.

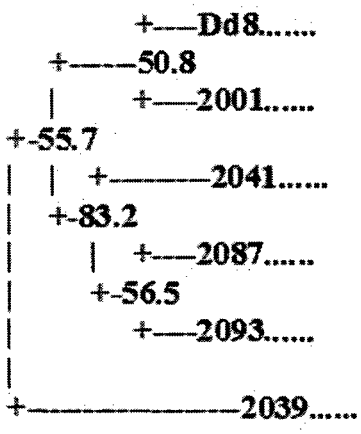


Figure 5.3: UPGMA cluster analysis of AFLP data generated by ten selective primer combinations for 6 clinical isolates of *L. donovani* depicting patterns of genetic diversity through WINBOOT.

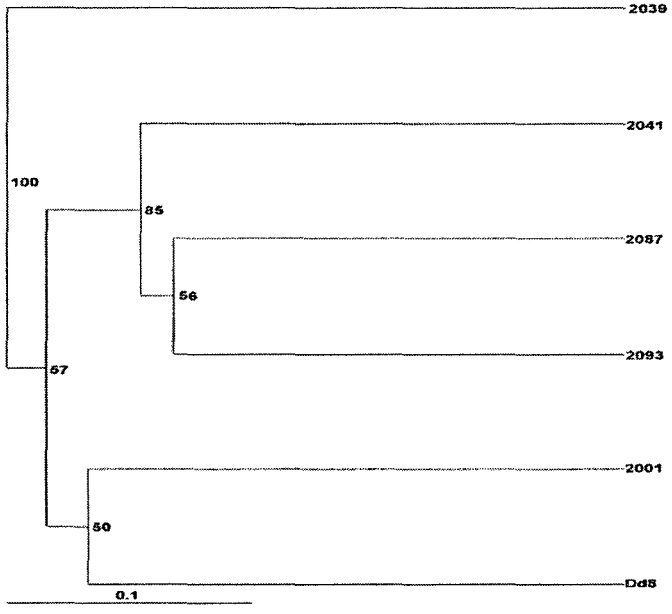


Figure 5.4: Dendrogram of *L. donovani* strains showing phylogenetic relationship among them using FREETREE software.

5.3.3. Principal component plot

The principal component plots generated from AFLP data have been shown in Figure 5.5. On the basis of principal component analysis of the AFLP data, the six strains of *L. donovani* were placed in 3 quadrants. In the first quadrant, there was occurrence of 3 species - 2041, 2087, and 2093. In the second one, both drug sensitive isolates - Dd8 and 2001 were placed but interestingly again, SAG resistant strain 2039 was placed as totally distinct from all other strains in the third quadrant.

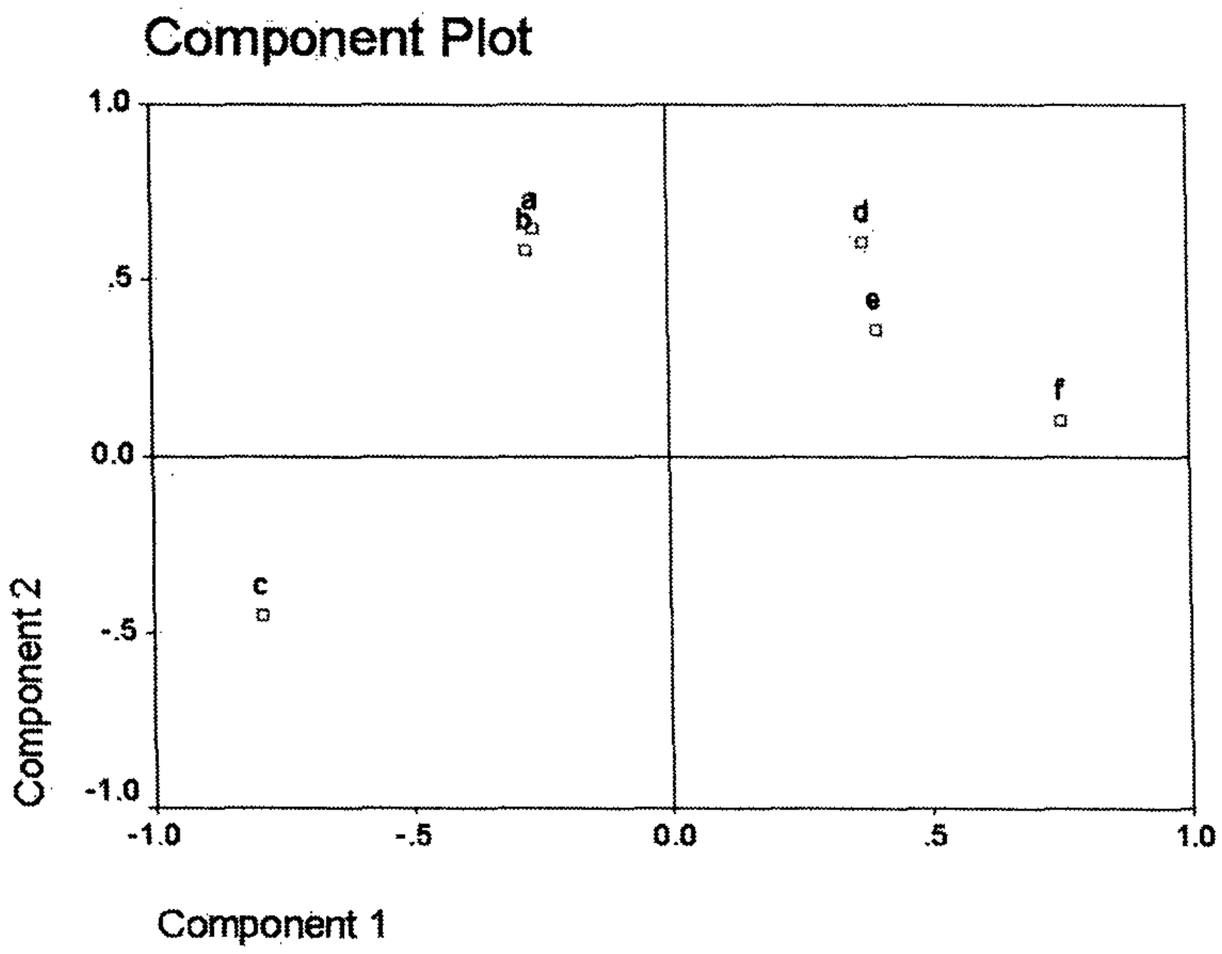


Figure 5.5: Principal component plot based on the AFLP data of six clinical isolates of *L. donovani* (a-Dd8, b-2001, c-2039, d-2041, e-2087 and f- 2093).

5.3.4. Polymorphism analysis

The primer combination MseI- CAT/ EcoRI-AGC revealed the highest percentage of polymorphic fragments (100%), while the lowest percentage (30%) was generated by the primer combination of MseI-CTT/ EcoRI-AAC (Table 5.1.). Some primers *viz*., MseI-CAA/ EcoRI-AGG, MseI-CAT/ EcoRI-ACA, MseI-CAT/ EcoRI-ACG, MseI-CAT/ EcoRI-AGC, MseI-CTC/ EcoRI-ACA, MseI-CTC/ EcoRI-ACG, MseI- CTC/ EcoRI-AGC, MseI-CAG/ EcoRI-ACG, and MseI-CTA/ EcoRI-ACA showed > 70% polymorphism (Figure 5.6.).

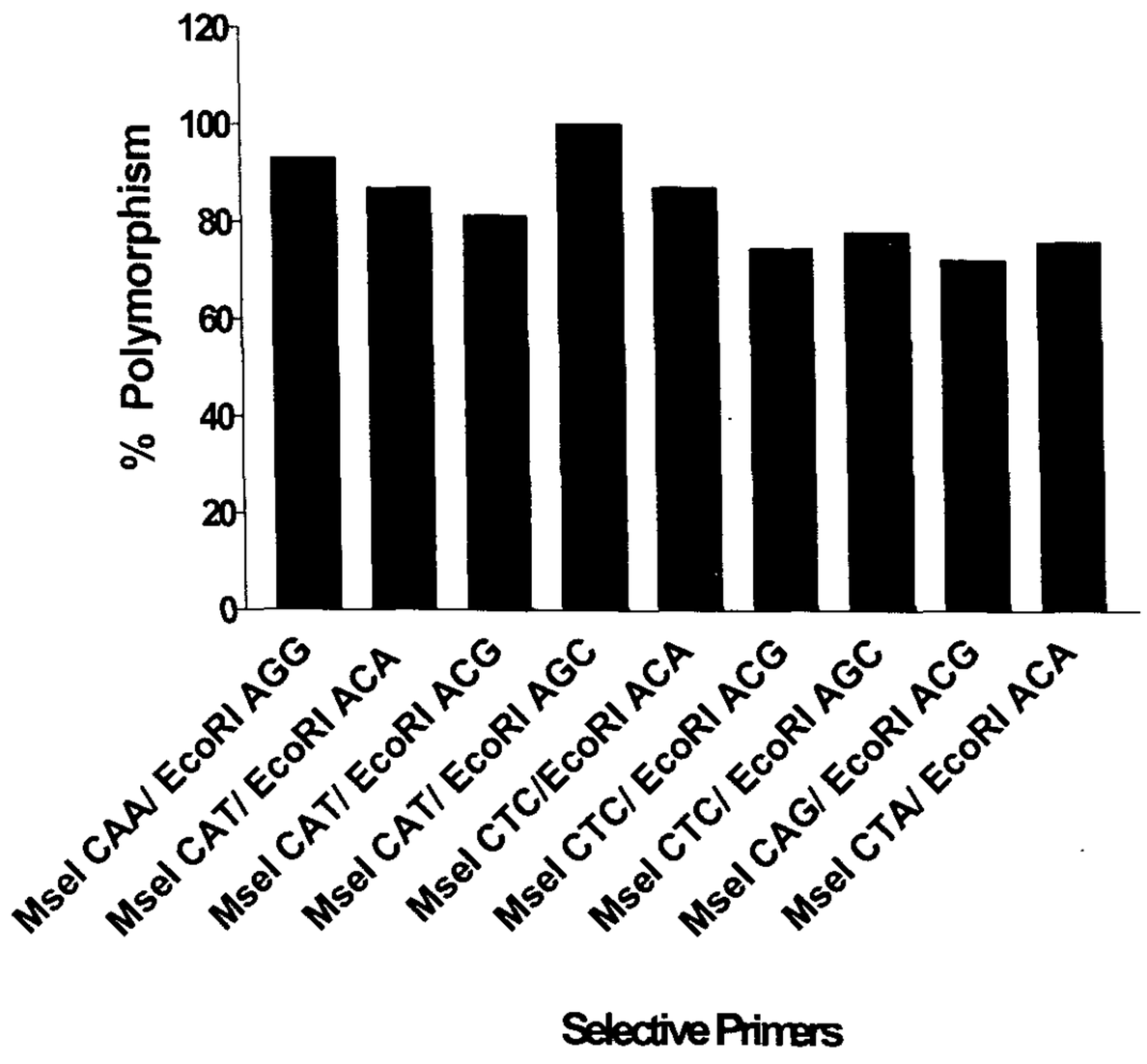


Figure 5.6: Selective primers showing more than 70% polymorphism.

5.3.5. SAG sensitive and resistant strain specific AFLP markers

A number of unique molecular marker bands specific for each strain of *Leishmania* were detected through AFLP analysis using ten primer combinations which has been listed in Table 5.2. Maximum number of unique bands (12) was observed in drug sensitive isolate - 2087 using primer combination of MseI CAA/ ECoRI AGC whereas among the drug resistant isolates maximum 5 unique bands was seen in 2039 using 3 different primer combinations- MseI-CTA/ EcoRI-ACA, MseI-CTA/ EcoRI-ACG and MseI-CTT/ EcoRI-AAC.

Table 5.2: Unique molecular marker bands (size in bp) specific for each SAG sensitive and resistance strain of *L. donovani* detected through AFLP.

Primer Combinations	Drug sensitive isolates			Drug resistant isolates		
	Dd8	2001	2087	2041	2039	2093
1) MseI CAA/ ECoRI ACA			101,247, 370		260, 307,335	382
1) MseI CAA/ ECoRI ACG			261			
1) MseI CAA/ ECoRI AGC	124, 153, 184, 284		105, 109, 110, 112, 113, 119, 128, 129, 130, 131, 183, 223	345	142, 392	
2) MseI CAC/ EcoRI ACA	379		114			
2) MseI CAC/ ECoRI ACG					212	321
2) MseI CAC/ ECoRI AGC	347	102		276, 277		245
3) MseICAA/ EcoRI ACT	134, 248, 274, 319	222, 280			121, 218, 275, 317	135,242, 250
3) MseICAA/ EcoRI AGG	319	227, 228, 259	119			
3) MseICAA/ EcoRI AAC			203			223
4) MseICAC/ EcoRI ACT	183, 251, 347		221		267	157, 215
4) MseICAC/ EcoRI AAG	260	172, 284	288		127,141	
4) MseICAC/ EcoRI AAC						
5) MseICAT/ EcoRI ACA	115, 251	333	101, 262, 304, 348	256	200	334, 339
5) MseICAT/ EcoRI ACG	253, 260, 318, 319, 321	112	235, 263	259	202, 213	233
5) MseICAT/ EcoRI AGC	321, 326, 334		215, 219, 307		203, 247, 336	344
6) MseICTC/EcoRI ACA	165, 337	123		245, 255		211
6) MseICTC/ EcoRI ACG	185	187,311, 322				232
6) MseICTC/ EcoRI AGC	190	311,313,31, 317, 318,321	173, 216	179, 274	213	
7) MseICAG/ EcoRI ACA	171, 223, 242		137, 343	107, 161		
7) MseICAG/ EcoRI ACG	172					243
7) MseICAG/ EcoRI AGC		107,164, 267		106,142,270	249	
8) MseICTA/ EcoRI ACA	175, 277		379	123	116,310, 312 363, 364	238, 297
8) MseICTA/ EcoRI ACG	112, 162		210		113.124,307, 310, 314	
8) MseICTA/ EcoRI AGC	378	217, 344, 367	102		262, 274, 315, 326	119
9) MseICTG/ EcoRI ACA	119, 179, 216, 217	210	101	162	205	207
9) MseICTG/ EcoRI ACG	166, 180, 197, 379				136, 208, 228, 374	195
9) MseICTG/ EcoRI AGC	112, 199, 308, 369	217		179	163, 277, 283	
10) MseICTT/ EcoRI ACT	208				348, 349, 350, 351	
10) MseICTT/ EcoRI AAG	189, 205, 209, 375				140, 348, 350	
10) MseICTT/ EcoRI AAC					202,348,349, 350,353	

5.3.6. Group specific AFLP markers of SAG sensitive and resistant strain

The primer combinations, as evident in Table 5.3 showed a number of group specific (SAG sensitive /resistant) AFLP markers which could be particularly helpful in detection of SAG resistant cases. The following selective primer combinations i.e. MseI-CAA/ EcoRI-ACA, MseI- CAC/ EcoRI-ACG, MseI-CAA/ EcoRI-ACT, MseI-CAC/ EcoRI-ACT, MseI-CAC/ EcoRI-AAG, MseI-CAT/ EcoRI-AGC, MseI-CAG/ EcoRI-AGC were found to be important for detection in respect of SAG resistant isolates and MseI-CAA/ EcoRI-AGG, MseI-CTC/ EcoRI-ACA, MseI-CTA/ EcoRI-AGC, MseI-CTG/ EcoRI-AGC for SAG sensitive ones.

Table 5.3: Group specific AFLP markers (size in bp) of SAG sensitive and resistance strains of *L. donovani.*

Primer Combinations	**SAG Sensitive strains specific bands (bp)**	**SAG Resistant strains specific bands (bp)**
MseI CAA/ ECoRI ACA		110, 240, 262, 390
MseI CAA/ ECoRI AGC	143, 215	273, 346, 352
MseI CAC/ ECoRI ACG		320
MseI CAA/ EcoRI ACT		269, 276, 346
MseI CAA/ EcoRI AGG	160, 240	
MseI CAC/ EcoRI ACT		277
MseI CAC/ EcoRI AAG		174, 264, 299
MseI CAT/ EcoRI ACA	202	332, 337, 340
MseI CAT/ EcoRI AGC		179, 218
MseI CTC/EcoRI ACA	117	
MseI CAG/ EcoRI AGC		274
MseI CTA/ EcoRI ACA	250	281, 314, 366
MseI CTA/ EcoRI ACG	135, 316	198
MseI CTA/ EcoRI AGC	113	
MseI CTG/ EcoRI ACA	161	202
MseI CTG/ EcoRI ACG	165	212
MseI CTG/ EcoRI AGC	378	
MseI CTT/ EcoRI ACT	174	206

5.4. Discussion

The main purpose of this study was to evaluate the polymorphism and phylogenetic relationships in SAG sensitive and resistant strains of *L .donovani* isolated from VL patients through AFLP technology, a high resolution method for DNA fingerprinting and co relating them with drug resistance. Documentation of genetic relatedness of SAG sensitive and resistant strains of *L. donovani* has been a significant impediment to advances in studying these pathogens. To the best of our knowledge, this is the first time that AFLP has been used to characterize genomic variability of clinical isolates of VL. AFLP accessed multiple independent sites within the genome and allowed a better definition of the phylogenetic relatedness of different *leishmania* isolates.

In the earlier study carried out in this lab, using six isolates, it was demonstrated that the Proteophosphoglycan (PPG) is differentially expressed in SAG sensitive and resistant isolates using FACS and Western blotting and was higher in SAG-resistant isolates (Samant *et al.*, 2007). In the present study AFLP analysis was carried out on similar six isolates to investigate whether the genetic variation among the clinical isolates is responsible for drug resistance. We may conclude that the differential expression in PPG in between SAG resistant and sensitive isolates may be due to the genetic variation. Estimates of variation obtained from large numbers of loci might be very accurate since AFLP was able to screen large numbers of unlinked or loosely linked loci (Rubio *et al.*, 2001). In addition, along with sample size, number of primer combinations taken for AFLP study is also an important issue to produce an accurate estimate of genetic relatedness (Ellis *et al.*, 1997). It is believed that by choosing the six combinations of primers, it is possible to explain more than 80 % of the expected relatedness (Chen *et al.*, 2004). In this study ten combinations of primers were taken that yielded 20 to 40 DNA fragments after the PCR, within the size range of 100 to 400 bp for more accuracy, reliability, and distinguish genetic relations in this result (Vos *et al.*, 1995). These DNA fragments were used to screen all the clinical isolates. The results obtained from a large number of primer combinations were utilized to check the robustness of the dendrogram, that clearly establish that the polymorphism revealed by AFLP is not only abundant but also statistically reliable (Chen *et al.*, 2004).

Moreover, these results demonstrate that genetic resolution provided by AFLP is amenable to phylogenetic analysis of closely related *Leishmania* species. The high sensitivity of the AFLP technique, which provides a novel and very powerful DNA

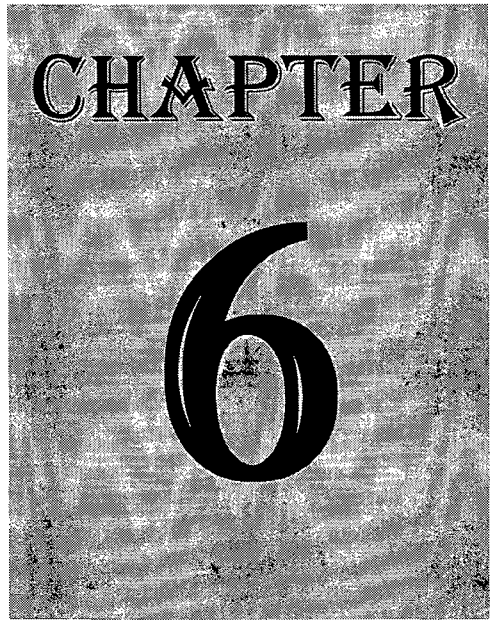
fingerprinting technique for DNAs of any origin or complexity (Vos *et al.*, 1995), alongwith its rich banding pattern (20 to 40 bands for each pair of selective primers) provided more information about polymorphisms among *Leishmania* clinical isolates, allowing them to be clustered based on percent similarity. These results demonstrated the usefulness of the AFLP technique as a reliable taxonomic tool for the genomic differentiation of *Leishmania* strains. This has also proved to be a powerful and reliable technique for the characterization of infra-specific polymorphisms among populations of clinical and environmental isolates of *Leishmania* and its reproducibility is reported to be very high (Vos *et al.*, 1995). A noteworthy observation, as evident from dendrogram, was that AFLP analysis, while displaying a higher rate of polymorphisms among *Leishmania* clinical isolates, further indicated towards the shifting of drug sensitive parasite to resistant condition.

AFLP techniques were able to extract considerable information about genome-wide nucleotide variability. Analyzed judiciously, such data can facilitate the rapid and minimally obtrusive assessment of genetic variability (Vos *et al.*, 1995). Emergence of resistance against SAG could result from allelic differences in single or multiple genes. The difficulty involved in the understanding related to SAG resistance of *Leishmania*, highlighted the need for a DNA marker system for easy detection and diagnosis against leishmaniasis. The estimates of genetic diversity for the AFLP data were very consistent between the different isolates and also across primer combinations within each isolate have also demonstrated the effectiveness of the present AFLP method for establishing genetic variation and indicated its applicability to other parasites of human health importance (de Gruijter *et al.*, 2005).

The ability to accurately detect polymorphisms and strain specific and group specific markers between SAG sensitive and resistance isolates is of major importance for the design of effective disease control strategies against this parasite. This study has established the AFLP conditions and primer combinations that permit the assessment of genetic diversity between *Leishmania* clinical isolates. It further demonstrated that the technique could be applied in diversity studies, with potential use for the identification of intra- or inter-specific genetic markers. AFLP fingerprinting may facilitate the identification of polymorphisms linked to parasite virulence factors and, thus, contribute to the understanding of host-parasite interactions at the molecular level.

This study further supports the hypothesis that SAG resistance strains evolve from the sensitive ones. From dendrogram it is clear that 2041 and 2093 strain (resistant) share homology with 2087 strain (sensitive) but their response against SAG is different. It indicates that due to point mutations, insertions, deletions, and/or other genetic rearrangements (Vos *et al.*, 1995; Tandon *et al.*, 2005) there is change in the genetic make-up of *Leishmania* strain which facilitates the conversion of parasite from sensitive to resistance condition. Indeed, this study too provides evidence suggesting that the *Leishmania* populations originated from a common ancestral species. This phylogenetic relationship among *Leishmania* species shows that all the lineages in this species converge to a single point before separating from other related species included as out grouped. These results further ascertain that 2039 strain of *Leishmania* is totally out-group indicating this strain is highly polymorphic and develop resistance against SAG. The variability observed among SAG sensitive and resistant populations based on AFLP analysis suggests that the SAG resistant strain may be a composite group. It can be derived that due to this genomic differentiation they acquire drug resistance. Further, it was evident from the component plots that the SAG resistant accessions 2039 are remarkably distinct from other SAG resistant accessions as it falls at the extremes of the plot. It again indicates about the highly polymorphic nature of 2039. Although 2041, 2093 are SAG resistant but degree of resistivity of 2039 is much more in comparison to other SAG resistant strain which is evident by its placement as out group. This finding correlated well to an earlier report in which, while using the same clinical isolates, it has been shown that expression of proteophosphoglycan (PPG) is highly upregulated in 2039 strain (Samant *et al.*, 2007). This has further validated the earlier results that the analyses of these two types of isolates (SAG resistant and sensitive) are directly comparable.

In conclusion, the results of this study emphasize that the AFLP technique described in this chapter was effective for showing inter-specific and intra-specific molecular variability between clinical isolates and demands more studies at proteomic level in order to investigate the degree of changes in protein profile of SAG sensitive and resistant strains. For this a comprehensive and differential proteomics studies need to be done by generating a proteome map of the both the strains of *Leishmania* species and then eventually compare the differential proteins identified using 2-D gel electrophoresis and mass spectrometry (MALDI-TOF/TOF) based approaches.



Comprehensive proteomic analysis of Leishmania donovani

"I have been impressed with the urgency of doing.
Knowing is not enough; we must apply.
Being willing is not enough; we must do".

- Leonardo da Vinci –

6. Proteomic analysis of the membrane as well as soluble proteins of *Leishmania donovani* promastigotes by Two-Dimensional Gel Electrophoresis (2-DGE) and Matrix Assisted Laser Desorption Ionization-Time of flight (MALDI-TOF/TOF) mass spectrometry

6.1. Introduction

Visceral leishmaniasis (VL) is the most overwhelming infectious disease caused by *L. donovani*, *L. infantum*, and *L. chagasi* and has significant antigenic diversity due to the digenetic life cycle of parasite. Since, due to the unsatisfactory treatment measures and increasing drug resistance there has been an urgent need to develop novel drug/vaccine targets against VL for which the primary goal should be to identify and characterize the structural and functional proteins. Modern genomics-based approaches such as proteomics offer increased hope for the discovery of promising new drug targets by virtue of their ability to characterize complex parasite biology and biochemistry. Therefore, the correspondence study between the genome and proteome are more effective for target identification and validation in parasite. Proteomics, being widely employed in the study of *Leishmania* seems to be a suitable strategy as the availability of annotated sequenced genome of *Leishmania major* has opened the door for dissection of both protein expression/regulation and function. Advances in clinical proteomic technologies have enable to enhance an understanding of virulence/pathogenicity/host–pathogen interactions, drug resistance thereby defining novel therapeutic/vaccine targets. Using proteomics approach, preliminary efforts made in this direction included the generation of a 2-D gel map of *L. donovani* promastigotes through MALDI-TOF/TOF. For better understanding of the mechanisms of drug resistance in *Leishmania* parasite, proteomic approaches shall be beneficial to identify proteins that are expressed in a stage-specific manner and should accelerate our understanding of key processes related to *Leishmania* biology (Kumari *et al.*, 2008). Keeping these things in mind proteomic analysis of membrane as well as soluble proteins has been done in fresh clinical isolates of *L. donovani* promastigotes. Till date, the proteome mapping of *L. donovani* was carried out in the whole extracts of *Leishmania* parasites (Bente *et al.*, 2003; Forgber *et al.*, 2006; Vergnes *et al.*, 2007). This study has been conducted for the first time in two separate protein fractions i.e. membrane and soluble that would provide a clearcut and sharp identification and characterization of protein profiles which will be able to highlight the relevant developments of targets discovery in the rapidly

emerging field of *Leishmania* proteomics. This chapter deals with the proteome mapping of Indian *L. donovani* clinical isolate.

6.2. Materials and Methods

6.2.1. Animal maintenance

Laboratory bred male golden hamsters (*Mesocricetus auratus*, 45-50 gm) were employed for experimental work and maintained as per description given in chapter 4.

6.2.2. Leishmania parasites isolation

The method of isolation and cultivation of a recent clinical isolate (2001) of *L. donovani* has been described in chapter 4.

6.2.3. Preparation of culture media for Leishmania culture

For cultivation of *Leishmania* parasites, various culture media i.e. cRPMI-1640 and cM199 were prepared as described in chapter 4.

6.2.4. Leishmania parasites culture

L. donovani clinical isolate (2001) was cultured as described previously in chapter 4.

6.2.5. Chemicals

Acrylamide, agarose, bis- acrylamide, biolytes, coomassie brilliant blue (CBB), dithiothreitol (DTT), iodoacetamide, IPG strips, mineral oil and other 2D standards were purchased from Bio-Rad, USA; trypsin, glycerol, thiourea, protease inhibitir cocktail (PIC), CHAPS, EDTA, TEMED, ammonium persulfate (APS), sodium chloride, mercaptoethanol, glucose, sorbitol, urea, Tris, SDS, TFA, Tri butyl phosphine (TBP), acetonitrile (AcCN) from Sigma Chemical Co., USA; α-cyano-4-hydroxycinnamic acid (CHCA), peptide calibration standard mixture (Cal mix.), Tri fluoro acetic acid (TFA) from Applied biosystem, USA, Triton X-100.

6.2.6. Isolation of membrane enriched proteins (MEPs) of L. donovani

Membrane enriched proteins fraction was isolated from promastigotes of *L. donovani* using the method described by Molloy *et al.*, (1998) and Pávková *et al.* (2005) with slight modifications. Briefly, metacyclic promastigotes (10^9) of *Leishmania* parasite culture were disintegrated using repeated cycles (10) of freeze-thawing in liquid nitrogen and the cell pellet was then lysed by ultrasonication (Soniprep 150, USA) at 10-15 micron amplitude for 10 cycles of 30 seconds in TNE buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 5mM EDTA) with Protease inhibitor cocktail (Sigma, USA). The cell lysate was then

incubated with TX100 (1%) for 30 min on ice. Finally, MEPs were collected by ultracentrifugation in a Beckman ultracentrifuge (CA, USA) at 120000 x g for 1 h at 4°C.

6.2.7. Isolation of soluble proteins (SPs) of L. donovani

SP was prepared as per method described by (Scott *et al.*, 1987). Briefly, metacyclic promastigotes (10^9) were harvested from culture, washed four times in cold PBS, resuspended in PBS containing protease inhibitors cocktail (Sigma, USA) and subjected to ultrasonication (Soniprep 150, USA) just like sample prepared for membrane enriched proteins and centrifugation at 40000xg for 30 min.

6.2.8. SDS-PAGE of membrane and soluble proteins

The MEP, SP and whole cell lysate (WCL) protein samples were estimated (Lowry, 1951) and prepared by heating them into equal volume of 2x SDS sample-loading buffer to denature the protein at 100°C for 5-10 min. MEP, SP, WCL of *L. donovani* were separated on SDS-PAGE containing 12% separating gels (Laemmli, 1970) at 12-24 mA current. The gel was removed from the apparatus into tray. It was now be fixed and stained with silver staining. For silver staining the gel was transferred to solution A (50% Acetone, 50% TCA, 37% HCHO) for 15min in order to fix the gel and then it was rinsed with TDW thrice for 5-10min. Gel was then dipped in 50ml of 50% acetone for 5-10min. Again the gel was rinsed with TDW thrice. The gel was soaked in 60ml of TDW containing 10µl of 30% $Na_2S_2O_3.5H_2O$ for 1 min. Gel was later on rinsed thrice with TDW and each washing must be of 5sec. The gel was stained in dark in Solution B (30% $AgNO_3$, 37% HCHO) for 15 min with constant gentle agitation on rocker. Gel was washed thrice with TDW for 5 min. Solution C (Na_2CO_3, 37% HCHO, $Na_2S_2O_3.5H_2O$) was added and gel was agitated gently till band appears. The reaction was stopped with 1% acetic acid after band appearance.

6.2.9. Protein sample preparation for two dimensional gel electrophoresis (2-DGE)

Membrane and soluble proteins of *L. donovani* was precipitated in trichloro-acetic acid (TCA) to a final concentration of 10% w/v and washed three times with acetone to eliminate contaminants like nucleic acid and salts (by employing PBS or Tris-HCl buffer). The dried sediments were reconstituted in water and stored lyophilized in aliquots after protein estimation. All protein estimations were done by Lowry method (1951). The dry pellet of MEP was solubilized in rehydration buffer [7 M urea, 2 M thiourea, 2mM Tributylphosphine (TBP), 40 mM Tris, 4% w/v CHAPS, 1% v/v biolyte (3–10), and 1% v/v

Triton X-100] and dry pellet of SP was solubilized in rehydration buffer [7 M urea, 4% w/v CHAPS, 100 mM DTT, 0.5% v/v biolyte (3–10), 0.5% v/v Triton X-100 and 40 mM Tris]. Dissolved MEPs and SPs fraction were vortexed and centrifuged at 10,000 g for 10 min to remove insoluble material.

6.2.10. Two dimensional gel electrophoresis

6.2.10.1. First dimension (IEF): 2-DGE was performed as described previously with minor modifications according to the manufacturer's manual (Bio-Rad, USA). Briefly the dry pellet was solubilized in rehydration buffer and immobilized on dry strips, *pI* 3–10, 17 cm (BioRad, USA) and proteins were loaded with 1.5 mg of MEPs & SPs separately and allowed to rehydrate for 18–22 h. Iso-electric focusing (IEF) was performed at 20°C using the Protean IEF cell (BioRad) according to the manufacturer's instructions after rehydration step. The IEF run was carried out using the following conditions: (i) conditioning step for 20 min at 250 V, (ii) liner voltage ramping for 2 hr and 30 min at 10,000 V, (iii) for the final focusing step 10,000 V constant until 40 kVh, and the current did not exceed 50μA/strip.

6.2.10.2. Second dimension: After IEF, the strip was equilibrated in solution A (0.375 M Tris, pH 8.8 containing 6 M urea, 2% SDS, 20% glycerol, 2% w/v DTT) and B (solution A without DTT, but with 2.5% w/v iodoacetamide) for 20 min at room temperature and inserted on to a 12% 2-D SDS-PAGE gel (20 x 22 cm) with wide range prestained protein molecular weight marker (Fermentas, USA) was used to identify and assess the molecular weights of protein bands observed in the gel. The gel was sealed with 1% agarose and SDS-PAGE was performed according to the Laemmli method (1970). Electrophoresis was performed at 16 mA/gel for the initial 30 minutes and then at 24 mA/gel at 14°C until the running dye reached the bottom. Standard SDS-PAGE markers were used to estimate the relative molecular weight of the protein spots in the gel. The gel was removed from the apparatus into the tray containing water for rinsing and then the gel was stained with CBB.

6.2.11. Gel staining and destaining

The gel was stained either upto 4 hrs or overnight with (0.25 g /100 ml, mentioned in appendix) colloidal coomassie brilliant blue [G-250, CBB (Bio- Rad, USA)]. Destaining was performed in destaining solution [1:1 (v/v) Methanol: TDW and 10 ml of acetic acid)].

6.2.12. Imaging and Image analysis

The stained gels were scanned and images were acquired by gel imaging and spot picking system (Investigator™ ProPic, Genomic solution, USA) and saved as tiff images.

6.2.13. Gel spot excision

The protein spots of interest were manually excised from the gels. The spots were transferred to microcentrifuge tubes, labelled properly and stored at -80°C.

6.2.14. In-gel digestion of proteins

The in-gel digestion of proteins and purification of peptides from gel was carried out according to the manufacturer's manual (Applied biosystem). Briefly, 2-DGE protein spots were excised and transferred into microcentrifuge tubes. Gel pieces were rinsed three times with RO water to remove SDS and buffer salts. Followed by 50% v/v acetonitrile (AcCN) in 50mM ammonium bicarbonate (ABC), pH 8.0 was added and the gel pieces were crushed using pestle. After centrifugation, supernatant was discarded, and above step was repeated 2 times. The gel pieces were shrunk by dehydration by adding 100µl AcCN and vacuum dried. Gel pieces containing proteins were rehydrated by adding 10-20 µg/ml digestion buffer containing sequencing grade modified 10 µg/ml trypsin (Sigma, USA). After 15 min, 25 µL of 50 mM ammonium bicarbonate (ABC) was added to keep the gel pieces wet during tryptic cleavage (37°C, overnight). To extract the tryptic peptides, 50% AcCN/0.3%TFA solution were added, and the samples were incubated for 15 min and vortexed. The separated liquid was dried under vacuum and the peptides were again dissolved in 10 µl 0.1% TFA. The peptides were purified with C-18 reversed-phase mini column filled in a micropipette tip, Zip-Tip C18 (Millipore, Bedford, MA, USA).

6.2.15. Mass spectrometry

The resulting peptides were mixed with double volume of matrix, α-cyano-4-hydroxycinnamic acid (Applied biosystem, Farmingham, USA) of 10 mg/ml in 50% AcCN, 0.1% TFA and spotted onto a MALDI sample plate and left for drying. MS and MS/MS spectrum were acquired in the positive ion mode on MALDI-TOF/TOF Mass Spectrometer, Applied Biosystems 4700 Proteomics Analyzer (Framingham, MA, USA). The instrument was operated in the delayed extraction mode with delay time of 200 ns. Spectra were obtained by accumulation of 1000 and 4000 consecutive laser shots respectively in MS and

MS/MS mode and laser intensity used were in the range of 5000 to 6000. Close external calibration for MS was performed with 4700 Cal Mix (Applied Biosystems, USA) a standard mixture of six peptides des-Arg1-Bradykinin (904.4681), Angiotensin I (1296.6853), Glu1-Fibrinopeptide B (1570.6774), ACTH [clip 1-17] (2093.0867), ACTH [clip 18-39] (2465.1989) and ACTH [clip 7-38] (3657.9294). Mass calibration for MS/MS spectra was performed by fragment masses of precursor Glu1-Fibrinopeptide B (1570.6774). Peak harvesting was carried out using 4000 Series Explorer ™ Software (Applied Biosystems, USA).

6.2.16. Database search

Database searching for protein identifications was performed with mass spectrometry data (MS or MS/MS) using Global Proteome Server v3.5 software (Applied Biosystems, USA) equipped with MASCOT (Matrix Science) search engine. Only monoisotopic masses were used for searching against the SWISS-PROT and NCBInr databases with a minimum number of matched masses set at 4. The maximum peptide precursor tolerance was set at 40 ppm and MS/MS fragment tolerance was defined as 0.2 kDa. At the most one missed cleavage for tryptic peptides was allowed, and the modifications accepted were carbamidomethyl cysteines as fixed modification and methionine oxidation as variable modification. Tandem MS was performed only in the cases where identification appeared ambiguous with MALDI-TOF-MS data. The criteria used to accept the identifications for Peptide mass fingerprint included the probabilistic protein score-based confidence interval %, the extent of sequence coverage, the number of peptides matched and whether *Leishmania spp.* or *Trypanosoma spp.* protein appeared as top candidates during the first search, when no restriction was applied to the species of origin. Identification criteria with MS/MS data were that peptides count should not be less than two and confidence interval % for the best ion score should be above 95 (significance level $P< 0.05$). Protein scores greater than 66 are significant ($p< 0.05$).

6.3. Results

6.3.1. Resolution of L. donovani proteins by SDS-PAGE

To evaluate the protein pattern of MEP, SP and WCL of *L. donovani*, SDS-PAGE was performed. The SDS-PAGE resolution revealed about the molecular weight of separated proteins in form of various bands (Figure 6.1). Protein bands which showed

almost same positions on gel *i.e.* were of almost same molecular weight. Protein pattern of above mentioned proteins (MEP, SP and WCL) were quite different that further indicated to study this differentiation through 2-D gel electrophoresis.

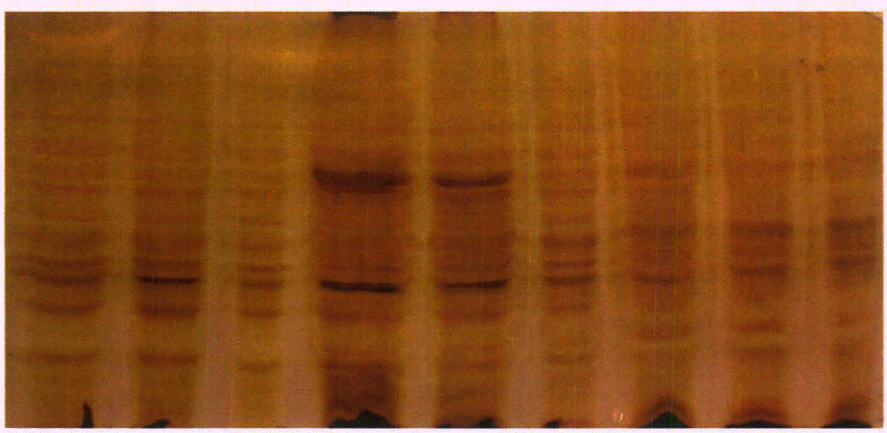


Figure 6.1: SDS-PAGE gel image of *L. donovani* (strain 2001); Soluble proteins (Lane 1, 2, 3), WCL (Lane 4, 5, 6) and membrane enriched proteins (Lane 7, 8, 9).

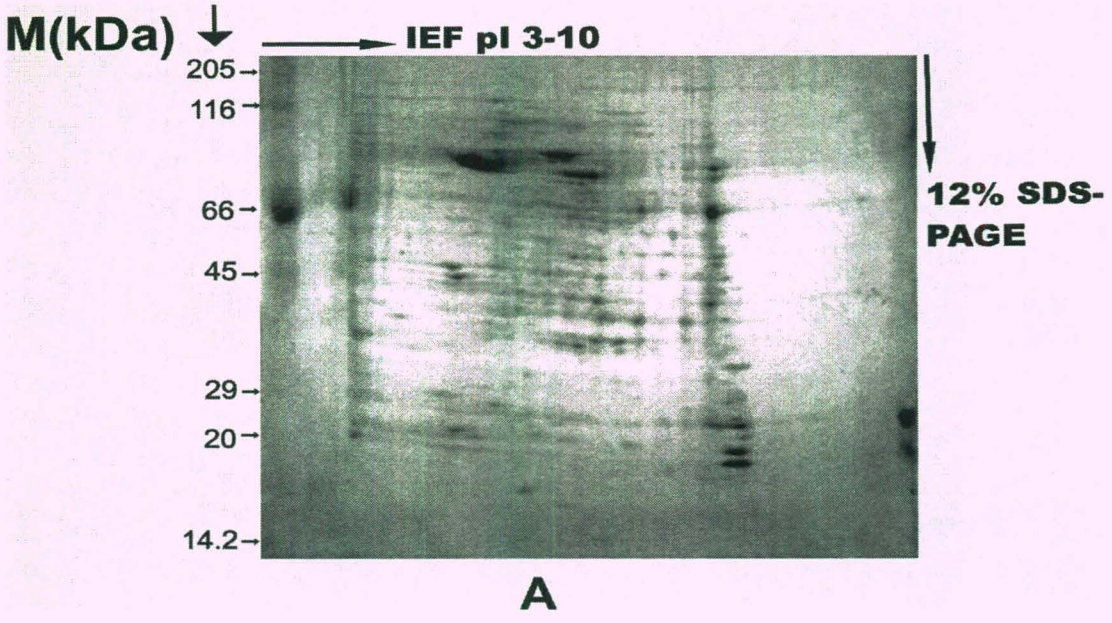
6.3.2. Resolution of 2-D separations of L. donovani proteins

MEPs and SPs of *L. donovani* were separated by high-resolution 2-DGE as shown in figure 6.2 and figure 6.3 respectively. The gels prepared with preparative protein loadings (1.5 mg) displayed good resolution with only minimal streaking. Coomassie blue staining showed several abundant protein groups and many other minor components suitable for MS analysis were also detected. Though silver staining might have detected more spots, it could have posed problems during the process of protein identification by MALDI-MS. The reproducibility of the 2-D patterns was confirmed and considered final when three consecutive runs produced identical patterns.

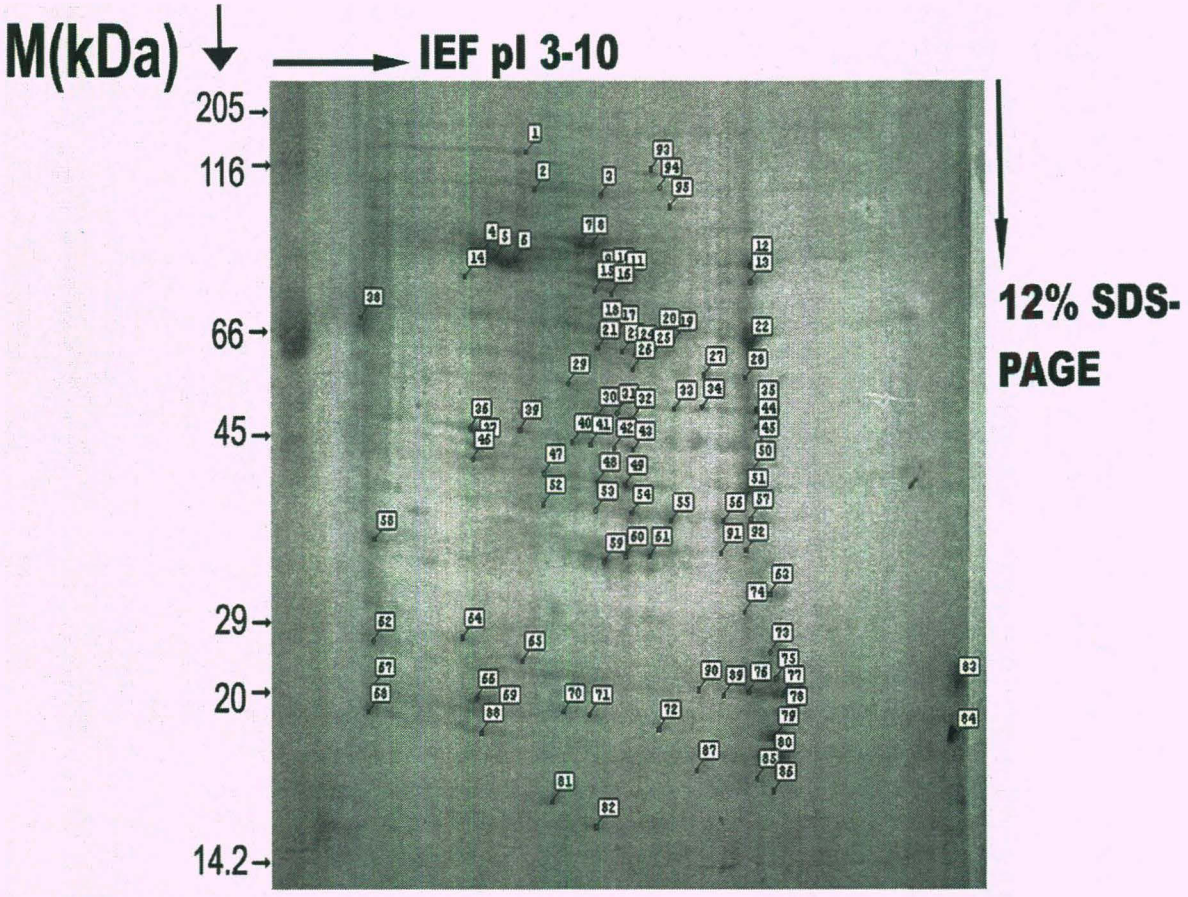
6.3.3. 2-D proteome map of L. donovani

Image analysis of 2-D gel revealed a highly reproducible proteome map generation for both MEP and SPs of L. donovani. As evident from gel picture, considerable differences were observed in proteome profiles of MEP and SP. Hence, mass spectrometric analysis was carried out to determine the protein identities. In total, 95 well resolved protein spots were detected in MEP fraction (Figure 6.2) of varying intensity in coomassie blue stained gel, within the pI range of 3 to 10. Out of these spots majority of them were detected around 4 to 8 pI i.e. acidic to slight basic pH range and only two were observed at higher basic range of pI 10 (spots Nos 83 and 84). On the other hand 184 spots were observed in SPs (Figure 6.3) all of which were within the pI range of 4 to 8 pI i.e. acidic to slight basic pI range. PMF spectrum of the some *L. donovani* identified proteins after MALDI-TOF/MS were shown in the figure 6.4.

Figure 6.2: **(A)** Representative Coomassie stained 2-D gel image of the membrane enriched proteins of *L. donovani*. The MEPs were loaded onto IPG strip pI 3–10 followed by SDS-PAGE (12%). **(B)** The numbered spots indicate the identified/ unidentified membrane proteins listed in Table 6.1.

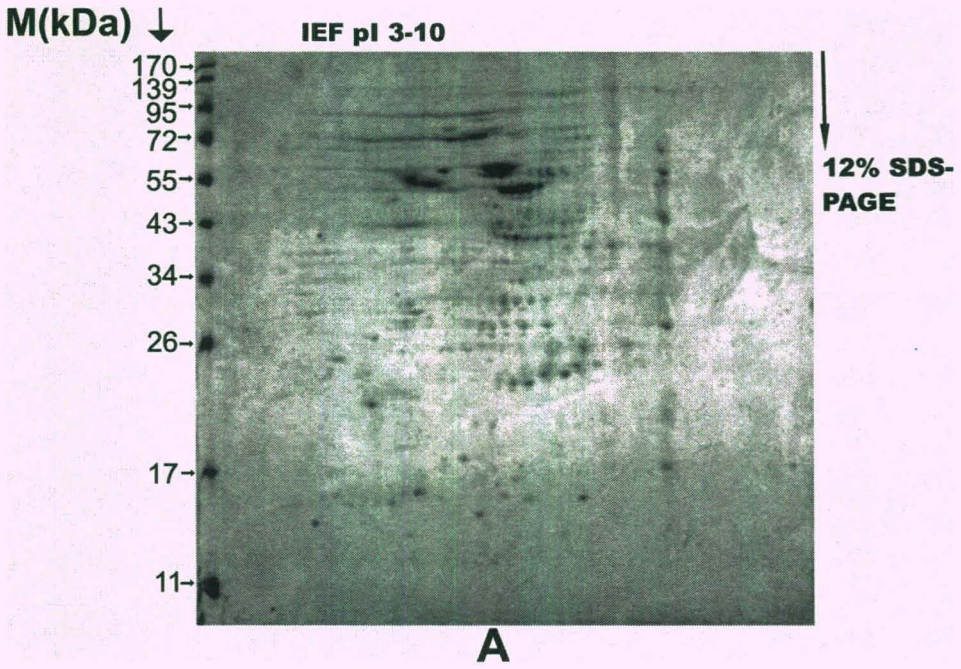


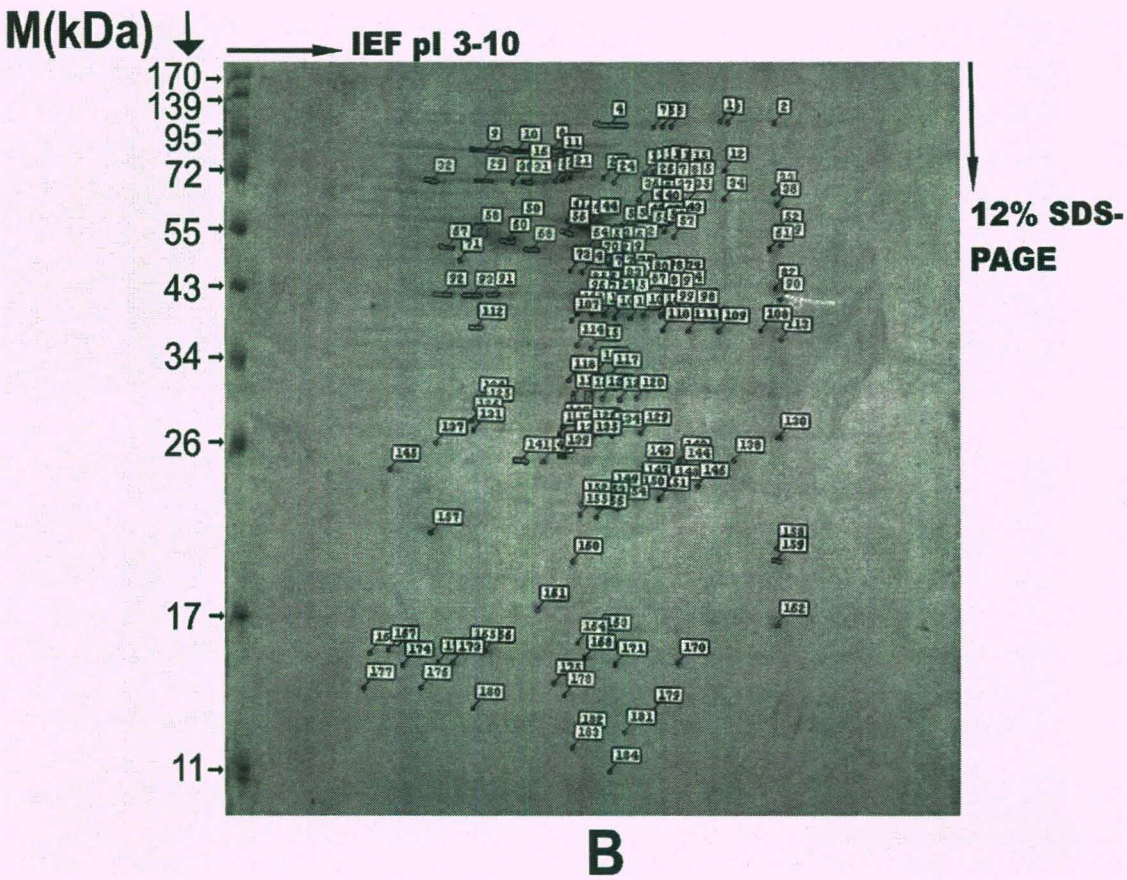
A

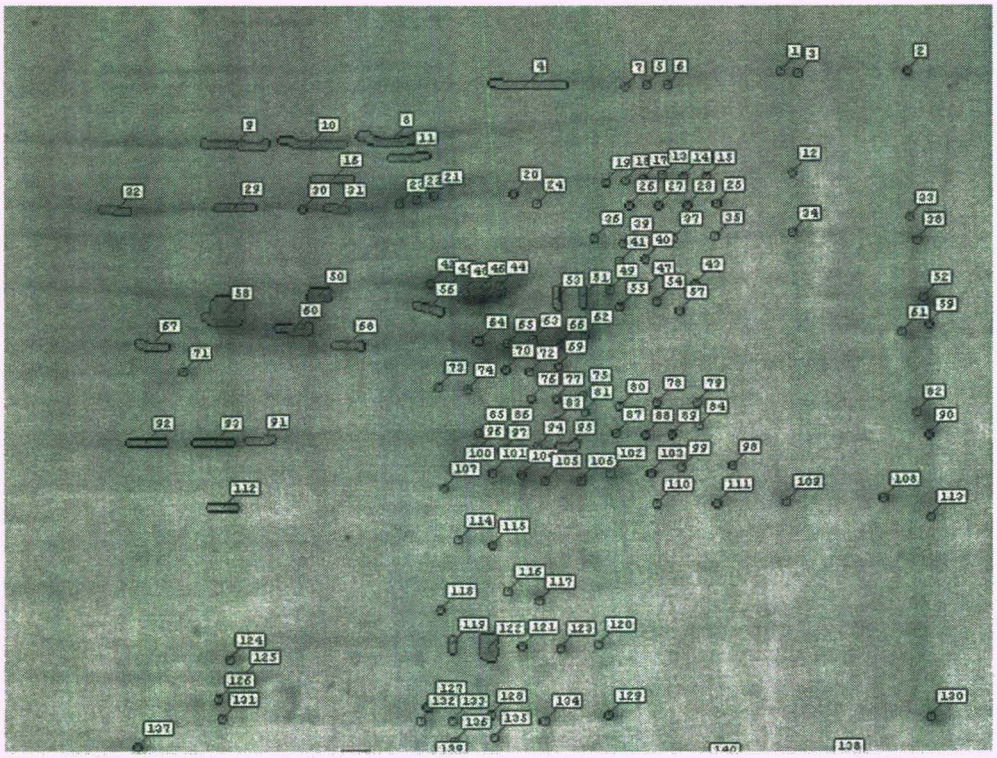


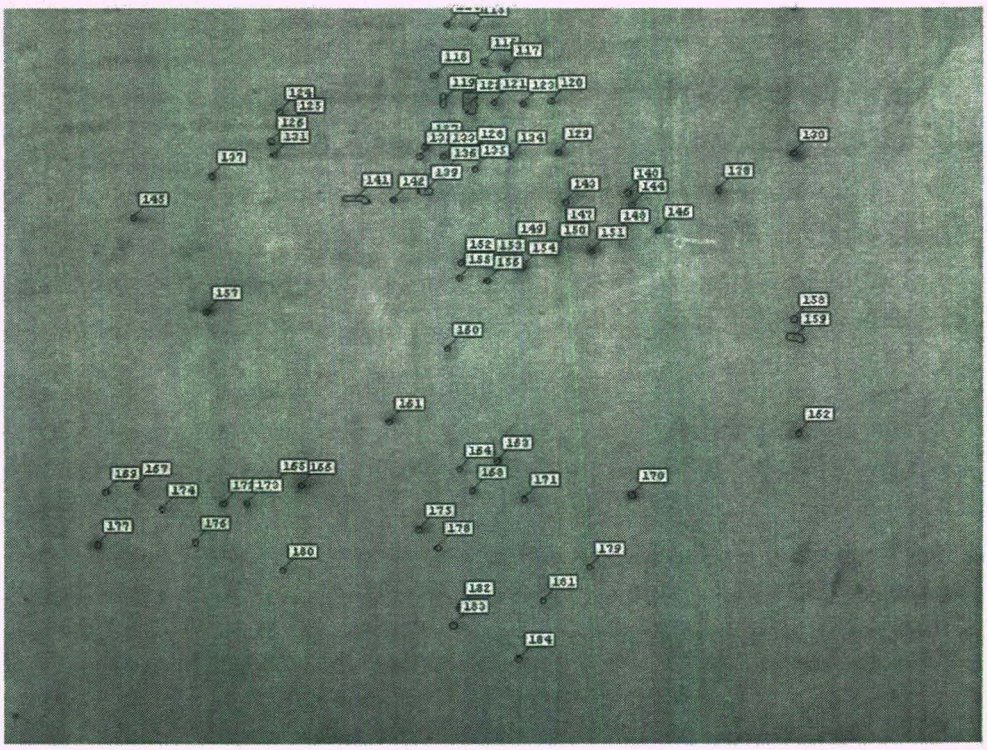
B

Figure 6.3: **(A)** 2-D gel map of soluble proteins (SPs) of *L. donovani*. The SPs were loaded onto IPG strip pI 3–10 followed by SDS-PAGE (12%). **(B)** The numbered spots indicate the identified/ unidentified soluble proteins listed in Table 6.3. **(C)** Respective partial enlarged spotted image of the same gel.

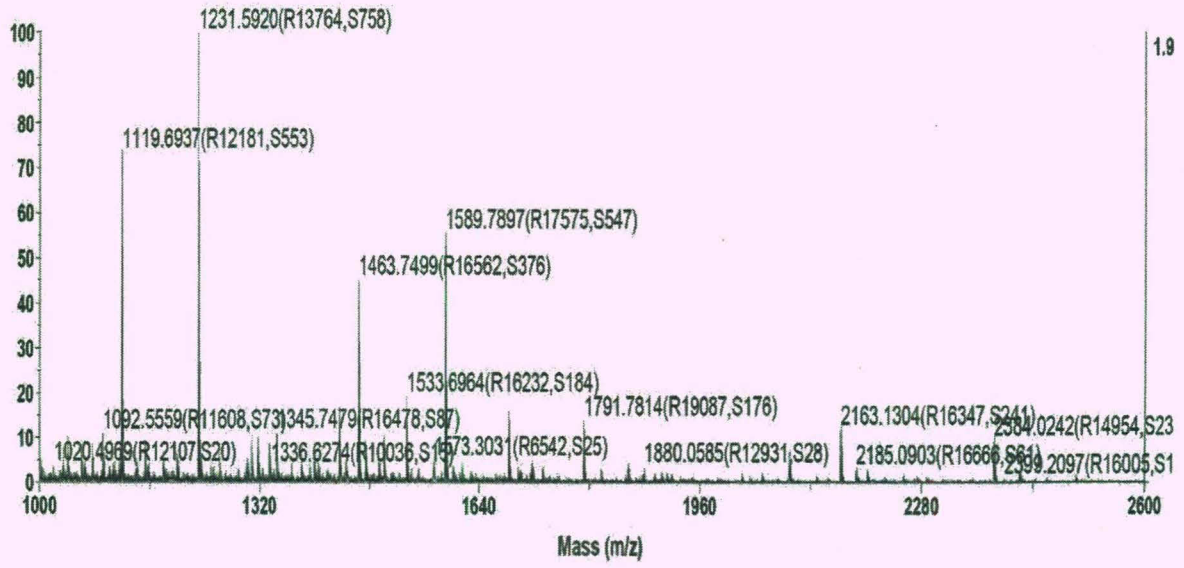


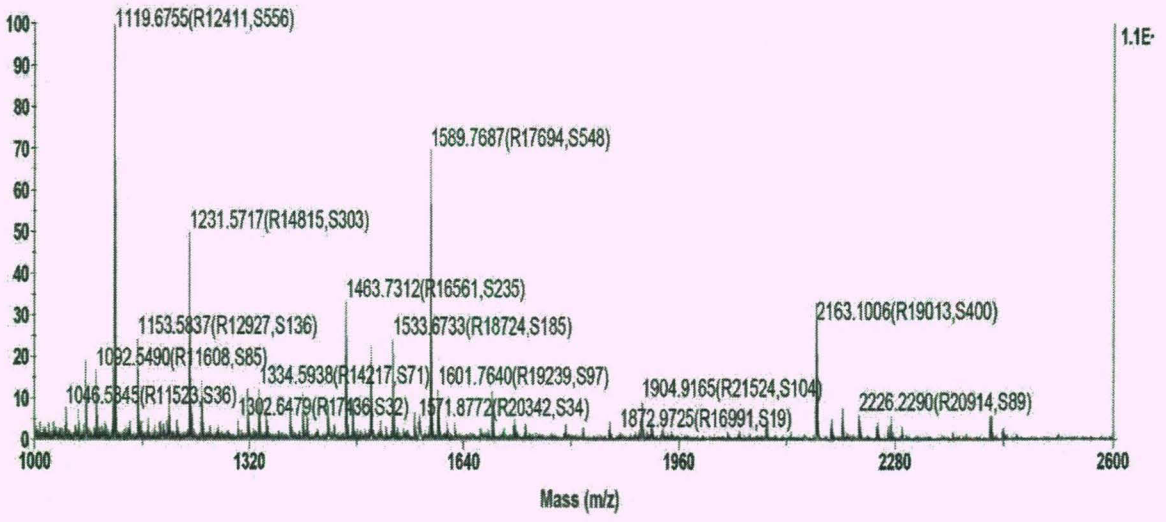


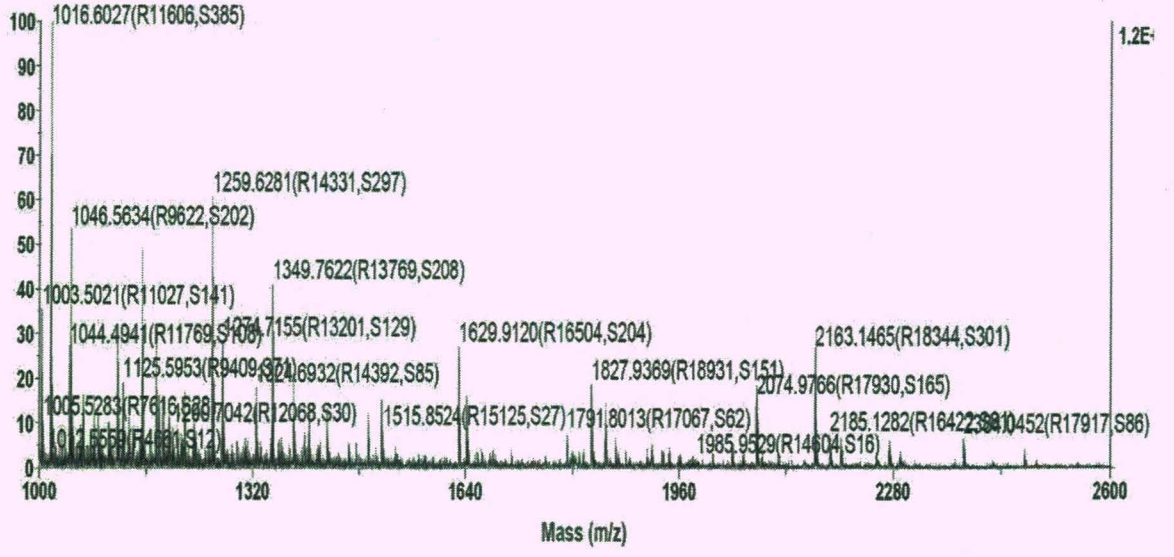




c


1231.5920(R13764,S758)
1119.6937(R12181,S553)
1589.7897(R17575,S547)
1463.7499(R16562,S376)
1533.6964(R16232,S184)
1791.7814(R19087,S176)
2163.1304(R16347,S241)
1880.0585(R12931,S28)
Mass (m/z)


1119.6755(R12411,S556)
1589.7687(R17694,S548)
1231.5717(R14815,S303)
1463.7312(R16561,S235)
2163.1006(R19013,S400)
1533.6733(R18724,S185)
1904.9165(R21524,S104)
2226.2290(R20914,S89)
Mass (m/z)


1016.6027(R11606,S385)
1259.6281(R14331,S297)
1046.5634(R9622,S202)
1349.7622(R13769,S208)
1629.9120(R16504,S204)
2163.1465(R18344,S301)
1827.9369(R18931,S151)
2074.9766(R17930,S165)
1515.8524(R15125,S27)
1791.8013(R17067,S62)
Mass (m/z)

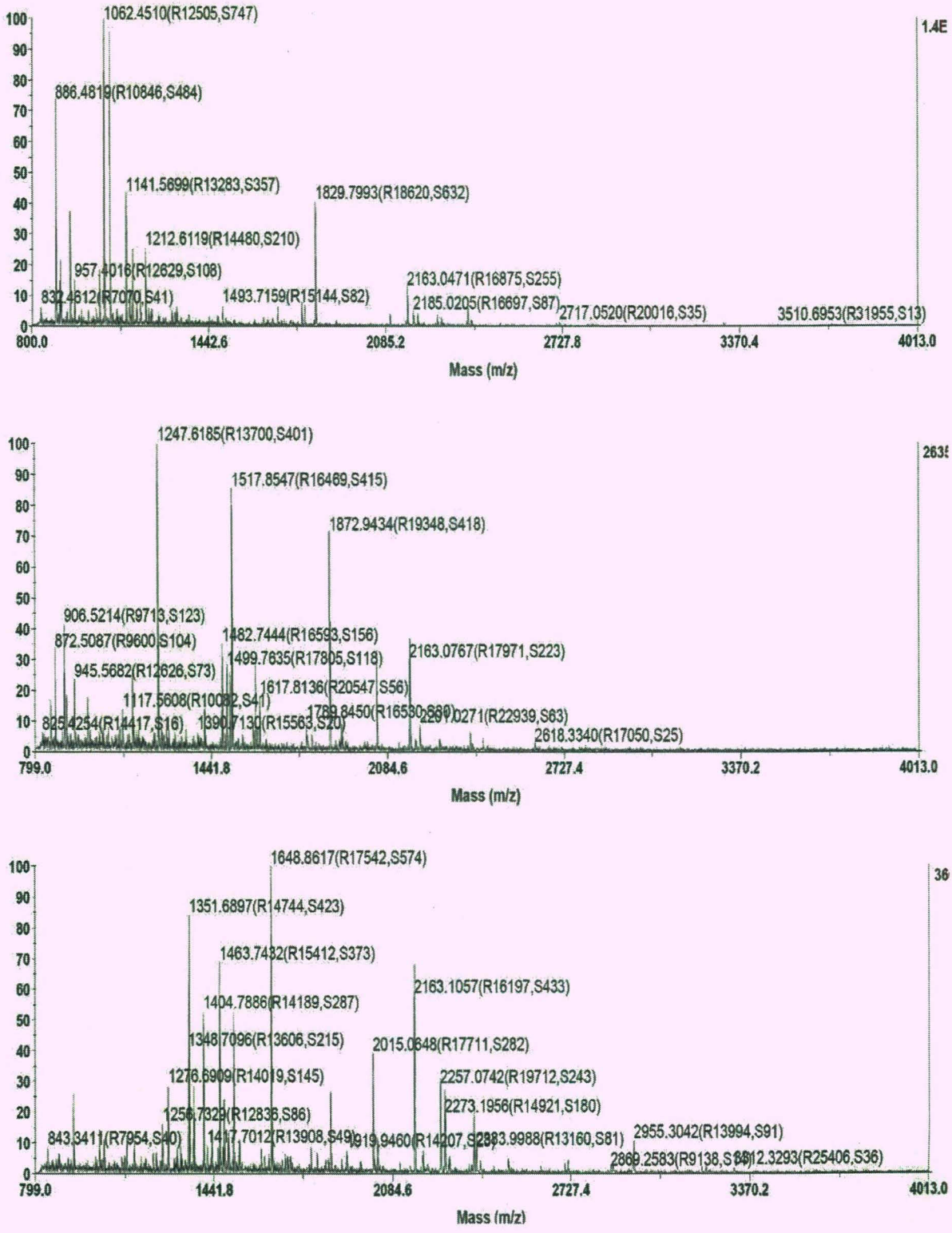


Figure 6.4: Peptide mass fingerprint (PMF) spectrum of the some *L. donovani* identified proteins after cutting the bands from gel, in gel digestion process and MALDI-TOF MS. Peptide precursor ions molecular mass is given on the top of peck. Y-axis represents the relative abundance of peptides and X-axis represents m/z ratio.

6.3.4. Identification of L. donovani membrane enriched and soluble proteins through mass spectrometric analysis

6.3.4.1 Membrane enriched proteins (MEPs):

In all, 95 well resolved protein spots excised from a coomassie blue stained gel of MEP fraction were analysed and 72 (76%) proteins were identified from spots by MS and MS/MS analysis (Table 6.1). The identified spots matched to a number of database entries. Minor identification failures could be due to sample amount, specific peptide characteristics and extensive post translational modifications (PTM) or significant divergence from sequenced strains (McNicoll *et al.*, 2006). A significant correlation between theoretical and experimental physicochemical properties of identified proteins (Mr and pI) was obtained, though some discrepancies existed which could be due to heavily prevalent post-translational protein modifications in protozoan parasite.

In addition, it was also noticed that the mass and charge of several proteins were different from those predicted by the leishmanial genome, which is hitherto reported to be a common feature of most proteomic analyses, probably reflecting the effect of protein 'maturation' events including co-or post-translational modifications (PTM) (McNicoll *et al.*, 2006; Sinha *et al.*, 2002). Further, in this study more than one protein was detected in a single spot for example, alpha-tubulin, tubulin-beta chain, gp63 etc. Some more examples are listed in Table 6.1. Similarly, in other studies of *Leishmania* also, predicted masses different from the observed masses have been seen where primarily tubulins and heat-shock proteins were found as multiple spots and have attributed that it is due to the degradation of proteins during sample preparation (Bente *et al.*, 2003; McNicoll *et al.*, 2006). Many of the identified proteins have either a well documented role in drug resistance (DR) or used as drug targets (DTs) and vaccine candidate (VCs) in many species (Table 6.1). Few proteins were diagnostic markers (DMs) also listed in table 6.1. Among identified proteins some were having unknown functions that may be the novel targets for leishmaniasis.

6.3.4.1.1 Functional classifications of identified membrane proteins of L. donovani

The identified proteins were classified on the basis of their biological function into ten functionally related groupings - Carbohydrate metabolism, Cell Signalling, Chaperons, Cytoskeletal organization, Dephosphorylation, Fatty acid synthesis, Protein synthesis, RNA processing, Transport, Unknown biological function (Table 6.2). Out of total 72 identified proteins 21 were the hypothetical ones.

Table 6.1: Proteins identified in membrane protein enriched fraction of *L. donovani*.

Spot[a] no.	Protein Identified[b]	Species[c]	Accession no.[d]	Mol. Mass[e] (Pred.)	pI[f] (Pred.)	Pm/ % of Sc/ Ms[g]	Function[h]	Cellular Localization[i]	Class/ Family[j]	Remarks[K]
1	ABC Protein	Lmj	gi|157874678	74.5	6.1	9/23/156	TP	MP	AT	DT
2	Hypothetical protein	Tb	gi|74025300	34.3	5.2	10/8/102	UN	UN	Unknown	??
3	Heat shock protein 100	Tb	CLP_TRYBB	96.8	6.1	12/10/91	CH	MT	clpA/clpB	??
4	Tubulin beta chain	Lmx	TBB_LEIME	50.0	4.7	17/14/98	CO	CS	Tubulin	Th1
5	Tubulin beta chain	Lmx	TBB_LEIME	50.0	4.7	19/15/99	CO	CS	Tubulin	Th1
6	Tubulin beta chain	Lmx	TBB_LEIME	50.0	4.7	15/11/103	CO	CS	Tubulin	Th1
7	Alpha tubulin	Lb	gi|154333816	53.5	5.1	11/17/123	CO	CS	Tubulin	Th1
8	Alpha tubulin	Lb	gi|154333816	53.5	5.1	9/14/109	CO	CS	Tubulin	Th1
10	Variant surface glycoprotein	Tb	Q38CQ7_9TRYP	54.2	6.4	13/11/129	UN	MP	Unknown	??
11	Protein tyrosine phosphatase	Ld	Q0PEE2_LEIDO	55.2	9.6	14/16/142	DP	MP	Hydrolase	IP
12	Variant surface glycoprotein phospholipase C	Tc	PHLC_TRYCR	42.7	5.3	9/12/104	CS	MP	Lyase	VC
13	Hypothetical protein	Tc	gi|71648861	22.8	8.8	12/14/117	UN	UN	Unknown	??
14	ABC transporter	Lmj	gi|157865529	32.9	5.3	9/15/99	TP	MP	AT	DT
15	Mucin-associated surface protein (MASP)	Tc	Q4DU24_TRYCR	27.5	4.1	18/12/111	UN	MP	Unknown	VC
16	Hypothetical protein	Tc	gi|71655193	81.0	5.7	15/9/117	UN	UN	Unknown	??
17	RNA binding protein	Li	gi|146093107	70.8	9.3	9/12/107	RP	CP	Unknown	DT
18	Putative MAP kinase kinase	Lmx	Q9Y074_LEIME	41.4	5.2	9/10/98	UN	MP	Protein kinase	DT
19	Coronin	Lmj	Q4QB38_LEIMA	56.6	6.6	9/11/96	AB	MP	Unknown	??
22	Heat shock protein 83	Ld	HSP83_LEIDO	52.6	5.4	15/19//125	CH	MP	Hydrolase	Th1
24	ABC1 transporter	Lmj	gi|157865765	20.0	5.2	9/12/111	TP	MP	AT	DT
26	Eukaryotic initiation factor 4A	Lb	IF4A_LEIBR	45.2	5.8	6/8/82	PS	CP	Helicase	Th1
28	Amino acid transporter	Lmj	Q4Q6N1_LEIMA	58.4	7.6	13/19/112	TP	MP	Unknown	DT
29	Hypothetical transmebrane protein	Tb	Q8WPU4_9TRYP	20.6	10.4	9/11/79	UN	MP	Unknown	??
30	RNA helicase	Li	gi|146085841	75.0	9.2	11/9/88	RP	NU	Helicase	DT
31	gp63, Leishmanolysin	Lb	A4H626_LEIBR	62.5	4.7	15/23/137	CS	MP	Hydrolase	DT
32	gp63, Leishmanolysin	Lb	A4H626_LEIBR	62.5	4.7	11/19/122	CS	MP	Hydrolase	DT

33	Surface antigen protein 2, putative	Li	A4HV45_LEIIN	43.9	5.1	19/21/117	CS	MP	Unknown	VC
34	LPG2	Ld	Q25266_LEIDO	37.1	9.7	6/9/77	TP	MP	ST	VC
35	GPI mannosyltransferase 1	Tb	PIGM_TRYBB	48.8	9.4	17/13/121	TP	ERM	Transferase	DT
36	p36 LACK protein	Ld	gi|13991854	34.3	6.1	6/21/106	CS	MP	Unknown	VC, DT
37	Hypothetical protein Fragment	Tc	Q4CPK5_TRYCR	27.8	6.1	8/11/107	UN	UN	Unknown	??
38	Small GTP-binding protein	Tb	Q580S0_9TRYP	20.6	6.1	10/15/121	PT	MP	GTP-binding	DT
40	Glycerol-3-phosphate dehydrogenase	Tc	gi|71649151	24.9	9.2	11/65/102	CM	CP	OR	DT
41	ATP synthase	Lmj	Q4QC61_LEIMA	54.0	5.6	14/18/132	TP	MP	ATPase	DT
42	Amino acid permease, putative	Lmj	gi|73544254	58.5	7.3	9/14/108	TP	MP	Permease	??
43	Hypothetical protein	Tb	Q388Z1_9TRYP	94.4	7.1	9/11/125	UN	UN	Unknown	??
44	Promastigote surface antigen (PSA)	Ld	Q4JI42_LEIDO	40.7	6.0	11/14/121	CS	MP	Unknown	VC
45	Promastigote surface antigen (PSA)	Ld	Q4JI42_LEIDO	40.7	6.0	13/19/127	CS	MP	Unknown	VC
47	Chaperonin HSP60, mitochondrial precursor	Lt	CH60_LEITA	50.2	11.8	8/14/135	CH	MT	Hydrolase	VC
48	Elongation factor Tu	Lmj	gi|7259177	51.5	6.9	9/18/137	PS	MP	GTP-binding	VC
51	Hypothetical protein	Lb	gi|154335425	14.4	5.0	9/15/138	UN	UN	Unknown	??
52	Hydrophilic acylated surface protein B	Lmx	Q9U1F9_LEIME	18.6	4.4	14/18/132	TP	MP	Unknown	??
53	Activated protein kinase-C receptor homolog (LACK)	Ld	O43942_LEIDO	30.6	6.6	9/11/101	CS	MP	AR	VC
54	Ubiquinol cytochrome-c reductase like protein	Li	A4I763_LEIIN	7.9	5.3	10/15/104	UN	MP	Unknown	??
55	HASPA Protein	Ld	O77299_LEIDO	8.4	4.4	17/13/141	CS	MP	Unknown	IP
56	Hypothetical protein	Li	gi|146102541	30.1	9.9	9/16/116	UN	UN	Unknown	??
58	Hypothetical protein	Tc	Q4DZ10_TRYCR	23.5	8.7	11/17/143	UN	UN	Unknown	??
59	Cytochrome c	Tb	COX2_TRYCR	24.1	4.8	14/12/92	TP	MT	OR	DT
60	Cytochrome c	Tb	COX2_TRYCR	24.1	4.8	11/19/93	TP	MT	OR	DT
61	Hypothetical protein	Lb	gi|154331852	19.7	8.6	13/14/132	UN	UN	Unknown	??
62	Hypothetical protein	Tc	Q4DZ10_TRYCR	23.5	8.7	11/17/143	UN	UN	Unknown	??
63	Hypothetical protein	Lmj	gi|157877088	19.2	9.7	9/14/103	UN	UN	Unknown	??
65	Hypothetical protein	Tb	Q4FKS2_9TRYP	10.8	9.9	9/19/106	UN	UN	Unknown	??
66	Maoc family dehydratase like protein	Lmj	gi|157864478	17.2	9.4	9/11/104	FS	CP	OR	??
67	RNA polymerase I subunit	Tb	gi|86438842	9.5	6.8	13/21/107	RP	CP	Unknown	DT
68	RNA polymerase I subunit	Tb	gi|86438842	9.5	6.8	12/16/99	RP	CP	Unknown	DT
69	Maoc family dehydratase like protein	Lmj	gi|157864478	17.2	9.4	10/14/119	FS	CP	OR	??
71	Hypothetical protein	Tb	Q4FKS2_9TRYP	10.8	9.9	9/19/106	UN	UN	Unknown	??
72	Hypothetical protein	Tb	Q4FKS2_9TRYP	10.8	9.9	9/19/106	UN	UN	Unknown	??
73	Hypothetical protein	Lb	gi|154344397	8.6	8.7	13/11/111	UN	UN	Unknown	??

74	Hypothetical protein	Lmj	gi|157877088	19.2	9.7	9/13/107	UN	UN	Unknown	??
77	Glyceraldehyde-3-phosphate dehydrogenase	Lmx	G3PG_LEIME	39.0	9.0	10/17/109	CM	GS	OR	DT
81	Hypothetical protein	Tb	Q4FKS2_9TRYP	10.8	9.9	10/17/111	UN	UN	Unknown	??
82	Ubiquinol cytochrome-c reductase like protein	Li	A4I763_LEIIN	7.9	5.3	11/15/110	UN	MP	Unknown	??
86	Hypothetical protein	Tc	Q4DZ10_TRYCR	23.5	8.7	15/12/121	UN	UN	Unknown	??
87	60S ribosomal protein L23a	Tb	RL23A_TRYBB	18.1	10.7	9/10/87	UN	RS	Unknown	??
90	Hypothetical protein	Tc	Q4CTS1_TRYCR	21.8	11.0	9/12/94	UN	UN	Unknown	??
91	HASPA Protein	Ld	O77299_LEIDO	8.4	4.4	17/12/129	CS	MP	Unknown	IP
92	Hypothetical protein	Tc	Q4DZ10_TRYCR	23.5	8.7	15/11/127	UN	UN	Unknown	??
93	Hexose transporter	Lmx	B1PLM1_LEIME	67.6	5.2	13/11/101	TP	MP	ST	DT
94	Glucose transporter	Lmx	O76486_LEIME	66.1	7.7	12/10/91	TP	MP	ST	DT
95	Cysteine-leucine rich protein	Li	gi|146098686	65.8	5.8	11/9/149	UN	MP	Unknown	??

The protein spots shown in Figure 6.2 were identified using Peptide Mass Fingerprint.

a) Protein spots no. indicated in Figure 6.2.
b) Name of identified protein.
c) Species: Lb: *Leishmania braziliensis*; Lc: *Leishmania chagasi*; Ld: *Leishmania donovani*; Li: *Leishmania infantum*; Lmj: *Leishmania major*; Lmx: *Leishmania mexicana*; Lt: *Leishmania tarentolae*; Tb: *Trypanosoma brucei;* Tc: *Trypanosoma Cruzi.*
d) Accession numbers of protein according to NCBI and Swiss-Prot accession number.
e) Predicted Molecular mass in kDa.
f) Predicted pI.
g) No. of peptides matched (Pm)/ Percentage of sequence coverage (Sc) /MOWSE score (Ms)
h) Function of identified proteins; AB: Actin binding, CH: Chaperon, CM: Carbohydrate metabolism, CO: Cytoskeletal organization, CS: Cell Signaling, DP: Dphosphorylation, FS: Fatty acid synthesis, NA: Nucleosome assembly, PS: Protein synthesis, RP: RNA processing, TP: Transport, UN: Unknown.
i) Cellular localization of identified proteins; CP: Cytoplasm, CS: Cytoskeleton, ERM: Endoplasmic reticulam membrane, GS: Glycosome, MP: Membrane protein, MT: Mitochondria, NU: Nucleus, RS: Ribosomal surface, Un: Unknown.
j) Class/Family; AR: Aldehyde reductase, AT: ABC transporter, OR: Oxidoreductase, ST: Sugar transporter.
k) Remarks; DM: Diagnostic Marker, DR: Drug Resistance, DT: Drug Targets, IP: Immunogenic protein, Th1: Th1 Stimulatory, VC: Vaccine Candidate, ??: Not described previously.

Note: Protein spots analyzed but not identified: 9, 20, 21, 23, 25, 27, 39, 46, 49, 50, 57, 64, 70, 75, 76, 78, 79, 80, 83, 84, 85, 88 and 89.

Table 6.2: Biological categories of identified proteins in membrane enriched fraction of *L. donovani.*

Sl. No.	Protein Categories and proteins	Total no. of identified proteins
1	**Carbohydrate metabolism**	**1**
	• Glyceraldehyde-3-phosphate dehydrogenase	1
2	**Cell Signalling**	**10**
	• Activated protein kinase C receptor homolog LACK	1
	• gp63, Leishmanolysin	2
	• HASPA Protein	2
	• p36 LACK protein	1
	• Promastigote surface antigen (PSA)	2
	• Surface antigen protein 2	1
	• Variant surface glycoprotein phospholipase C	1
3	**Chaperons**	**4**
	• Chaperonin HSP 60	1
	• Coronin	1
	• Heat shock protein 83	1
	• Heat shock protein 100	1
4	**Cytoskeletal organization**	**5**
	• Alpha tubulin	2
	• Tubulin beta chain	3
5	**Dephosphorylation**	**1**
	• Protein tyrosine phosphatase	1
6	**Fatty acid synthesis**	**2**
	• Maoc family dehydratase like protein	2
7	**Protein synthesis**	**2**
	• Elongation factor Tu	1
	• Eukaryotic initiation factor 4A	1
8	**RNA processing**	**4**
	• RNA binding protein	1
	• RNA helicase	1
	• RNA polymerase I subunit	2
9	**Transport**	**14**
	• ABC Protein	3
	• ATP synthase	1
	• Amino acid permease	1
	• Amino acid transporter	1
	• Cytochrome c	2
	• Glucose transporter	1
	• GPI mannosyltransferase 1	1
	• Hexose transporter	1
	• Hydrophilic acylated surface protein B	1
	• LPG2	1
	• Small GTP-binding protein	
10	**Unknown biological function**	**27**
	• 60S ribosomal protein L23a	1
	• Cysteine-leucine rich protein	1
	• Hypothetical Proteins	21
	• Mucin-associated surface protein (MASP)	1
	• Ubiquinol-cytochrome-c reductase-like protein	2
	• Variant surface glycoprotein	1

6.3.4.1.2 Cellular localization

The identified proteins were also classified on the basis of their cellular localization (Table 6.1). Most of the proteins were located on membrane but some identified proteins were cytoplasmic, cytoskeletal, endoplasmic reticular, glycosomal, mitochondrial, nuclear and ribosomal also. Many identified proteins could not be classified into any category because they were hypothetical proteins and their functional and metabolic roles are yet to be deciphered.

6.3.4.1.3 Membrane proteins classes/ families

Identified membrane proteins belong to many class/ family which were summarised in table 6.1. The class/family of some of the identified proteins are still unknown.

6.3.4.2 Soluble proteins

Out of 184 well resolved protein spots excised from a coomassie blue stained gel 132 (71.7%) protein spots were identified by MS and MS/MS analysis (Table 6.3) in the SPs fraction of *L. donovani* which included 29 hypothetical proteins. Among these, most of the proteins have also been reported as potential drug targets (DTs) *viz.*, ADP-ribosylation factor GTPase activating protein, DEAD/DEAH box helicase, Protein kinase, Leishmanolysin precursor, HSP-90, Triosephosphate isomerase, Protein Disulfideisomerase, Actin, Pyruvate kinase, Cofactor independent phosphoglycerate mutase, Adenosylhomocysteinase, Trypanothione reductase, ATP synthase, Glutamate dehydrogenase, Proteinphosphatase-2C, Phosphoenolpyruvate carboxy kinase (ATP), Dihydrolipoamide dehydrogenase, Mitochondrial DNA polymerase I protein A, Ubiquitin, NAD-dependent deacetylaseSIR2 homolog, Adenosylhomocysteinase, Cytochrome b domain protein, Ubiquitin, Cell division control protein-2 -homolog-1. Some proteins were identified as immunogenic proteins (IPs)/Th1 immunostimulatory type *viz.*, Calreticulin, Enolase, Kinesin, Heat shock 70-related protein1, Elongation factor-2, Tubulin beta chain, Eukaryotic translation initiation factor, Hsp83, Triosephosphate isomerase, calreticulin, Histones and have been evaluated as vaccine candidates (VCs) such as Phosphoglycerate kinase, Elongation factor-2, Tubulin beta chain, Carboxypeptidase, Triosephosphate isomerase, Activated protein kinase C receptor homolog (LACK). Protein Disulfideisomerase (PDI) and Fructose-1, 6-bisphosphate aldolase were reported as DT and

VC both. Kinesin is a diagnostic marker (DM) for *Leishmania* diagnosis. Some proteins were involved in drug resistance mechanism such as Alfa Tubulin, Cytochrome b domain protein.

Further, in this study more than one protein was also detected in a single spot for example, Heat shock 70-related protein1, Pyruvate kinase, enolase etc. Some more examples are listed in Table 6.3. Many proteins showed a good correlation in predicted and obtained values in terms of their Mr and pI, while many others showed a considerable variation. The identification of the same protein at more than one location within the same gel was observed like Actin, Tubulin, Calreticulin etc. The multiple spots identifying same protein can be accounted for post translational modifications which might have taken place either in the living system or presence of truncated/ degraded portions of the proteins, as previously reported (Bente *et al.*, 2003; Cuervo *et al.*, 2007). These observations suggested that PTMs are widely prevalent in this organism. Among identified proteins some were having unknown function listed in table 6.3 that may be the novel targets for leishmaniasis.

6.3.4.2.1 Biological categories of identified SPs of L. donovani on the basis of function

The identified proteins were classified on the basis of their biological function into 17 functionally related groups - Amino acid metabolism, Apoptosis/ Cell cycle, ATP synthesis/ hydrolysis, Calcium Binding Protein, Carbohydrate metabolism, Chaperons/ Protein folding, Cytoskeletal organization, Dephosphorylation, DNA replication, Nucleic Acid Metabolism, Nucleosomal assembly Protein synthesis, Proteolysis, RNA processing, Transcription, Transport, Unknown biological function (Table 6.4).

6.3.4.2.2 Cellular localization

The identified proteins were also classified on the basis of their sub-cellular localization (Table 6.3). Identified proteins were localized in cytoplasm, endoplasmic reticulum, glycosome, mitochondria, nucleus and ribosome. Many identified proteins could not be classified into any category because they were hypothetical proteins and their functional, biochemical and metabolic roles are yet to be deciphered.

6.3.4.2.3 Class/ family of soluble protein

Identified soluble proteins belong to so many classes/ families which were summarised in table 6.3. Class/ family of some identified proteins were still unknown.

Table 6.3: Proteins identified in soluble fraction of *L. donovani*.

Spot No[a]	Protein Identified[b]	Species[c]	Accession No.[d]	Mol. Mass[e] (Pred.)	pI[f] (Pred.)	Pm/ % of Sc/ Ms[g]	Function[h]	Sub-cellular Localization[i]	Class/ Family[j]	Remarks[K]
1	Phosphoglycerate kinase	Lmx	PGKC_LEIME	51.4	8.8	11/47/129	CM	CP	Transferase	VC
2	Phosphoglycerate kinase	Lmx	PGKC_LEIME	51.4	8.8	9/18/104	CM	CP	Transferase	VC
3	Hypothetical protein	Tc	Q4DDH4_TRYCR	73.8	6.1	15/34/110	UN	UN	Unknown	??
4	Hypothetical protein	Tb	Q57UX3_9TRYP	25.9	6.9	8/17/98	UN	UN	Unknown	??
6	Hypothetical protein	Li	gi|146097028	74.5	8.2	12/33/143	UN	UN	Unknown	??
7	T-complex protein 1 gamma subunit	Tb	Q57YZ8_9TRYP	60.7	6.7	13/45/156	CH	CP	Hydrolase	??
10	Hypothetical protein	Tc	gi|71412647	65.0	8.8	11/28/92	UN	UN	Unknown	??
11	ADP-ribosylation factor GTPase activating protein	Tb	gi|71746258	45.6	5.8	16/38/157	ER-GT	CP	Unknown	DT
12	DEAD/DEAH box helicase (RNA helicase)	Lmj	Q4Q1G8_LEIMA	77.5	9.1	14/31/136	NM	NU	Helicase	DT
14	RNA pseudouridylate synthase	Tc	Q4CQR7_TRYCR	52.0	8.2	14/40/111	NM	NU	PS	??
15	Protein kinase	Tc	Q4CRN6_TRYCR	41.9	6.1	15/25/117	CM	CP	Transferase	DT
18	Hypothetical ORF-2 protein	Lt	gi|83986	10.6	10.7	11/38/99	UN	MT	Unknown	??
19	Retrotransposon hot spot protein	Tc	gi|71397664	68.5	5.9	12/43/131	TC	NU	Unknown	??
20	Hypothetical protein	Tb	gi|72390734	17.8	5.1	11/27/107	UN	UN	Unknown	??
21	Heat shock 70-related protein1	Lmj	HSP71_LEIMA	68.2	5.5	12/31/138	CH	MT	Hydrolase	Th1
22	Heat shock 70-related protein1	Lmj	HSP71_LEIMA	68.2	5.5	15/436/144	CH	MT	Hydrolase	Th1
23	Heat shock 70-related protein1	Lmj	HSP71_LEIMA	68.2	5.5	12/31/138	CH	MT	Hydrolase	Th1
24	Leishmanolysin precursor	Lc	GP63_LEICH	63.8	6.8	9/19/78	PL	CP	Hydrolase	DT
25	60S ribosomal protein L17	Tc	Q4CRX5_TRYCR	19.1	10.8	9/17/89	PS	RS	Transferase	??
26	Hypothetical protein	Lmj	Q4FXB5_LEIMA	42.2	6.7	11/31/156	UN	UN	Unknown	??
27	Kinesin	Tb	gi|71746254	74.5	6.7	9/15/101	CC	NU	Hydrolase	DM
30	Elongation factor-2	Lmj	gi|11244578	73.0	7.2	15/ 29/114	PS	CP	Kinase	VC,Th1
31	Protein Disulfideisomerase, PDI	Lmj	gi|25990151	52.0	5.2	9/23/104	CH	ER	Isomerase	DT,VF,VC
32	Tubulin beta chain	Lmx	TBB_LEIME	50.6	4.7	9/11/102	CO	CP	Tubulin	VC,Th1
33	Heat shock protein-90	Ld	gi|323030	53.0	5.6	15/37/119	CH	CP	Hydrolase	DT
35	Hypothetical protein	Lmj	gi|12311835	84.0	5.4	11/25/94	UN	UN	Unknown	??
36	Hsp83	Li	gi|362545	81.0	5.1	15/21/79	CH	CP	Hydrolase	Th1
37	Triosephosphate isomerase	Tc	TPIS_TRYCR	76.0	6.6	13/18/132	CM	CP	Isomerase	DT

38	Protein Disulfideisomerase, PDI	Lmj	gi|25990151	52.0	5.2	8/22/91	CH	ER	Isomerase	DT
39	Hypothetical protein	Li	gi|146097028	74.5	8.2	12/26/81	UN	UN	Unknown	??
40	Actin	Lmj	ACT_LEIMA	41.9	5.1	9/29/79	CO	CP	Actin	DT
41	Actin	Lmj	ACT_LEIMA	41.9	5.1	11/33/97	CO	CP	Actin	DT
42	Pyruvate kinase	Lmx	gi|577072	55.0	6.0	11/28/94	CM	CP	Transferase	DT
43	Pyruvate kinase	Lmx	gi|577072	55.0	6.0	15/26/92	CM	CP	Transferase	DT
44	Pyruvate kinase	Lmx	gi|577072	55.0	6.0	11/23/86	CM	CP	Transferase	DT
45	Pyruvate kinase	Lmx	gi|577072	55.0	6.0	11/28/104	CM	CP	Transferase	DT
46	Pyruvate kinase	Lmx	gi|577072	55.0	6.0	13/27/92	CM	CP	Transferase	DT
48	Cofactor independent phosphoglycerate mutase	Lmx	gi|28400787	61.0	5.4	12/22/116	AM	CP	Isomerase	DT
49	Actin	Lmj	ACT_LEIMA	41.9	5.1	9/32/107	CO	CP	Actin	DT
50	Adenosylhomocysteinase	Ld	gi|1710837	48.0	5.7	6/ 31/148	AM	CP	Hydrolase	DT
52	Trypanothione reductase	Lmj	gi|7677022	53.0	5.8	8/13/123	AM	CP	Isomerase	DT
53	p45	Lmj	gi|6274526	41.0	6.6	11/26/96	ET	MT	Oxidoreductase	VC,TC
54	Centromere/microtubule binding protein cbf5	Lmj	gi|157869177	48.4	9.3	9/22/99	CO	CP	Unknown	??
55	Centromere/microtubule binding protein cbf5	Lmj	gi|157869177	48.4	9.3	11/29/132	CO	CP	Unknown	??
57	Hypothetical protein	Tc	Q4DSF8_TRYCR	26.6	6.6	15/22/91	UN	UN	Unknown	??
58	Beta tubulin	Lmj	gi|157869199	49.8	4.5	11/32/97	CO	CP	Tubulin	Th1
59	DNA repair protein	Lmj	gi|157871611	83.0	9.9	8/24/125	CC	NU	Transferase	??
60	Alfa Tubulin	Ld	gi|606648	49.0	4.9	8/17/134	CO	CP	Tubulin	DR
62	Enolase	Lmj	gi|8388689	46.0	6.6	13/25/97	CM	CP	Enolase	IP
63	Enolase	Lmj	gi|8388689	46.0	6.6	16/27/104	CM	CP	Enolase	IP
64	Enolase	Lmj	gi|8388689	46.0	6.6	16/27/104	CM	CP	Enolase	IP
65	Enolase	Lmj	gi|8388689	46.0	6.6	15/25/103	CM	CP	Enolase	IP
66	Enolase	Lmj	gi|8388689	46.0	6.6	15/25/103	CM	CP	Enolase	IP
67	Carboxypeptidase	Lmj	gi|157866450	50.1	5.3	13/15/119	PL	CP	Metallopeptidase	VC
68	Beta tubulin	Lmj	Q4QBZ6_LEIMA	49.8	4.5	11/30/103	CO	CP	Tubulin	Th1
69	ATP synthase	Lmj	gi|157869069	54.0	6.0	6/30/152	AS	MT	Transferase	DT
70	Actin	Lmj	ACT_LEIMA	41.9	5.1	11/32/129	CO	CP	Actin	DT
71	Glutamate dehydrogenase	Tc	gi|3080751	49.0	6.5	19/27/116	AM	CP	Oxidoreductase	DT
72	Actin	Lmj	LmjF04.1230	42.0	5.3	9/32/107	CO	CP	Actin	DT
73	Fructose-1,6-bisphosphate aldolase	Lmx	gi|5834626	47.0	7.9	14/24/123	CM	CP	Lyase	VC,DT
74	Fructose-1,6-bisphosphate aldolase	Lmx	gi|5834626	47.0	7.9	15/22/120	CM	CP	Lyase	VC,DT
76	Beta tubulin	Lmj	Q4QBZ6_LEIMA	49.8	4.5	7/19/91	CO	CP	Tubulin	Th1
77	Beta tubulin	Lmj	Q4QBZ6_LEIMA	49.8	4.5	11/30/103	CO	CP	Tubulin	Th1

78	Proteinphosphatase-2C	Ld	gi|2665676	45.0	7.8	12/36/110	DP	CP	Hydrolase	DT
79	Hypothetical protein	Lmj	gi|6580519	79.0	8.0	15/21/96	UN	UN	Unknown	??
81	Kinesin-like protein K39	Lc	KINL_LEICH	106.1	4.9	7/32/98	CH	CP	Hydrolase	DM
82	Dihydrolipoamide dehydrogenase	Lmj	gi|44804791	51.0	6.4	11/19/122	CM	CP	Oxidoreductase	DT
83	Aminomethyl transferase	Lmj	gi|157877282	41.3	9.2	12/31/119	AM	CP	Transfersae	DT
84	Calreticulin	Lmj	gi|5263289	33.0	4.7	9/28/99	CH	CP	Calreticulin	VF,IP
85	Isoleucin tRNA synthetase	Ld	Q9BMG3_LEIDO	124.3	6.1	7/15/66	PS	CP	Transferase	??
86	Phosphoenolpyruvate carboxy kinase (ATP)	Tb	PPCK_TRYBB	52.4	8.2	11/27/100	CM	GS	Lyase	DT
87	NADP-dependent alcohol hydrogenase	Lmj	gi|6066457	39.0	5.8	13/25/151	AM	CP	Oxidoreducase	??
88	Hypothetical protein	Tc	Q4DSF8_TRYCR	26.6	6.6	15/ 31/159	UN	UN	Unknown	??
89	Hypothetical protein	Lmj	gi|157873031	51.8	9.0	6/10/71	UN	UN	Unknown	??
90	Dihydrolipoamide dehydrogenase	Lmj	gi|44804791	51.0	6.4	10/13/122	CM	CP	Oxidoreductase	DT
91	Phosphoenolpyruvate carboxy kinase(ATP)	Tb	PPCK_TRYBB	52.4	8.2	8/17/93	CM	GS	Lyase	DT
92	Calreticulin	Lmj	gi|5263289	33.0	4.7	10/28/71	CH	CP	Calreticulin	VF,IP
93	Hypothetical protein	Tb	gi|72393541	96.3	5.4	10/21/74	UN	UN	Unknown	??
94	Mitochondrial DNA polymerase I protein A	Lb	gi|154336355	138.2	8.2	11/27/139	DR	MT	DNA polymerase	??
96	Hypothetical protein	Tc	Q4E141_TRYCR	40.8	8.0	8/11/83	UN	UN	Unknown	??
98	Ubiquitin conjugating enzyme e2	Lmj	gi|71746028	16.9	5.9	9/47/121	PL	UN	Hydrolase	??
99	Hypothetical protein	Lb	gi|123484417	24.2	9.3	9/21/97	UN	UN	Unknown	??
101	Hypothetical protein	Tc	Q4D7T1_TRYCR	33.7	7.6	10/34/144	UN	UN	Unknown	??
102	Hypothetical protein	Lmj	Q4QAB5_LEIMA	11.9	5.7	13/27/127	UN	UN	Unknown	??
104	Ubiquitin	Tb	UBIQ_TRYBB	8.5	6.5	9/24/90	PL	CP	Ubiquitin	??
105	Hypothetical protein	Li	gi|146093231	77.0	9.7	11/26/117	UN	UN	Unknown	??
106	60S acidic ribosomal protein P2-1	Li	RLA2_LEIIN	10.5	4.4	6/17/73	PS	CP	Transferase	??
107	Hypothetical protein	Lmj	gi|157874766	16.0	6.0	11/19/95	UN	UN	Unknown	??
108	Chaperone protein DNAJ	Tb	gi|72391984	29.8	9.2	12/26/137	CH	CP	DnaJ homolog B	??
111	Hypothetical protein	Lmj	gi|12311865	29.0	6.8	11/29/89	UN	UN	Unknown	??
112	Proliferative cell nuclear antigen (PCNA)	Lmj	gi|157867015	37.1	4.8	12/15/107	DR	NU	PCNA	DR
113	Hypothetical protein	Lmj	gi|157876232	20.4	9.6	6/22/101	UN	UN	Unknown	??
114	Alanine transaminase/ amino transferase	Lmj	gi|15426123	55.0	5.6	9/22/121	PS	CP	Transferase	??
115	Proteasome alpha 5 subunit	Tc	gi|71420529	27.3	4.9	10/17/117	PL	CP	Peptidase T1A	DR
116	NAD-dependent deacetylaseSIR2 homolog	Lmj	SIR2_LEIMA	43.0	5.6	14/28/69	AM	CP	Sirtuin	DT
117	NAD-dependent deacetylase SIR2 homolog	Lmj	SIR2_LEIMA	43.0	5.6	13/16/97	AM	CP	Sirtuin	DT
118	Hypothetical protein	Lmj	gi|73544526	22.3	4.5	9/31/84	UN	UN	Unknown	??
119	Arginine kinase	Tc	gi|3831705	44.0	6.7	7/44/103	CM	CP	Transferase	??

122	Triosephosphate isomerase	Li	gi|20378696	27.0	8.3	12/34/153	CM	CP	Isomerase	Th1, VC
124	Aldolase epimerase related protein 2	Lm	gi|9954713	41.0	6.0	14/49/97	CM	CP	Isomerase	??
125	Hypothetical protein	Lm	gi|7677014	42.0	4.6	7/26/72	UN	UN	Unknown	??
126	Calreticulin	Lmj	gi|5263289	33.0	4.7	14/29/79	CH	CP	Calreticulin	VF,IP
127	DNA-directed RNA polymerase subunit	Tb	Q387N1_9TRYP	14.4	5.1	11/23/129	TC	CP	RPB	??
128	Hypothetical protein	Lmj	gi|157868073	28.3	8.5	11/36/86	UN	UN	Unknown	??
129	Calcium motif P-type ATPase (TBCA2)	Tb	gi|4884978	37.0	5.3	9/41/147	CB	CP	Hydrolase	??
130	Cytochrome b domain protein	Lmj	gi|71745895	24.1	6.4	9/27/93	ET	MT	Cytochrome b5	DT,DR
132	Eukaryotic translation initiation factor	Lmj	Q4Q813_LEIMA	37.7	7.3	17/31/98	PS	CP	EIF- 4E	EA
133	Hypothetical protein	Lmj	Q4Q813_LEIMA	16.4	4.0	9/32/77	UN	UN	Unknown	??
134	Uridine kinase like protein	Lmj	Q4Q614_LEIMA	34.1	9.2	12/37/94	PS	CP	Kinase	??
135	40S ribosomal protein SA	Lmj	Q4Q0Q0_LEIMA	27.5	7.8	9/22/101	PS	RS	Transferase	??
136	Hypothetical protein	Lmj	Q4Q9T6_LEIMA	11.6	7.8	11/36/144	UN	UN	Unknown	??
138	Small nuclear Ribonucleo-protein	Lmj	Q4Q224_LEIMA	16.0	10.7	7/21/123	RP	NU	Unknown	??
139	Tyrosine phosphatase like protein	Lmj	Q4QEZ5_LEIMA	19.5	7.7	8/32/97	AM	CP	Hydrolase	DT
140	Activated protein kinase C receptor homolog, LACK	Ld	gi|13991854	30.0	6.6	14/41/153	AP	CP	Kinase	VC
142	Ubiquitin	Tb	UBIQ_TRYBB	8.5	6.5	9/36/134	PL	CP	Ubiquitin	??
143	Eukaryotic initiation factor 5a	Li	gi|8977987	18.0	4.8	6/30/78	PS	RS	EIF	
144	Activated protein kinase C receptor homolog, LACK	Ld	gi|13991854	30.0	6.6	12/39/96	AP	CP	Kinase	VC
145	Centrin	Lmj	Q4Q0E9_LEIMA	18.8	4.3	11/23/124	CB	CR	Unknown	??
146	Eukaryotic initiation factor 5a	Li	gi|8977987	18.0	4.8	6/30/114	PS	RS	Kinase	DT
147	Hypothetical protein	Lmx	gi|2131001	48.0	7.1	11/22/68	UN	UN	Unknown	??
149	Possible RAN binding protein	Lm	gi|11061633	17.0	5.6	10/23/91	PT	CP	Unknown	??
150	Nucleoside diphosphate kinase	Li	gi|12055485	18.0	7.8	10/21/107	NM	NU	Kinase	??
151	Alcohol dehydrogenase like protein	Lmj	gi|9989041	28.0	6.3	13/28/142	CM	CP	Oxidoreductase	??
152	Hypothetical protein	Lmj	Q4QAB5_LEIMA	11.9	5.7	9/41/104	UN	UN	Unknown	??
154	Adenosylhomocysteinase	Ld	gi|1710837	48.0	5.7	11 /21/123	AM	CP	Hydrolase	DT
157	Cell division control protein-2 -homolog-1	Tc	CC2H1_TRYCO	34.0	7.7	14/29/87	CC	NU	Protein kinase	DT
158	Histone H2A	Tc	H2A_TRYCR	14.3	11.2	8/11/77	NA	NU	Histone	VC
159	Histone H2B	Li	gi|2143266	12.2	11.2	4/9/81	NA	NU	Histone	VC
160	Ribosomal protein L14	Lmj	Q4Q2G1_LEIMA	21.5	11.6	12/31/83	PS	CP	Unknown	??
161	Ribosomal protein S12	Lt	RT12_LEITA	10.0	4.6	9/31/112	PS	CP	Unknown	??
162	Histone H4	Lt	gi|5738233	11.5	10.7	6/9/101	NA	NU	Histone	VC
165	RNA polymerase I subunit	Tb	gi|86438842	9.5	6.8	13/32/118	CC	NU	Unknown	??

The protein spots shown in Figure 6.3 were identified using Peptide Mass Fingerprint.

a) Protein spots no. indicated in Figure 6.3.

b) Name of identified protein.

c) Species: Lb*: Leishmania braziliensis*; Lc: *Leishmania chagasi*; Ld: *Leishmania donovani,* Li: *Leishmania Infantum*; Lmj: *Leishmania major*; Lmx: *Leishmania mexicana*; Lt: *Leishmania tarentolae*; Tb: *Trypanosoma brucei;* Tc*: Trypanosoma Cruzi.*

d) Accession numbers of protein according to NCBI and Swiss-Prot accession number.

e) Predicted Molecular mass in kDa.

f) Predicted p*I.*

g) No. of peptides matched (Pm)/ Percentage of sequence coverage (Sc) /MOWSE score (Ms)

h) Function of identified proteins; AM: Amino acid Metabolism, AP: Apoptosis, AS: ATP synthesis, CB: Calcium binding, CC: Cell Cycle, CH: Chaperon, CM: Carbohydrate metabolism, CO: Cytoskeletal organization, CS: Cell Signaling, CR: Centrosome DP: Dephosphorylation, DR: DNA replication, ER-GT: Endoplasmic reticulum-Golgi transport, ET: Electron transport, FS: Fatty acid synthesis, NA: Nucleosome assembly, NM: Nucleic acid metabolism, PS: Protein synthesis, RP: RNA processing, PL: Proteolysis, PS: Protein synthesis, PT: Protein Transport, RP: RNA processing, TC: Transcription, TP: Transport, UN: Unknown.

i) Cellular localization of identified proteins; CP: Cytoplasm, CS: Cytoskeleton, ER: Endoplasmic reticulum, GS: Glycosome, MM: Mitochondrial membrane, MT: Mitochondria, NU: Nucleus, RS: Ribosomal, Un: Unknown.

j) Class/Family; EIF: RNA poly-merase β 4E, PS: Pseudouridine synthase, RPB: RNA poly-merase beta.

k) Remarks; DM: Diagnostic marker, DR: Drug Resistance, DT: Drug Targets, EA: Exacebatory Antigen, IP: Immunogenic protein, Th1: Th1 Stimulatory, VC: Vaccine Candidate, VF: Virulence factor, ??: Not described previously.

Note: Protein spots analyzed but not identified: 5, 8, 9, 13, 16, 17, 28, 29, 30, 34, 47, 51, 56, 61, 75, 80, 95, 97, 100, 103, 109, 110, 120, 121, 123, 131, 137, 141, 148, 153, 155, 156, 163, 164, 166-184.

Table 6.4: Biological categories of identified soluble proteins of *L. donovani*.

Sl. No.	Protein Categories	Total no. of identified Proteins
1	**Amino acid metabolism**	**10**
	• Adenosylhomocysteinase	2
	• Aminomethyl transferase	1
	• Glutamate dehydrogenase	1
	• Cofactor independent hosphoglycerate mutase	1
	• Trypanothione reductase	1
	• NADP-dependent alcohol hydrogenase	1
	• NAD-dependent deacetylase SIR2 homolog	2
	• Tyrosine phosphatase like protein	1
2	**Apoptosis/ Cell cycle**	**6**
	• Activated protein kinase C receptor homolog LACK	2
	• Kinesin	1
	• DNA repair protein	1
	• Cell division control protein-2 -homolog-1	1
	• RNA polymerase I subunit	1
3	**ATP synthesis/hydrolysis**	**1**
	• ATP synthase	1
4	**Calcium Binding Protein**	**2**
	• Centrin	1
	• Calcium motif P-type ATPase (TBCA2)	1
5	**Carbohydrate metabolism**	**24**
	• Phosphoglycerate kinase	2
	• Protein kinase	1
	• Triosephosphate isomerase	2
	• Pyruvate kinase	5
	• Enolase	5
	• Fructose-1,6-bisphosphate aldolase	2
	• Dihydrolipoamide dehydrogenase	2
	• Phosphoenolpyruvate carboxy kinase (ATP)	2
	• Alcohol dehydrogenase like protein	1
	• Arginine kinase	1
	• Aldolase epimerase related protein 2	1
6	**Chaperons/ Protein folding**	**13**
	• T-complex protein 1 gamma subunit	1
	• Heat shock 70-related protein1	3
	• Protein Disulfideisomerase, PDI	2
	• Heat shock protein-90	1
	• Hsp83	1
	• Kinesin-like protein K39	1
	• Calreticulin	3
	• Chaperone protein DNAJ	1

7	**Cytoskeletal organization**	**13**
	• Actin	5
	• Centromere/microtubule binding protein cbf5	2
	• Beta tubulin	5
	• Alfa Tubulin	1
8	**Dephosphorylation**	**1**
	• Proteinphosphatase-2C	1
9	**DNA replication**	**2**
	• Mitochondrial DNA polymerase I protein A	1
	• Proliferative cell nuclear antigen (PCNA)	1
10	**Nucleic Acid Metabolism**	**3**
	• DEAD/DEAH box helicase (RNA helicase)	1
	• RNA pseudouridylate synthase	1
	• Nucleoside diphosphate kinase	1
11	**Nucleosomal assembly**	**3**
	• Histone H2A	1
	• Histone H2B	1
	• Histone H4	1
12	**Protein synthesis**	**12**
	• 60S ribosomal protein L17	1
	• Elongation factor-2	1
	• Isoleucin tRNA synthetase	1
	• 60S acidic ribosomal protein P2-1	1
	• Alanine transaminase/ amino transferase	1
	• Eukaryotic translation initiation factor	1
	• Uridine kinase like protein	1
	• 40S ribosomal protein SA	1
	• Eukaryotic initiation factor 5a	2
	• Ribosomal protein L14	1
	• Ribosomal protein S12	1
13	**Proteolysis**	**6**
	• Carboxypeptidase	1
	• Leishmanolysin precursor	1
	• Proteasome alpha 5 subunit	1
	• Ubiquitin conjugating enzyme e2	1
	• Ubiquitin	2
14	**RNA processing**	**1**
	• Small nuclear Ribonucleoprotein	1
15	**Transcription**	**2**
	• DNA-directed RNA polymerase subunit	1
	• Retrotransposon hot spot protein	1
16	**Transport**	**4**
	• Cytochrome b domain protein	1
	• ADP-ribosylation factor GTPase activating protein	1
	• Possible RAN binding protein	1
	• p45	1
17	**Unknown biological function**	**29**
	• Hypothetical Protein	29

6.4. Discussion

Proteome analysis by 2-DGE together with MALDI-TOF is a powerful approach for the resolution and identification of proteins in complex biological samples. Although *L. donovani* is a well-studied pathogen that causes fatal VL, the comprehensive proteome profile of membrane as well as soluble proteins of *L. donovani* has remained unexplored so far. Proteomics-based dissection of *L. donovani* provides a comprehensive inventory of the parasitic proteins (membrane as well as soluble proteins). This information can be instrumental in the development of novel drug targets, new immune monitoring and diagnostic devices (Dea-Ayuela *et al.*, 2006) and also enhance our knowledge in drug resistance mechanism (Thiel and Bruchhaus, 2001; Acestor *et al.*, 2002; Drummelsmith *et al.*, 2003; El Fakhry *et al.*, 2002). Henceforth, as per our knowledge, this is the first study related to proteome mapping of membrane as well as soluble proteins of *L. donovani*. On the basis of results obtained here, a number of proteins identified were classified in various biological classes and most of the identified proteins can be targeted and expected to be far more effective of the outcome of the disease.

At present, in ~33.6 Mb genome (http://www.genedb.org/leish/index.jsp), 8300 protein coding genes of *L. major* have been sequenced (Ivens *et al.*, 2005). In consequence, it was necessary to take an attempt and identify *L. donovani* proteins by reference of all known *Leishmania spp.* sequences in the public database. This strategy has been applied with success for other *Leishmania* species that lack a fully sequenced genome (Juttner *et al.*, 1998; Chiurillo *et al.*, 2000).

Proteome analysis of the cell membrane is considered a daunting task, mainly due to problems encountered at two levels: (a) isolation of membrane protein enriched fraction and (b) solubilization of membrane proteins in a manner amenable to Isoelectric focusing (Santoni *et al.*, 2000). This study constitutes the first membrane proteomic analysis of *L. donovani* and to achieve this combination of rehydration buffers was used to solublise the membrane proteins which have been isolated through ultracentrifugation. This method was successfully used for the isolation of membrane enriched proteins in case of gram-negative facultative intracellular pathogen *Francisella tularensis* by Pávková *et al.* (2005). A clearcut differentiation of MEPs and SPs of L.donovani was observed in the SDS PAGE analysis alongwith whole cell lysate (WCL) (Figure 6.1)

Result of this **comprehensive membrane proteomics** analysis has revealed many new cellular targets against leishmaniasis. Two copies of Glyceraldehyde-3-phosphate dehydrogenase protein (involved in carbohydrate metabolism) were identified in membrane eeriched proteins of *L. donovani* which has been reported as a potent DT in trypanosoma by Lakhdar-Ghazal *et al.,* (2002). Interestingly it was found that seven proteins in membrane fraction were belonging to **cell signalling**. Out of these, Activated protein kinase C receptor homolog (LACK) (Bente *et al.,* 2003; Saxena *et al.,* 2007), Promastigote surface antigen (PSA) (Handman *et al.,* 1995), HASPA Protein (Stager *et al.,* 2000), Variant surface glycoprotein phospholipase C (Paturiaux-Hanocq *et al.,* 1997), Surface antigen protein 2 (Handman *et al.,* 1995) were proposed as VCs. According to Soteriadou *et al.,* (1993) gp63 was observed to be involved in cell signaling and proposed it as DT. LACK is critical for effective mammalian parasitization and thus represents a potential drug target for leishmaniasis (Kelly *et al.,* 2003). Stager *et al.,* (2000) showed that immunization with a recombinant stage regulated surface protein from *L. donovani* induces protection against VL. *L. donovani* p36 DNA vaccine has been found to be highly immunogenic but not protective against experimental VL (Melby *et al.,* 2001).

Four proteins which belong to **molecular chaperons** are Heat shock proteins (HSPs). It is well known fact that HSPs are immunostimulants predominantly inducing a Th1 type response. Chaperonin HSP-60 (Ohashi *et al.,* 2000), HSP-83 (De Andrade *et al.,* 1992; Tascon *et al.,* 1996) are immunogenic so they can act as VCs. However, there are so far no studies addressing a putative role of coronin and HSP-100 in the virulence of these pathogens.

Studies indicated that Alpha- and beta-tubulin (Probst *et al.,* 2001) belonging to **cytoskeletal organization** category were reported to be highly immunogenic (Li *et al.,* 2006) so they can also be an effective VCs. Nascimento *et al.,* (2006) reported the involvement of protein-tyrosine phosphatase - a dephosphorylating agent, in virulence of *Leishmania.* Maoc family dehydratase like protein play a vital role in fatty acid synthesis and may be an effective novel targets.

Elongation factor Tu (Dallo *et al.,* 2002) and Eukaryotic initiation factor 4A (Probst *et al.,* 1997), known as key molecules involved in **protein synthesis**, have been also reported as novel VCs. A number of identified proteins have been found to be associated with RNA processing *viz.*, RNA binding protein (Xavier *et al.,* 2000), RNA helicase (Cho *et*

al., 1998; Frick, 2003) and RNA polymerase I subunit (O'Neill *et al.,* 2000) were reported as DTs. These all are an attractive drug targets and could be panacea for leishmaniasis.

The most noteworthy and interesting proteins among the identified proteins in membrane enriched fraction of *L. donovani* are the presence of **transporter proteins** particularly ATP-binding cassette (ABC) proteins which are known to be involved in drug resistance in several parasitic protozoans BoseDasgupta *et al.,* 2008). The role of ABC transporters in drug targeting was confirmed by Castanys-Munoz *et al.*, (2008). ATP synthases, one of the transporter proteins which is widely distributed in bacteria and eukaryotes and are highly conserved multi-subunit complexes also might serve as a drug target (Maeda, 2008). Amino acid transporters are the signaling machineries and have been proposed as DT by Eiichi *et al.,* (2005). The roles of Amino acid permease- a small GTP-binding protein and Hydrophilic acylated surface protein B as any target have not been described previously but may be considered as significant molecules if studied extensively. Vaidya *et al.,* (2008) advocated Cytochrome c another transporter protein as a potent anti-malarial DT. There are so many studies that have proven Cytochrome c as an attractive and important target (Reed and Purohit, 2001).

In order to attract attention for the search of novel drug targets they must be shown to be essential for parasite survival. In this regard, one of the identified protein-glucose transporter in membrane enriched proteins of *L. donovani*, is known to be involved in the pathway for transport of glucose to the *P. falciparum* parasite and has attracted increasing interest as a target for antimalarial chemotherapy (Krishna *et al.*, 2002). Another protein identified in this study - glycosylphosphatidylinositol (GPI) anchors are reported to be indispensable for survival of *P. falciparum* therefore the GPI biosynthetic pathway has offered potential targets for novel anti-malarial drugs (Delorenzi *et al*, 2002). The hexose transporter, identified in this study, has been validated as a novel drug target in *P. falciparum* (Joet *et al.*, 2003). Both glucose and hexose transporters may be potent DTs in case of *Leishmania*. GPI mannosyltransferase have also been strongly advocated as a novel DT (Basagoudanavar *et al.*, 2007). Burchmore and Landfear (1998) showed the differential regulation of multiple glucose transporter genes in *L. mexicana*. LPG2 was reported as potent VC by many workers (Descoteaux *et al.*, 1995; Spath *et al.*, 2004).

No detailed informations are available about the biological functions of 60S ribosomal protein L23a, Cysteine-leucine rich protein, Mucin-associated surface protein

(MASP), Ubiquinol-cytochrome-c reductase-like protein, Variant surface glycoprotein and the hypothetical proteins. These may be representing as novel targets for drug development or putative vaccine candidates in future for Leishmaniasis.

In case of **soluble proteins** of *L. donovani*, eight among the identified ones, were found to be involved in **amino acid metabolism**. Some of these proteins like Adenosylhomocysteinase (Henderson *et al.,* 1992; Gupta *et al.*, 2007), Glutamate dehydrogenase (Werner *et al.,* 2005), Cofactor independent phosphoglycerate mutase (Zhang *et al.,* 2004), Trypanothione reductase (Drummelsmith *et al.,* 2003), Tyrosine phosphatase like protein (Hooft van Huijsduijnen *et al.*, 2002) have been reported as DTs in different organisms. According to TDR targets database (http://qa.tdrtargets.org/targets/view?gene_ id=26658), Aminomethyl transferase was proposed as a strong DT. There has been no documentation available on NADP-dependent alcohol hydrogenase and NAD-dependent deacetylase SIR2 homolog protein.

A number of identified proteins have been reported to be associated with **apoptotic pathway**. Activated protein kinase C receptor homolog (LACK) was reported as a VC (Melby *et al.,* 2001) in case of *L. donovani.* Kinesin (Ayan Dey *et al.*, 2008) has been reported as an important diagnostic marker of leishmaniasis. Function of DNA repair protein, Cell division control protein-2 -homolog-1, RNA polymerase I subunit have not been reported previously in case of *Leishmania.* ATP synthase has been documented to be involved in ATP synthesis/ hydrolysis. Zhang *et al.*, (2006) reported ATP synthase as VC in case of *L. viannia.* No significant remarks or data are available on Centrin and Calcium motif P-type ATPase (TBCA2) which belong to calcium binding protein.

Some other proteins that are detected in soluble proteins fraction from MALDI analysis involved in **carbohydrate metabolism** *viz.*, Fructose-1, 6-bisphosphate Aldolase, Enolase, Triosephosphate isomerase (TPI) and Phosphoglycerate kinase which may be considered as potential VCs, since they have been reported to be immunogenic in other organisms (Certa *et al.*, 1988; Sundstrom and Aliaga, 1992, 1994; Reynolds *et al.*, 1994; Hooker and Brindley, 1996; Mitsutake *et al.*, 1996; Gomez *et al.*, 1997; Piper *et al.*, 1999; McCarthy *et al.*, 2002; Pal-Bhowmick *et al.*, 2004; Saha *et al.*, 2006). Aldolase has been previously reported as a potential VC for the prevention of infection with the filarial nematode *Onchocerca volvulus* (McCarthy *et al.,* 2002). Extensive work has already been carried out with many glycolytic enzymes identified in this study, like aldolase, enolase,

triosephosphate isomerase of *Plasmodium falciparum,* as promising vaccine candidates, diagnostic tools and drug targets (Certa *et al.*, 1988; Gomez *et al.*, 1997; Piper *et al.*, 1999; Pal-Bhowmick *et al.*, 2004; Saha *et al.*, 2006). TPI was found to be a potential vaccine against *Schistosoma mansoni* by inducing IL-2 and IFN-γ production (Th1 responses) (Hooker and Brindley, 1996; Reynolds *et al.*, 1994; Sundstrom and Aliaga, 1994). Studies by Sundstrom (Sundstrom and Aliaga, 1994) have reported enolase as an immunodominant antigen in an experimental model of *Candida albicans* infection (Mitsutake *et al.*, 1996). These glycolytic enzymes may be considered as novel parasite proteins as none of these have been reported previously as T cell-stimulating antigens from *Leishmania donovani* and therefore, can be exploited as novel vaccine candidate against VL. Protein kinase (Parsons *et. al.,*1993), Pyruvate kinase (Gupta *et al.*, 2007), Dihydrolipoamide dehydrogenase (Krauth-Siegel and Schoneck, 1995), Phosphoenolpyruvate carboxy kinase (Mottram and Coombs, 1985), have been reported as potential DTs. Alcohol dehydrogenase like protein, aldolase epimerase related protein 2, Arginine kinase have not been explored as yet.

Interestingly, among the identified proteins in SPs the presence of **chaperons** was most noteworthy as was observed in membrane portion. Emerging evidence indicates that Heat shock proteins (HSPs) such as HSP-70, HSP-83, HSP-90 are the set of highly evolutionary conserved proteins. High-molecular-weight HSP participates in a large number of biochemical and immunological pathways. They behave as chaperones (Hartl, 1996; Mayer and Bukau, 1998), as immunodominant antigens (Srivastava *et al.*, 1998) and are also implicated in the antigen-processing pathway (Schirmbeck *et al.*, 1997; Panjwani *et al.*, 1999) . HSPs have been shown to act as immunostimulant of antigen-specific T cells predominantly of Th1 type characterized by induction of cytotoxic T lymphocyte activity and cytokine secretion e.g. chimeric HSP70 stimulated strong immunostimulatory response in mammals (Blachere *et al.,* 1997), *Mycobacterium tuberculosis* (Suzue *et al.,* 1997) and *L. infantum* (Rico *et al.,* 1998). *Leishmania* HSP 70, known to potentiate a Thl-type response and their importance as potent immunogens in leishmaniasis have also been recognized (de Andrade *et al.,* 1992). Moreover, they have also been implicated in immunoprotection (Tascon *et al.,* 1996). Among the other identified proteins elongation factors-2 and p45 have been reported to induce proliferative response in cured CL patients' PBMCs as well as leishmanial parasite-specific T cell lines derived from an immune donor (Probst *et al.,* 2001).

Another interesting stress shock protein identified in the present study is calreticulin, a chaperonin involved in glycoprotein folding. It is an important multifunctional immunodominant calcium (Ca^{+2})-binding protein (Joshi *et al.,* 1996). Calreticulin in *Leishmania* may affect the targeting of proteins that are associated with the virulence of the parasite (Debrabant *et al.,* 2002). Another protein responsible for virulence factor, identified in the present study, was Protein Disulfide Isomerase (LmPDI) an excreted/secreted antigen (Chenik *et al.,* 2005), which has been reported previously (Ben Achour *et al.,* 2002). Earlier work has shown that PDI plays a potential role in the pathogenicity of various microorganisms. It was believed that LmPDI represented a new potential component of novel immunogenic or vaccine preparations aimed at conferring immunity in humans or animals against *Leishmania* (Kebaier *et al.*, 2001). Kinesin-like protein K39 has been reported as an important diagnostic marker of leishmaniasis because kinesin is already a diagnostic marker for this disease. T-complex protein 1 gamma subunit and Chaperone protein DNAJ are undefined targets in *Leishmania* research till date. Both can be possible interesting target molecule for this disease.

Four identified proteins from the soluble fraction have also been reported to be involved in the **cytoskeletal organization**. Alfa- and beta-tubulin have been shown as T-cell stimulating antigens Probst *et al.*, (2001) and in several studies have also been reported as molecular marker (Gonzalez *et al.*, 2002; Mendoza-Leon *et al.*, 2002; Isaza *et al.*, 2008). Actin, first identified in non-muscle cells only about three decades ago and considered as a potential target for cancer drug development (Rao and Li, 2004), has also been reported as DT in *Leishmania* spp. (Kapoor *et al.,* 2008). Centromere/microtubule binding protein cbf5 may be a significant molecule but remain unexplored till date.

Hanada *et al.*, (2001) and Bente *et al.*, (2003) reported Proteinphosphatase-2C as an inportant DT in *Saccharomyces cerevisiae*. Mitochondrial DNA polymerase I protein A, is reported to play a role in **DNA replication** and has been proposed as a strong DT in cancer therapy (Dias and Bailly, 2005). Proliferative cell nuclear antigen (PCNA) is an auxiliary protein of DNA polymerase delta and is involved in the control of eukaryotic DNA replication by increasing the polymerase's processibility during elongation of the leading strand. PCNA are involved in mitogenic signal transduction and cellular proliferation pathway so its possible role in drug resistance may be considered (Maiti *et al.,* 2008). DEAD/DEAH box helicase (RNA helicase) was considered as a DT by Tuteja and Pradhan,

(2006) in *P. falciparum*. It would be really interesting to clone and characterize other members of the 'DEAD-box' family and understand their role in the replication and transmission of the parasite. These detailed studies may help to identify a parasite-specific enzyme, which could be a potential drug target to treat malaria. The various steps at which this probable drug can act are also discussed. RNA pseudouridylate synthase and Nucleoside diphosphate kinase is still unexplored molecule.

Histones are very important molecule that directly involve in the phenomena of chromatin remodeling. Histone post-translational modifications in tumorigenesis are increasingly recognized as important epigenetic changes controlling cancer. Phosphorylation, acetylation, ubiquitination, methylation and prenylation are the post-translational modifications (PTMs) of histones, which regulate DNA-histone binding and hence expression of target genes (Leifso *et al.,* 2007). Histone H2A, Histone H2B and Histone H4 (Soto *et al.,* 1999; Chenik *et al.,* 2006) have been identified in this study were reported as immunogenic protein in many studies. Histones are mainly involved in nucleosomal assembly and have been reported as vaccine candidate in VL and CL (Carrion *et al.,* 2007 and 2008).

Among all the identified proteins a large number of proteins were found to be involved in **protein synthesis process**. Eukaryotic initiation factor 5a was projected as a novel therapeutic target in BCR-ABL-positive leukemias identified by a proteomics approach Balabanov *et al.,* (2007). Elongation factor-2 have been reported to induce proliferative response in cured CL patients PBMCs as well as leishmanial parasite-specific T cell lines derived from an immune donor (Probst *et al.,* 2001). Elongation factor-2 is well known immunostimulatory or immunogenic type and has been evaluated as VCs. Eukaryotic translation initiation factor was proposed as exacerbatory antigen in vaccination study by Stober *et al.,* (2006) and is also reported in http://www.genedb.org as VC. The roles of 60S ribosomal protein L17, Isoleucin tRNA synthetase, 60S acidic ribosomal protein P2-1, Alanine transaminase/ amino transferase, Uridine kinase like protein, 40S ribosomal protein SA, Ribosomal protein L14 and Ribosomal protein S12 have not been described previously. They may represent novel targets for drug development or putative vaccine candidates.

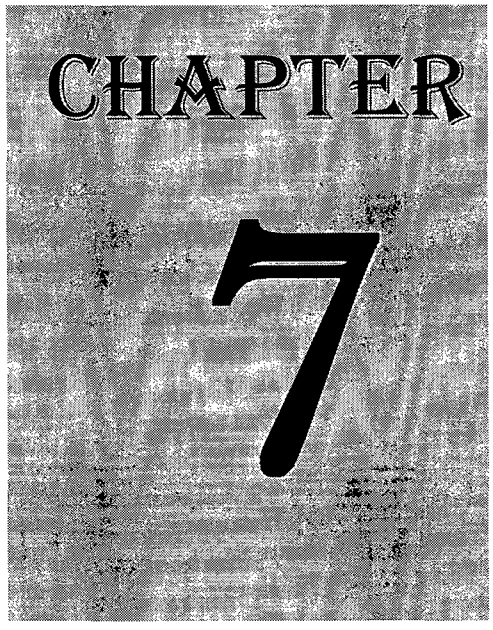
Five proteins identified in this study act as **proteolytic agents**. They are carboxypeptidase, Leishmanolysin precursor, Proteasome alpha 5 subunit, Ubiquitin

conjugating enzyme e2 and Ubiquitin. Carboxypeptidase has been reported as VC in *L. major* (Isaza *et al.*, 2008). Carboxypeptidases B of *Anopheles gambiae* had been found to be a blocking vaccine targets in *Plasmodium falciparum* (Lavazec *et al.,* 2007). Santos *et al.*, (2007) and TDR targets database advocated Leishmanolysin precursor as a DT. Proteasome, a multicatalytic proteinase complex is characterized by its ability to cleave peptides with Arg, Phe, Tyr, Leu, and Glu adjacent to the leaving group at neutral or slightly basic pH. The proteasome has an ATP-dependent proteolytic activity that may be the cause of drug resistance (Wang *et al.,* 2003). Ubiquitins and Ubiquitin conjugating enzyme e2 are involved in the selective degradation of short-lived and abnormal polypeptides by the ubiquitin-proteasome pathway. The occurrence of ubiquitin-proteasome pathway has previously been directly demonstrated by the partial purification of proteosome complexes from *L. mexicana* (Robertson, 1999), *T. brucei* (Huang *et al.,* 1999) and *T. cruzi* (de Diego *et al.,* 2001). Both proteins have not been studied on molecular level in *Leishmania*. Since they are involved in ubiquitin-proteasome pathway, they would be persuasive targets.

Small nuclear Ribonucleoprotein is implicated in **RNA processing** which is not described previously. Molecular characterization of DNA-directed RNA polymerase subunit and Retrotransposon hot spot protein has not been done in *Leishmania*. Both are involved in transcription process of the cell. Four identified proteins belong to **transport family**. p45 is a major immunostimulatory protein and have been reported as potent vaccine candidates by Gupta *et al.,* (2007). Cytochrome b domain protein (McFadden *et al.,* 2000) and ADP-ribosylation factor GTPase activating protein (Cho *et al.,* 2000) were evaluated as DTs. The recent identification of GTPase regulatory proteins in directing downstream signalling from small GTPases, and detailed structural information on the GTPases themselves suggests new possibilities for the development of effective and selective anti-inflammatory drugs (Benard *et al.,* 1999). The role of Cytochrome b domain protein in the mechanism of atovaquone-resistance in *Toxoplasma gondii* (McFadden *et al.,* 2000) have also been reported but no documentations are available on Possible RAN binding protein which may serve as a novel target if its role is properly corroborated. Most of the proteins identified were from 15 functionally related groupings, but total 29 hypothetical proteins identified in this proteomics study fall outside these categories and may be valuable for the diagnosis of leishmaniasis.

It was interesting to note that some identified proteins were **common in membrane and soluble protein fractions** of *L. donovani*. They were HSP 83, Alfa-tubulin, beta-tubulin, elongation factor and eukaryotic initiation factor. Since these proteins have been found to serve as DTs/VCs, they might be of major significance and importance from novel *Leishmania* target point of view.

Notably, the proteomic study presented here also documents a large number of completely **unknown or hypothetical proteins** of the parasite in membrane as well as soluble fractions of *L. donovani*, which may represent as potential drug targets and putative vaccine candidates after doing extensive study on hypothetical molecule. Although the amastigote form of the parasite is responsible for clinical manifestations, proteomic studies on the promastigote form are relevant because about 90% of the proteome remains qualitatively unchanged throughout the life cycle of the parasite (Cuervo *et al.,* 2007). Taken together, proteomics data presented here and similar studies like this will enable the identification of novel drug targets, virulence factors and vaccine antigens as identification of antigenic or immunogenic proteins for vaccine development is a key goal of immunovaccinology for use in leishmaniasis disease control as well as in the understanding of drug resistance in *Leishmania* parasites. Additional molecular biological studies such as cloning and expression of the best antigenic/ drug targets, are needed to characterize these new proteins identified through proteomics study. This report represents the first comprehensive analysis of the *L. donovani* proteome and the first extensive proteomic examination of late log phase of the parasite. It demonstrates the feasibility of the 2-DGE/MS approach for protein identification even in the absence of the *L. donovani* genome sequence.



Differential expression proteomics study of Leishmania donovani

"The capacity to blunder slightly is the real marvel of DNA. Without this special attribute, we would still be anaerobic bacteria and there would be no music".

-Lewis Thomas -

7. Identification of differentially expressed membrane and soluble proteins between sodium antimony gluconate (SAG) sensitive and SAG resistant *L. donovani* isolates by 2-DGE and MALDI-TOF/TOF

7.1. Introduction

Mechanism by which *Leishmania* acquires resistance to antimonials has been leading to contradictory results for several decades. The high level resistance to antimony observed in *Leishmania* can be due to simultaneous selection of loss in metal reduction, decreased drug uptake, increased glutathione and trypanothione synthesis, and increased transport (sequestration or efflux) of thiol-metal conjugates (Croft *et al.*, 2006c). This does not exclude that the parasite could have other mechanisms that confer metal resistance. In contrast to *in vitro* selected strains, resistance to Sb(V) in *Leishmania* field isolates is not well understood. Since, the available treatment for leishmaniasis poses many problems, research need to be focused on how antimonial drugs work and why they sometimes fail which would be instructive in the development of new therapies. In order to elucidate the basis for drug resistance, a comparative analysis of differentially expressed membranous and soluble proteins was done in SAG sensitive and resistant *L. donovani* strains isolated from VL patients using 2-DGE and MALDI-TOF/TOF.

In the last chapter it was observed that 72 and 132 proteins have been identified in membrane as well as soluble portion of promastigote respectively belonging to SAG sensitive clinical isolate (2001) of *L. donovani*. This has provided a basis to compare the protein profile which is differentially expressed in SAG resistant isolate. In the present study, a comparative proteome profile of soluble and membranous proteins of SAG-resistant (2039) and -sensitive (2001) Indian *L. donovani* field isolates was carried out to characterize the proteins which are differentially expressing in these clinical isolates representing their probable role in drug resistance. This comparative and differential proteomic expression studies shall provide a lead in identifying the molecular determinants of *Leishmania* parasite drug resistance and virulence, as well as discovering new drug targets for this disease.

7.2. Materials and Methods

7.2.1. Animal maintenance

Laboratory bred male golden hamsters (*Mesocricetus auratus*, 45-50 gm) were employed for experimental work and maintained as per description given in chapter 4.

7.2.2. Leishmania parasites isolation

The method of isolation and cultivation of a recent clinical isolate (2001) of *L. donovani* has been described in chapter 4.

7.2.3. Preparation of culture media for Leishmania culture

For cultivation of *Leishmania* parasites, various culture media i.e. cRPMI-1640 and cM199 were prepared as described in chapter 4.

7.2.4. Leishmania parasites culture

L. donovani SAG sensitive isolate (2001) as well as resistant isolate (2039) were cultured as described previously in chapter 4.

7.2.5. Chemicals

The requisite chemicals for the experiment have been described in chapter 6.

7.2.6. Isolation of membrane enriched proteins (MEPs) of L. donovani

Isolation of membrane enriched proteins of both isolates (2001, 2039) was done according to chapter 6.

7.2.7. Isolation of Soluble Protein (SPs) of L. donovani

Isolation of SP of both isolates (2001, 2039) was done as described in chapter 6.

7.2.8. Two dimensional gel electrophoresis (2-DGE) and Mass spectrometry

Protein sample prepration and estimation, IEF, 2-D gel electrophoresis, gel staining and destaining, gel imaging, images analysis [AlphaImager HP (Alpha Innotech, CA, USA)], gel spot excision, in-gel digestion of proteins and MALDI-TOF/TOF were carried out as per methods described previously in chapter 6.

7.3. Results

7.3.1. Identification of differentially expressed proteins in SAG sensitive as well as resistant isolates of L. donovani

7.3.1.1 Membrane enriched proteins (MEP)

Membrane enriched proteins from the SAG sensitive and resistant isolates of *L. donovani* was separated by high-resolution 2-DE as shown in Figure 7.1. The gels prepared with preparative protein loadings (1.5 mg) displayed good resolution with only minimal streaking. The reproducibility of the 2-D patterns was confirmed and considered final when three consecutive runs produced identical patterns. Gel spots were considered to represent differentially expressed proteins if they were upregulated by $\geq$ 1.5-fold in three independent experiments. There were a total of 12 well-resolved reproducibly overexpressed protein spots that were differentially detected in the membranous proteins (Figure 7.1) of SAG resistant isolate of CBB stained gels with pI ranging from 3 to 10.

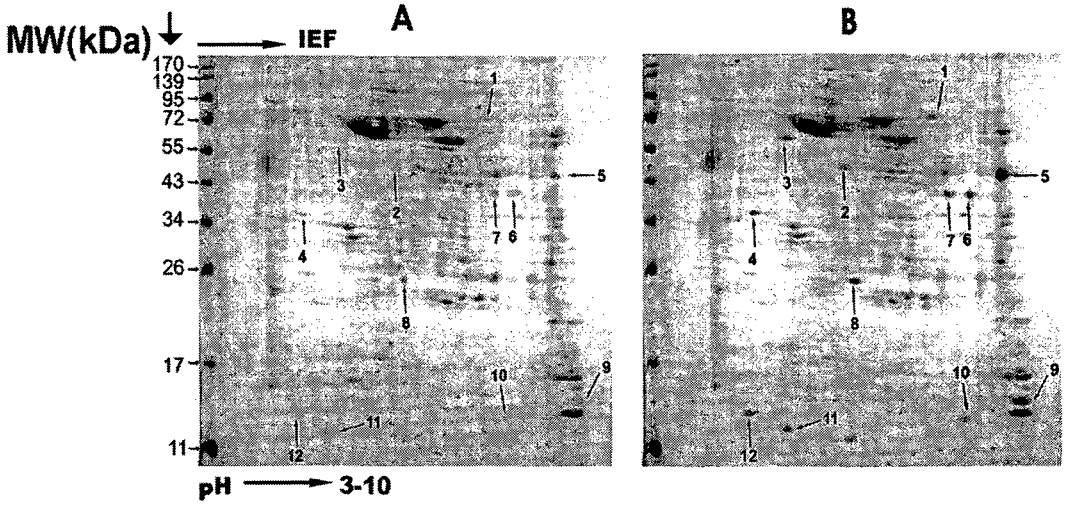


Figure 7.1: Two-dimensional gel electrophoresis separation of membrane enriched protein extract from SAG sensitive isolate 2001 (A) and SAG resistant isolate 2039 (B) of *L. donovani*. The numbered spots represent up and down-regulated proteins between these two isolates identified by peptide mass fingerprinting (Table 7.1).

Mass spectrometry analysis demonstrated that 6 out of 12 protein spots were identified as differentially over expressed in membrane enriched protein fraction of SAG resistant parasite and they are listed in detail in Table 7.1. Minor protein identification failures could be due to sample amount, specific peptide characteristics and extensive post-translational modification or significant divergence from sequenced strains (McNicoll *et al.*, 2006).

Table 7.1: Differentially overexpressed membrane enriched proteins of SAG resistant *L. donovani* isolate (2039) identified through two-dimensional gel electrophoresis and MALDI-TOF/TOF mass spectrometery.

Spot no.[a]	Protein Identified[b]	Species[c]	Accession no.[d]	Mol. Mass[e] (Pred.)	pI[f] (Pred.)	Pm/ % of Sc/ Ms[g]	Fold change[h]	Function[i]	Subcellular Localization[j]	Class/Family[K]
1	Cysteine-leucine rich protein	Li	gi|146098686	65.8	5.8	11/9/149	1.86	UN	MP	Unknown
2	ABC1 transporter	Lmj	gi|157865765	20.0	5.2	9/12/111	2.97	TP	MP	ABC transporter
3	ABC transporter	Lmj	gi|157865529	32.9	5.3	9/15/99	3.17	TP	MP	ABC transporter
5	Heat shock protein 83 (Fragment)	Ld	HSP83_LEIDO	52.6	5.4	15/19//125	5.30	CH	MP	Hydrolase
6	GPI protein transamidase	Lmx	Q9U5N7_LEIME	38.8	5.5	8/11/101	3.71	PL	MP	Cystein type Endopeptidase
10	60S ribosomal protein L23a(L25)	Tb	RL23A_TRYBB	18.1	10.7	5/10/87	2.03	UN	RS	Unknown

The differentially expressed protein spots shown in Figure 7.1 were identified using Peptide Mass Fingerprint

a) Protein spots no. indicated in Figure 7.1.
b) Name of identified protein.
c) Species: Ld: *Leishmania donovani*; Li: *Leishmania Infantum*; Lmj: *Leishmania major*; Tb: *Trypanosoma brucei.*
d) Accession numbers of protein according to NCBI and Swiss-Prot accession number.
e) Predicted Molecular mass in kDa.
f) Predicted pI.
g) No. of peptides matched (Pm)/ Percentage of sequence coverage (Sc) /MOWSE score (Ms).
h) Fold change of overepressed protein in SAG resistant isolates (2039).
i) Function of identified proteins; CH: Chaperon , PL: Proteolysis, TP: Transport, UN: Unknown.
j) Sub cellular localization; MP: Membrane protein, RS: Ribosomal surface.
K) Class/Family.

Note: Protein spots analyzed but not identified: 4, 7, 8, 9, 11, 12.

Among these, major proteins such as HSP 83, and ABC transporter were reported to

Table 7.2: Proteins identified by 2-D gel electrophoresis and MALDI-TOF/TOF mass spectrometry as being differentially overexpressed in soluble protein content of

Mass spectrometry analysis identified 14 spots out of 18 in SP fraction as listed in detail in Table 7.2. Minor protein identification failures could be due to sample amount, specific peptide characteristics and extensive post-translational modification or significant divergence from sequenced strains (McNicoll *et al.,* 2006). Due to the extremely small number of *L. donovani* protein sequences in databases, reference was made to the more comprehensive *on other species of Leishmania* using MASCOT. Among these, major proteins such as Proliferative cell nuclear antigen, Proteasome alpha 5 subunit, Carboxypeptidase, HSP 70, Tubulin-beta chain were reported to be involved in drug resistance mechanism. Some of the other proteins including some enzymes from carbohydrate metabolism, proteolysis have also been reported as potential drug targets *viz.*, fructose-1, 6-bisphosphatealdolase, enolase. In addition, it has also been observed that the mass and charge of several proteins were different from those predicted by the leishmanial genome, which is reported to be a common feature of most proteomic analyses, probably reflecting the effect of protein maturation events including co- or post-translational modification (McNicoll *et al.,* 2006; Sinha *et al.,* 2002). The identified proteins were also classified on the basis of their function, sub cellular localization, class/family (Table 7.2).

6.4. Discussion

Current armament against *Leishmania* infection relies on few chemotherapeutic drugs including Sb(V), amphotericin B, miltefosine and pentamidine. The use of the standard pentavalent antimonial Sb(V) drugs for VL, such as sodium stibogluconate, is threatened by the development of drug resistance which has reached epidemic proportions in some parts of India. Variation in the efficacy of drugs in the treatment of leishmaniasis is frequently due to the differences in drug sensitivity of *Leishmania* species, the immune status of the patient, or the pharmacokinetic properties of the drug. Most of the forms of leishmaniasis are zoonotic, where acquired drug resistance is not an important consideration. In areas with anthroponotic visceral leishmaniasis, especially India, acquired resistance to pentavalent antimonials has occurred and effective monitoring of drug resistance is needed. No molecular markers of resistance are available for currently used antileishmanial drugs (Croft *et al.,* 2006; Croft *et al.*, 2006c). Antimonial resistance mechanisms are starting to be understood in lab isolates but are less clear in clinical isolates. The expression levels of several genes known to be altered *in vitro* resistant isolates were unchanged in the selected pair of sensitive and resistant clinical isolates suggesting that the

resistance mechanisms in these field parasites may differ from those of lab-resistant mutants

organisms that range from bacteria to man. They include hormone receptors, tyrosine kinase

immunostimulants of antigen-specific T cells (Suto *et al.,* 1995) and are predominantly Th1

(Sundstrom *et al.*, 1992; Mitsutake *et al.*, 1994; Sundstrom *et al.*, 1994; Mitsutake *et al.*, 1996) and Fructose-1, 6-bisphosphate aldolase (Gomez *et al.*, 1997; Certa *et al.*, 1988; McCarthy *et al.*, 2002) are involved in carbohydrate metabolism and have also been reported as potential drug targets & vaccine candidate. Both proteins were identified in multiple copies and upregulated by more than 2 fold in SAG resistant isolate. Due to the over expression of these proteins, metabolic process of parasite get enhanced and parasite became resistant towards drugs. Proliferative cell nuclear antigen is an auxiliary protein of DNA polymerase delta and is involved in the control of eukaryotic DNA replication by increasing the polymerase's processibility during elongation of the leading strand. Its two fold increased expression indicated the shifting of parasite towards resistant condition. The proteasome is a multicatalytic proteinase complex which is characterized by its ability to cleave peptides with Arg, Phe, Tyr, Leu, and Glu adjacent to the leaving group at neutral or slightly basic pH. The proteasome has an ATP-dependent proteolytic activity that may be the cause of drug resistance (Wang *et al.*, 2003). The differential expression proteomics study on clinical isolates has pinpointed involvement of various proteins that are differentially expressed in membranous as well as soluble proteins of *L. donovani.* This study, supported by the fact that most of the proteins which are over-expressed are found to be involved in drug resistant mechanism in other parasites, indicate that they may play a major role in resistance mechanisms allowing the emergence of new mutations or may constitute primary resistance mechanisms.

Till date, most of the studies carried out to identify proteins involved in resistant mechanism were confined to one or two proteins. This study has provided for the first time the cumulative proteomic analysis of most of the over expressed proteins in drug sensitive and resistant clinical isolates of *L. donovani* indicating their possible role in antimony resistance of the parasite and provide a vast field to be exploited to find much needed novel treatment strategies against visceral leishmaniasis such as cloning and over expression of these targets could be done to produce recombinant therapeutic / prophylactic proteins.

Summary

"I may not have gone where I intended to go, but I think I have ended up where I needed to be".

-Douglas Adams-

SUMMARY

Visceral leishmaniasis (VL) or Kala-azar is the most devastating type well-recognized major infectious disease among the complex of leishmaniasis (cutaneous, mucocutaneous, and visceral) and is caused by the hemoflagellate protozoan parasite, *L. donovani* through the invasion of the reticuloendothelial system (spleen, liver and bone marrow). Although VL is widely distributed throughout the tropics, it is rampant in the Indian subcontinent and Southwest Asia. Annually, about 500,000 cases of VL occur worldwide, of which 90% occurs in India, Sudan, Nepal, Bangladesh and Brazil. A recent WHO report shows that an estimated 350 million people are at risk of infection and approximately twelve millions are infected. In India, VL is endemic in North-East part especially in Bihar, West Bengal, Assam and certain pockets of eastern Uttar Pradesh. Annually, about 100,000 cases of VL are estimated to occur in India. Of these, the state of Bihar accounts for more than 90 percent of the cases. VL is characterized by intermittent fever, massive hepatosplenomegaly, anemia, thrombocytopenia, and polyclonal B-cell activation with hypergammaglobulinemia. The skin becomes dark gray, corresponding to the name of the disease kala-azar or black sickness. In most acute infections death may occur within a few weeks; in sub acute cases within a year and in chronic cases within 2 to 3 years. VL is often fatal if not treated properly. Patients, who recovered from VL, usually have life-long immunity to re-infection but occasionally relapses may occur. The incomplete treatment of the patient may also lead to a condition usually referred to as post kala-azar dermal leishmaniasis.

There has been little advancement in the area of drug development against leishmaniasis and the age old drugs which have benefited the patient for over 40 years are still the only hope of patients. In Bihar, about 37-70% of cases are currently reported to be non- responsive to SAG (Sundar *et al.,* 2000; Croft *et al.,* 2006). Further treatment failures reported with this drug worldwide have often been interpreted as SAG drug resistance. A second line drug, amphotericin B, has numerous side effects and costly. Pentamidine is associated with disturbance of glucose metabolism and other toxicity problems. Miltefosine is the first orally effective antileishmanial drug registered in 2002 for clinical use in India but can cause teratogenicity and not advised to women of child bearing age. So new drug targets are urgently required to combat the disease and in addition to the therapeutic

measures, suitable and effective vaccine candidate identification is the point of serious consideration. As evident above, the treatment modalities available against VL are not entirely satisfactory and hence necessitate the development of better, effective and cheap drugs. To date, there are no vaccines against human VL. However, there is consensus that in the longer term, vaccines ought to become a major tool in the control of this group of diseases. Unfortunately, the development of vaccines has been hampered by significant antigenic diversity and the fact that the parasites have a digenetic life cycle in at least two hosts (sand fly vector and human/animal reservoir). The evidence cited above indicates that VL did not get the attention that it deserved because, until the late 1940s the disease was a local problem and the world's attention was engaged in tackling the highly fatal and epidemic bacterial and viral infections.

Due to the alarming rise of drug resistance there is an urgent need to identify and characterized parasite targets for the development of novel drug/vaccine for effective therapies. The mechanism of resistance is now partly understood in laboratory bred isolates but it is still lagging behind in respect of field or clinical isolates. Recent studies with field isolates (Singh *et al.,* 2003; Decuypere *et al.,* 2005) suggest similarities and differences with laboratory resistant isolates but it is not yet clear whether any of the highlighted mechanisms is central to the resistance phenotype of field isolates. In this study a number of five clinical isolates were taken from the patients in *Leishmania* endemic areas *viz.*, Muzzafarpur, the epicenter of VL as well as Varanasi and were maintained in the laboratory. Of these three belong to the patients who did not respond to antimonial therapy i.e. 2039, 2041 and 2093. The rest of the two isolates- 2001 & 2087 were from those who responded to SAG therapy. These isolates alongwith the reference strain Dd8 were subjected to SAG sensitivity both *in vitro* and *in vivo*. It was observed the isolates 2039, 2041 and 2093 did not respond to SAG treatment in laboratory conditions also. In order to investigate whether this unresponsiveness to SAG is due to genomic variations a comparative genomic differentiation analysis of these strains was obligatory.

Therefore, in this study, the above stated clinical isolates were analyzed on the basis of their genetic diversity, molecular variance and phylogenetic structure using for the first time, a fingerprinting approach – amplified fragment length polymorphism (AFLP). This high throughput molecular technique analysis is able to detect genetic variation and polymorphisms without prior genome sequence knowledge using PCR. Altogether 2338

informative AFLP bands were generated using 10 selective primer combinations. Percentage of polymorphism was 55.35%. A number of unique AFLP markers (217) were also identified in these strains. It was deduced that a higher rate of variations occurred among *Leishmania* clinical isolates which indicate the shifting of drug sensitive nature of parasite towards resistant condition. This approach of genomics is undertaken in order to develop DNA marker of SAG sensitive and resistant strains of *L. donovani.* These results ascertain that 2039 strain of *Leishmania* is totally out-group indicating that this strain is highly polymorphic and develop resistance against SAG. The variability observed among SAG sensitive and resistant populations based on AFLP analysis suggests that the SAG resistant strain may be a composite group. It might be inferred that due to this genomic differentiation they acquire drug resistance. Further, it was evident from the component plots that the SAG resistant accessions 2039 are remarkably distinct from other SAG resistant accessions as it falls at the extremes of the plot.

Thus, the AFLP technique was effective in showing inter-specific and intra-specific molecular variability between clinical isolates and demands more studies at proteomic level in order to investigate the degree of changes in protein profile of SAG sensitive and resistant strains. For this, a comprehensive and differential proteomics studies need to be done by generating a proteome map of *Leishmania donovani* and then eventually compare the differentially expressed proteins identified using 2-D gel electrophoresis and mass spectrometry (MALDI-TOF) based approaches. Modern genomics-based approaches such as proteomics offer increased hope for the understanding of virulence/pathogenicity/host–pathogen interactions, drug resistance thereby defining novel therapeutic/vaccine targets by virtue of their ability to characterize complex parasite biology and biochemistry. Therefore, the correspondence study between the genome and proteome are more effective for target identification and validation in parasite. Proteomics, being widely employed in the study of *Leishmania* seems to be a suitable strategy as the availability of annotated sequenced genome of *Leishmania major* has opened the door for dissection of both protein expression/regulation and function.

Using proteomics approach, preliminary efforts in this direction include the generation of a partial 2-D gel map of *L. donovani* and analysis of differentially expressed proteins through MALDI-TOF was pinpointed. Proteomic methods has been applied to study the sub-cellular localization, function, changes in protein expression patterns in dug

resistant and sensitive condition i.e. differential proteomic expression studies. Keeping these things in mind proteomic analysis of membrane as well as soluble proteins has been done in

Based on proteome profile of membrane and soluble proteins of SAG sensitive isolate 2001 a differential proteomic expression study with totally outgroup, highly polymorphic SAG resistant strain *viz.*, 2039 was carried out which provided lead towards identification of at least those proteins which are involved in drug resistance mechanism.There were six out of 12 protein spots which were identified as differential proteins in the SAG resistant membrane enriched protein fraction whereas 14 out of 18 from soluble fractions were identified. Major proteins were *ATP-binding cassette (ABC) transporters, GPI protein transamidase, proteasome and proliferative cell nuclear antigen, Tubulin-beta chain* which are usually involved in drug resistance in several parasitic protozoans and can be the effective DTs for Leishmaniasis. HSPs may be termed as strong VCs for this disease. Enolase and Fructose-1, 6-bisphosphate aldolase is involved in carbohydrate metabolism and has also been reported as potent drug targets & vaccine candidate both. Some of the differentially expressed proteins, the role of which were not known to be invoved in drug resistance are Cysteine-leucine rich protein and 60S ribosomal protein L23a (L25) unique molecules, overexpressed in membrane enriched protein fraction of SAG resistant isolate of *Leishmania*. Notably, the proteomic study presented here also documents completely unknown or hypothetical proteins of the parasite, which may represent potential targets for putative vaccine candidates. These studies has provided first time the cumulative proteomic analysis proteins in drug sensitive and resistant clinical isolates of *L. donovani* indicating their possible role in antimony resistance of the parasite and provide a vast field to be exploited to find much needed novel treatment strategies against visceral leishmaniasis.

In a nut shell, the combined genomic and proteomic approaches, spanning the bridge between clinical and parasitological profile of VL, elucidates the possible mechanism of drug resistance and in the process identify a number of markers and novel drug/vaccine targets with the hope to contribute new therapeutics in *Leishmania* research. Future work such as cloning and over expression of these targets could be done to produce recombinant therapeutic/ prophylactic tool for diagnostic purpose of VL. The overall result of this study demonstrate how a multidisciplinary approach considering a clinical, biological and molecular aspects of *Leishmania* parasite population can provide valuable lesson for the design of future novel drugs/vaccines for VL control and management.

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"The process of scientific discovery is, in effect, a continual flight from wonder."

- Albert Einstein -

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Appendices

"Discovery consists of looking at the same thing as everyone else and thinking something different"

- Albert Szent-Gyorgyi -

Composition of Buffers, Reagents and Solutions

1. Acrylamide stock:

29 g of acrylamide and 1 g bis-acrylamide were dissolved in 60 ml triple distilled water (TDW) and was made up to 100 ml. The stock solution was stored at 4^0C.

2. 1.5 M Tris-HCl (pH 8.8):

18.15 g of Tris was dissolved in 85 ml TDW. The pH was adjusted to 8.8 with 1 N HCl. The solution was made up to 100 ml and stored at 4^oC.

3. 0.5 M Tris-HCl (pH 6.8):

6.0 g of Tris was dissolved in 70 ml TDW. The pH was adjusted to 6.8 with 1N HCl. The solution was made up to 100 ml and stored at 4^oC.

4. 10% SDS:

10 g of SDS was dissolve in 100 ml TDW and stored at room temperature.

5. 10% Ammonium per Sulphate (APS):

2 g of APS was dissolved in 20 ml TDW, aliquoted and stored at 4^oC.

6. Tris-glycine electrophoresis buffer (pH8.3): store at room temperature

Tris (25 mM)	*3.0 g*
Glycine (250 mM)	*18.4 g*
SDS (0.1%)	*1.0 g*
TDW (Final Volume)	*1000 ml*

7. 2X SDS-PAGE Sample buffer: store at 4-8 ^0C

Tris-HCl (100 mM)	*1.21 g*
DTT (200 mM)	*3.09 g*
SDS (4%)	*4.0 g*
Bromophenol blue (0.2%)	*0.2 g*
Glycerol (20%)	*20 ml*
TDW (Final Volume)	*100 ml*

8. Coomassie Blue Stain:

0.25 g of Coomassie Brillient Blue (R-250) was dissolved in 90 ml of methanol and TDW (1:1). Finally 10 ml of glacial acetic acid was added followed by filtration.

9. Destaining solution:

90 ml of 1:1 (v/v) Methanol: TDW and 10 ml of acetic acid was dissolved.

10. Phosphate buffer saline (PBS, pH 7.2):

NaCl	-	8.00 gm
KCl	-	0.20 gm
Na_2HPO_4	-	1.15 gm
KH_2PO_4	-	0.20 gm

The above ingredients were dissolved in 1000 ml distilled water.

11. Giemsa buffer:

Sol A-	
Na_2HPO_4	*0.95 g*
TDW	*100 ml*
Sol B-	
KH_2PO_4	*0.907 g*
TDW	*100 ml*
Working solution	*Sol A: 7.2 ml*
	Sol B: 2.8 ml and TDW: 90 ml

12. NET buffer for chromosomal DNA isolation:

NaCl (0.1 M)	*0.58 g*
Tris (10 mM)	*0.121 g*
EDTA (1.0 mM)	*20 µl of 0.5 M solution*

13. RBC lysis buffer:

Sol A-	
Tris base	*2.06 g*
Water	*100 ml*
Sol B-	
NH_4Cl	*8.3 g*
Water	*100 ml*
Working solution	*Sol A: 10 ml*
	Sol B: 90 ml

14. Reagents for protein estimation by modified Lowry method:

Reagent-A: *Solution of 2% sodium carbonate (Na_2CO_3) in 0.1N NAOH.*
Reagent-B: *0.5% copper sulphate ($CuSO_4$) in 1% sodium potassium tartarate ($NaKC_4O_6$-Freshly prepared).*
Reagent-C: *Alkaline $CuSO_4$ solution. Mix 50 ml A + 1 ml of B just before use.*
Reagent-D: *1 N Folin-ciocalteau (F.C.) reagent and stored in dark bottle.*
Standard protein: *BSA (Bovine Serum Albumin), 1mg/ml (Stock).*

15. TAE (50X) buffer:

Tris base	*121.0 g*
Glacial Acetic Acid	*28.6 ml*
0.5 M EDTA (pH 8.0)	*50 ml*
TDW (Final Volume)	*500 ml*

16. Solutions for silver staining:

Solution A	***Solution B***	***Solution C***
50% Acetone- 60ml	*30% $AgNO_3$ -0.8ml*	*Na_2CO_3 –1.2gm, TDW-6ml*
50% TCA-1.5ml	*37% HCHO-0.5ml*	*37% HCHO- 25µl*
37% HCHO-25µl	*TDW- 60ml*	*$Na_2S_2O_3.5H_2O$- 25µl*

Resarch Publications

"Research is to see what everybody else had seen

and to think what no body else had thought".

RESEARCH PUBLICATIONS

RESEARCH PAPERS PUBLISHED IN PEER REVIEWED JOURNALS

1. **Awanish Kumar,** Brijesh Sisodia, Pragya Misra, Ajit Kumar Shasany and Anuradha Dube (2009). "Comprehensive proteomic analysis of membrane enriched proteins fraction of *Leishmania donovani* promastigotes". (Communicated in **Proteomics**).
2. **Awanish Kumar**, Vijay Raju Boggula, Shyam Sundar, Ajit Kumar Shasany and Anuradha Dube (2009). "Identification of genetic markers in Sodium Antimony Gluconate (SAG) sensitive and resistant Indian clinical isolates of Leishmania donovani through amplified fragment length polymorphism (AFLP)". **Acta Tropica;** 110: 80-85.
3. Pragya Misra, **Awanish Kumar,** Swati Gupta, Prashant Khare, Nikhil Kumar and Anuradha Dube (2009). "Antileishmanial activity of *Piper betle Linn.* Landrace Bangla Mahoba mediated by apoptosis". **J. of Medical Microbiology**: (Accepted).
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2. **Awanish Kumar** (2006). "**General Studies:** Section- 1(General Science)", Page No. 1-210. Editor: Tomar, R.K.S., **Vishwas Publication,** Ahemdabad, Gujrat, India.
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Short communication

Identification of genetic markers in Sodium Antimony Gluconate (SAG) sensitive and resistant Indian clinical isolates of *Leishmania donovani* through amplified fragment length polymorphism (AFLP)

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ABSTRACT

Sodium Antimony Gluconate (SAG) is currently used worldwide as the first-line drugs for the treatment of visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) since 1940s. Unfortunately, the resistance of *Leishmania* parasite to this drug is increasing in several parts of the world. The mechanism of drug resistance in clinical isolates is still not very clear. Earlier, we have established a differentiation between six clinical isolates as sensitive and resistant on the basis of their sensitivity to SAG *in vitro* and *in vivo* as well as expression of proteophosphoglycan contents. In this preliminary study, we have further analyzed these isolates on the basis of their genetic diversity, molecular variance and phylogenetic structure using for the first time, a fingerprinting approach – amplified fragment length polymorphism (AFLP). Altogether 2338 informative AFLP bands were generated using 10 selective primer combinations. Percentage of polymorphism was 55.35%. A number of unique AFLP markers (217) were also identified in these strains. It was deduced that a higher rate of variations occurred among *Leishmania* clinical isolates which indicate the shifting of drug sensitive nature of parasite towards resistant condition.

1. Introduction

Leishmania donovani, a causative agent of visceral leishmaniasis (VL) or kala-azar poses a serious health threat on human populations and endemic areas in large parts of the world (Murray et al., 2005). The disease is usually fatal if not treated properly. The life cycle of *Leishmania* is digenetic with vertebrates as definitive host and *Phlebotamine* sand fly as intermediate one. Due to the lack of an effective vaccine, the control of VL relies mostly on chemotherapy. The pentavalent antimonials such as Sodium Antimony Gluconate (SAG) are the main drugs used for the treatment of the infection of *L. donovani* but unfortunately, the incidence of parasite becoming resistant to these drugs is increasing in several parts of the world including South America (Rojas et al., 2006), Europe (Carrio et al., 2001), Middle East (Hadighi et al., 2006) and most notably in India (Sundar et al., 2000). In India it is most prevalent in Bihar, West Bengal, Orissa, Assam, and Eastern Uttar Pradesh. The mechanism of resistance is now partly understood in laboratory bred isolates but it is still lagging behind in respect of field or clinical isolates. Recent studies with field isolates (Decuypere et al., 2005; Singh et al., 2003) suggest similarities and differences with laboratory resistant isolates but it is not yet clear whether any of the highlighted mechanisms is central to the resistance phenotype of field isolates.

The previous studies in our laboratory have established the sensitivity of recently isolated Indian clinical isolates of *L. donovani* to SAG the first-line antileishmanial drug. On the basis of their responses to SAG both *in vitro* and *in vivo* three resistant and three sensitive isolates were identified (Dube et al., 2005). Further characterization also revealed that a mucin like glycoconjugate proteophosphoglycan (PPG) is differentially expressed in these isolates (Samant et al., 2007) suggesting the possibilities of involvement of PPG in drug-resistant mechanisms and of using PPG abundance as a marker for identifying drug-resistant clinical isolates in Indian kala-azar. In this study we have further tried to explore these SAG sensitive and resistant clinical isolates at genomic level. A comparative analysis of genomic study of SAG sensitive and resistant *L. donovani* strains isolated from kala-azar patients was done using amplified fragment length polymorphism (AFLP) which is a DNA fingerprinting technique, for the study of genetic polymorphism across the whole genome. Since, prior sequence knowledge is not necessary to conduct AFLP experiments; hence, this method is particularly applicable to organisms for which no substantia-

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A pro-apoptotic effect of Landrace of *Piper betle*- Bangla Mahoma on *Leishmania donovani* may be due to high content of eugenol

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Running Title: *P. betle* induces apoptosis in *Leishmania*

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Abstract

In the absence of effective and safe treatment of Kala-azar (VL) - a most devastating parasitic disease caused by *Leishmania donovani* a need for the search of antileishmanial from natural resources, in common use, is imperative. Recently, a comparative *in vitro* antileishmanial activity of methanolic extract of two landraces of *Piper betle*- Bangla Mahoba (PB-BMM) and Kapoori Vellaikodi (PB-KVM) has been reported. Herein, the putative pathway responsible for death induced by extract of effective landrace PB-BMM in promastigotes as well as intracellular amastigotes form of *L. donovani* was assessed using various biochemical approaches. It was observed that PB-BM is capable of selectively inhibiting both the stages of *Leishmania* parasites by accelerating apoptotic events by generation of ROS targeting mitochondrion without any cytotoxicity to macrophages. Study was extended to reason out the presence/absence of activity in PB-BMM and PB-KVM on the basis of differences in essential oil composition present in methanolic extract assessed by gas chromatography and mass spectra. The oil in PB-BME was found to be rich in eugenol as compared to PB-KVE. The antileishmanial efficacy of PB-BMM mediated through apoptosis is probably due to the higher content of eugenol in active landrace. This observation emphasizes the need of extending the studies related to traditional medicines from bioactive plants below species to gender/landrace level for better efficacy and reproducibility.

Keywords: *Piper betle*, landrace, *Leishmania donovani*, antileishmanial, apoptosis

Discovery of Novel Vaccine Candidates and Drug Targets Against Visceral Leishmaniasis Using Proteomics and Transcriptomics

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Abstract: Among the three clinical forms (cutaneous, mucosal and visceral) of leishmaniasis visceral (VL) one is the most devastating type caused by the invasion of the reticuloendothelial system of human by *Leishmania donovani, L. infantum* and *L. chagasi.* India and Sudan account for about half the world's burden of VL. Current control strategy is based on chemotherapy, which is difficult to administer, expensive and becoming ineffective due to the emergence of drug resistance. An understanding of resistance mechanism(s) operating in clinical isolates might provide additional leads for the development of new drugs. Further, due to the lack of fully effective treatment the search for novel immune targets is also needed. So far, no vaccine exists for VL despite indications of naturally developing immunity. Therefore, an urgent need for new and effective leishmanicidal agents and for this identification of novel drug and vaccine targets is imperative.

The availability of the complete genome sequence of *Leishmania* has revolutionised many areas of leishmanial research and facilitated functional genomic studies as well as provided a wide range of novel targets for drug designing. Most notably, proteomics and transcriptomics have become important tools in gaining increased understanding of the biology of *Leishmania* to be explored on a global scale, thus accelerating the pace of discovery of vaccine/drug targets. In addition, these approaches provide the information regarding genes and proteins that are expressed and under which conditions.

This review provides a comprehensive view about those proteins/genes identified using proteomics and transcriptomic tools for the development of vaccine/drug against VL.

Key Words: Vaccine/Drug targets, Visceral Leishmaniasis, Proteomic and Transcriptomic tools.

INTRODUCTION

Leishmaniasis is endemic in 88 countries with approximately 12 million infected and 350 million people at risk (http://www.who.int/en/). The disease is caused by parasites of the genus *Leishmania*, a group of kinetoplastid protozoans that are transmitted by sandflies as flagellated promastigotes. With the bite of the female vector the parasites are injected into the host to enter and multiply in the phagolysosomes of macrophages as amastigotes [1, 2]. Dependent on the *Leishmania* species and the immune response of the host, there are three basic clinical manifestations of the disease: cutaneous, mucocutaneous and visceral leishmaniasis (VL) [3, 4]. VL is the most severe and often fatal syndrome. *L. donovani, L. infantum* and *L. chagasi* are the major species responsible for VL. *L. infantum* and *L. chagasi* are of veterinary importance, as they are not only the cause of zoonotic VL in man but also of a severe chronic wasting disease in dogs: canine VL [5]. The disease, restricted to areas which are heavily infested by the sandfly (*Phlebotomus spp.*), particularly in Indian subcontinent, Africa, South America and Mediterranean regions [6].

VL in India, known as Kala azar, is caused by *L. donovani* has resulted in >100,000 deaths in the recent epidemics of Sudan and India. With the advent of the HIV epidemics VL has emerged as an important opportunistic infection in AIDS patients. In India, high incidence has been reported from the states of Bihar, Assam, West Bengal and Eastern Uttar Pradesh where resistance and relapse are on the increase. A recent survey in Bihar has recorded an alarming 1,000,000 cases with 10,000 unresponsive to antimonials [7]. It is characterized by irregular bouts of fever, substantial weight loss, hepatosplenomegaly and anemia [8]. Drugs are the major weapons against leishmaniasis and there is some hope that drug treatment of infected individuals could lead to eradication for *L. donovani*, which has no reservoir host. Like many other parasitic diseases VL is a disease of poverty and to worsen matters, in the endemic areas treatment is increasingly failing due to resistance of the parasites to the most common anti-leishmanial drug, Pentavalent Antimony (Sb^V) which has been used, often with poor supervision and compliance. A recent survey in Bihar, an epicenter of VL has recorded an alarming 100,000 cases with 10,000 unresponsive to antimonials [7, 9]. Newer drugs such as liposomal Amphotericin B and Miltefosine are prohibitively expensive for the most affected populations. Besides they are toxic, have serious side effects and are associated with numerous relapses. In all cases, the evolution of resistance on the part of a parasite determined to survive is predictable and new drug discovery must stay a step ahead of increasing ineffectiveness of those in current use. Consequently, discovery of new genes essential for parasite survival that can be targeted by new drugs for treatment of these diseases is imperative.

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REVIEW

Proteomic approaches for discovery of new targets for vaccine and therapeutics against visceral leishmaniasis

Shraddha Kumari[1], Awanish Kumar[1], Mukesh Samant[1], Shyam Sundar[2], Neeloo Singh[3] and Anuradha Dube[1]

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Visceral leishmaniasis (VL) is the most devastating type caused by *Leishmania donovani*, *Leishmania infantum*, and *Leishmania chagasi*. The therapeutic mainstay is still based on the antiquated pentavalent antimonial against which resistance is now increasing. Unfortunately, due to the digenetic life cycle of parasite, there is significant antigenic diversity. There is an urgent need to develop novel drug/vaccine targets against VL for which the primary goal should be to identify and characterize the structural and functional proteins. Proteomics, being widely employed in the study of *Leishmania* seems to be a suitable strategy as the availability of annotated sequenced genome of *Leishmania major* has opened the door for dissection of both protein expression/regulation and function. Advances in clinical proteomic technologies have enable to enhance our mechanistic understanding of virulence/pathogenicity/host–pathogen interactions, drug resistance thereby defining novel therapeutic/vaccine targets. Expression proteomics exploits the differential expression of leishmanial proteins as biomarkers for application towards early diagnosis. Further using immunoproteomics efforts were also focused on evaluating responses to define parasite T-cell epitopes as vaccine/diagnostic targets. This review has highlighted some of the relevant developments in the rapidly emerging field of leishmanial proteomics and focus on its future applications in drug and vaccine discovery against VL.

Keywords:
Diagnostics / Drug resistance / Drug targets / Vaccine / Visceral Leishmaniasis

1 Introduction

Leishmania species are obligatory intracellular protozoan parasites responsible for a wide spectrum of diseases ranging from local self-healing cutaneous disease to potentially fatal visceral infection of the spleen and liver [1]. This genus is known to have 21 species. The disease is an important global public health problem with an estimated 350 million people at risk of infection (http://www.who.int/en/). Depending on the *Leishmania* species and the immune response of the host, there are three basic clinical manifestations of the disease: cutaneous (CL), mucocutaneous (MCL), and visceral leishmaniasis (VL) [2, 3]. *Leishmania major*, *Leishmania amazonensis*, *Leishmania guynanensis*, *Leishmania panamensis*, and *Leishmania tropica* are usually associated with the cutaneous forms of the disease whereas VL is caused by *Leishmania donovani*, *Leishmania infantum*, and *Leishmania chagasi*. *L. infantum* and *L. chagasi* are of veterinary importance, as they are not only the cause of zoonotic

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Abbreviations: SP, soluble proteins, **VL,** visceral leishmaniasis; **WCP,** whole cell protein

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SHORT COMMUNICATION

Evaluation of antileishmanial potential of *Tinospora sinensis* against experimental visceral leishmaniasis

Nasib Singh · Awanish Kumar · Prasoon Gupta · Kailash Chand · Mukesh Samant · Rakesh Maurya · Anuradha Dube

Received: 9 August 2007 / Accepted: 23 November 2007 / Published online: 13 December 2007

Abstract The chemotherapeutic interventions against visceral leishmaniasis (VL) are limited and facing serious concerns of toxicity, high cost, and emerging drug resistance. There is a greater interest in new drug developments from traditionally used medicinal plants which offers unprecedented diversity in structures and bioactivity. With this rationale, ethanolic extract of *Tinospora sinensis* Linn and its four fractions were tested in vitro against promastigotes and intracellular amastigotes and in vivo in *Leishmania donovani* infected hamsters. Ethanolic extract exhibited an appreciable activity against promastigotes (IC_{50} 37.6±6.2 μg/ml) and intracellular amastigotes (IC_{50} 29.8±3.4 μg/ml). In hamsters, it resulted in 76.2±9.2% inhibition at 500 mg/kg/day×5 oral dose level. Among fractions, *n*-butanol imparted highest in vitro and in vivo activities. Ethanolic extract and butanol fraction also enhances reactive oxygen species (ROS) and nitric oxide (NO) release. The results indicate that *T. sinensis* may provide new lead molecules for the development of alternative drugs against VL.

Introduction

Visceral leishmaniasis (VL) or kala-azar is a fatal systemic infection caused by *Leishmania donovani*, an obligate intracellular protozoan parasite belonging to family Trypanosomatidae. It is endemic in 62 countries, primarily in the developing world, and the population at risk is estimated at 200 million (Guerin et al. 2002). Approximately 100,000 new cases of VL occur in India annually, and the state of Bihar accounts for 90% of these (Sundar 2001). If left untreated, the mortality rate for VL is near 100%. A major emerging problem is *Leishmania*/HIV coinfections, which present difficulty in diagnosis and treatment (Cruz et al. 2006). Resistance to sodium stibogluconate (SSG), the first line treatment, is increasing in Bihar state of India, where up to 60% previously untreated patients are unresponsive to its recommended dose regimen (Croft et al. 2006). Despite the development and registration of miltefosine, an oral drug for VL treatment in India (Sundar et al. 2002), search for new active compounds including those of natural origin is greatly encouraged, as there has been little progress toward development of vaccines against VL.

Tinospora sinensis (Lour.) Merrill (syn *Tinospora malabarica*) belonging to the family Menispermaceae, is commonly known as Gurch in Hindi and Sudarsana in Sanskrit (Jain and DeFilipps 1991). It is a large deciduous climber with rambling stems, bearing aerial roots from branches and is found almost throughout India, ascending to an altitude of 1,000 m. It has been used in traditional Ayurvedic medicine for treating debility, dyspepsia, fever, syphilis, bronchitis, jaundice, urinary, skin, and liver diseases (Wealth of India 1976; Chopra et al. 1956). It is also reported to possess anti-inflammatory (Li et al. 2003) and antidiabetic (Yonemitsu et al. 1993) activities. It also inhibits cyclophosphamide induced anemia, increase WBC count in mice, and possess immunomodulatory activity (Manjrekar et al. 2000). Stem of this plant is used as a brain tonic, in chronic rheumatism and for fumigation in piles and ulcerated wounds (Li et al. 2003). A white starchy

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JAC

Antileishmanial activity mediated by apoptosis and structure-based target study of peganine hydrochloride dihydrate: an approach for rational drug design

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***Objectives*: The aim of this study was to resolve the putative pathway responsible for death induced by peganine hydrochloride dihydrate isolated from *Peganum harmala* seeds at cellular, structural and molecular level in *Leishmania donovani*, a causative agent of fatal visceral leishmaniasis.**

***Methods*: The mode of action was assessed using various biochemical approaches including phosphatidylserine exposure, estimation of mitochondrial transmembrane potential and *in situ* dUTP nick end labelling staining of nicked DNA in the parasite. Molecular modelling and molecular dynamics studies were conducted with DNA topoisomerase I to identify the target of peganine hydrochloride dihydrate mediating apoptosis. Further, DNA topoisomerase I inhibition by peganine hydrochloride dihydrate was also assessed using an *L. donovani* topoisomerase I relaxation assay.**

***Results*: Peganine hydrochloride dihydrate, besides being safe, was found to induce apoptosis in both the stages of *L. donovani* via loss of mitochondrial transmembrane potential. Molecular docking studies suggest that a binding interaction with DNA topoisomerase I of *L. donovani* (binding energy of −79 kcal/mol) forms a stable complex, indicating a possible role in apoptosis. The compound also inhibits *L. donovani* topoisomerase I.**

***Conclusions*: The compound induces apoptosis in *L. donovani* and inhibits DNA topoisomerase I.**

Keywords: apoptosis, mitochondrial transmembrane potential, TUNEL assay, molecular docking

Introduction

Most antileishmanial drugs are expensive, toxic and have unacceptable side effects; dosing is also complicated by the fact that they are given parenterally.[1] Moreover, cases of drug resistance are on the rise.[2] This has caused a renewed interest in the study of medicinal plants as a source for new antiparasitic leads. Further, understanding the mode of action and binding modes of these natural products to specific target sites may be used to design potent, novel, selective and less toxic antileishmanial analogues of these compounds on a structural basis.

In our earlier studies, activity-guided fractionation and isolation from *Peganum harmala* seeds led to the identification of oral antileishmanial activity in peganine hydrochloride dihydrate (**1**), a small molecule with simple structural features [Figure S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)], easily synthesizable and non-toxic to hosts. In this paper, we show that the leishmanicidal effect of **1** seems to be the consequence of induction of programmed cell death (PCD) both in the intracellular amastigote and extracellular promastigote forms of *Leishmania* cells targeting DNA topoisomerase I.

Materials and methods

Enzyme and chemicals

Recombinant type I DNA topoisomerase was prepared as described previously.[3] Camptothecin (Sigma, St Louis, MO, USA) was

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ORIGINAL PAPER

Age-influenced population kinetics and immunological responses of *Leishmania donovani* in hamsters

Nasib Singh · Mukesh Samant · Shraddha K. Gupta · Awanish Kumar · Anuradha Dube

Received: 27 December 2006 / Accepted: 23 April 2007 / Published online: 7 May 2007

Abstract Susceptibility of animals to infections depends upon various factors including sex and age of the host, which plays a pivotal role. In this communication, we have investigated the "intake" of *Leishmania donovani* infection in young (3–4 weeks old) and adult (15–16 weeks old) golden hamsters. The splenic parasite load in young hamsters on day 15 post infection (p.i.) was 54±4 amastigotes/100 macrophage nuclei and increased to 106.3±3.5 on day 30 p.i. However, adult group showed 2.2-($P<0.001$) and 1.75-fold ($P<0.001$) lesser parasite burden on these days, respectively. But as the disease progresses further, differences in parasite burden become less significant, as revealed by comparable levels of parasite loads at 2 months p.i. Spleen weight measurements correspond to the above observations. In the young group, the levels of antileishmanial antibody rise two and 4.5 times on days 15 and 30 p.i., respectively, as compared to only 1.3 and 2.3 times increase in their respective adult counterparts. However, after 2 months of infection both groups recorded analogous (12-fold) rise in antibody levels. Both mitogenic and antigenic responses in adult hamsters were less suppressed compared to young hamsters on days 15 and 30 p.i. However, both groups exhibited highly suppressed cell-mediated immune (CMI) responses after 2 months of infection. These findings implicate that age of the host may influence the susceptibility and resistance to *Leishmania* infection.

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Introduction

Visceral leishmaniasis (VL) also known as kala-azar is a vector-borne parasitic disease caused by an obligate intracellular kinetoplastid protozoan parasite, *Leishmania donovani*. It is characterized by prolonged fever, splenomegaly, hepatomegaly, substantial weight loss, progressive anemia, pancytopenia, and hypergammaglobulinemia. It is almost always fatal if left untreated. An estimated 500,000 new cases of VL occur annually worldwide, of which 90% occurs in only five countries—India, Nepal, Bangladesh, Sudan, and Brazil. The Bihar state of India accounts for more than 90% of total 100,000 cases of VL occurring annually. In India, 88% of leishmaniasis patients have a daily income of less than US$ 2, meaning they belong to the poor economic level (Murray 2004).

Malnutrition and age contribute greatly to the risk of developing the visceral disease, adversely affecting the clinical picture and resulting in increased morbidity and mortality. High incidence of VL is found to be associated with malnutrition, and undernourished children are highly prone to severe disease due to poor immune status (Dye and Williams 1993; Sharma et al. 1990). Approximately 63.4% of VL cases are aged between 10 and 29 years. The disease is least common in persons above 40 years of age (Thakur 1984). Morbidity in aging individuals is associated with an increase in susceptibility to many kinds of tumors, autoimmune disase, and infectious pathogens. The decline in the immune response toward infectious microbes in senescence is associated with a dysregulation of certain macrophage functions, such as NO release or a reduced generation of oxygen radicals (Ding et al. 1994; Miller 1996; Kissin et al. 1997). Aging has also been associated with a decline in immunocompetence and resistance to infection, partially

1: FJ014501. Reports Leishmania donova...[gi:201066707] Links

- Features
- Sequence

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LOCUS       FJ014501                  882 bp    DNA     lin
DEFINITION  Leishmania donovani isolate 2039 proliferative
            (PCNA) gene, complete cds.
ACCESSION   FJ014501
VERSION     FJ014501.1  GI:201066707
KEYWORDS    .
SOURCE      Leishmania donovani
  ORGANISM  Leishmania donovani
            Eukaryota; Euglenozoa; Kinetoplastida; Trypano
            Leishmania.
REFERENCE   1  (bases 1 to 882)
  AUTHORS   Kumar,A., Khare,P., Misra,P. and Dube,A.
  TITLE     Cloning and overexpression of Leishmania donov
            2039 proliferative cell nuclear antigen (PCNA)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 882)
  AUTHORS   Kumar,A., Khare,P., Misra,P. and Dube,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (12-AUG-2008) Parasitology, Central
            Institute, M.G. Road, Lucknow, U.P. 226001, In
FEATURES             Location/Qualifiers
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ORIGIN
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       61 aacggcctgg tgaatgaggc gaacttcgac tgcaacccgg gcgg
      121 atggactcca gccacgttgc cctcgtccac atgctgctgc gcga
      181 tatcagtgcg ggcgcaacag catcctcggc ctcaacctcg cctc
      241 aagattgtag acagcaatga cagcctcagc cttcgtcacg acga
      301 acgctgacat ctgaaaaccc ggagaagaca cgcaagtgcg agta
      361 gagatcgagg cagagtcgat gggcattccg gagatggact atcg
      421 aactcggccg agtttgcgaa gattgtgcgg gatatgcagg tgtt
      481 attgcaatca gcaaggaggg cgtgaagttc agctcgtcgg gcga
      541 acctttctgc aagcagctgg tgtgtcggac cgcagcacga agag
      601 gtgaaggcag aggcgcggga cgacgacgag gagccgctct cgcg
      661 gacagcagcg ccaacgcgat tggagtcgag gtgacgatgg agga
      721 tttgcgctgc ggttcatggg tatcttcgcg aagggctcga cgct
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1: FJ014500. Reports Leishmania donova...[gi:201066705] Links

- Features
- Sequence

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LOCUS       FJ014500                  735 bp    DNA     lin
DEFINITION  Leishmania donovani isolate 2039 20S proteasom
            gene, complete cds.
ACCESSION   FJ014500
VERSION     FJ014500.1  GI:201066705
KEYWORDS    .
SOURCE      Leishmania donovani
  ORGANISM  Leishmania donovani
            Eukaryota; Euglenozoa; Kinetoplastida; Trypano
            Leishmania.
REFERENCE   1  (bases 1 to 735)
  AUTHORS   Kumar,A., Misra,P., Khare,P. and Dube,A.
  TITLE     Cloning and overexpression of Leishmania donov
            2039 20S proteasome subunit alpha 5 gene
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 735)
  AUTHORS   Kumar,A., Misra,P., Khare,P. and Dube,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (12-AUG-2008) Parasitology, Central
            Institute, M.G. Road, Lucknow, U.P. 226001, In
FEATURES             Location/Qualifiers
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                     /isolate="2039"
                     /db_xref="taxon:5661"
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     CDS             1..735
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ORIGIN
        1 atgttcacct caaagtctga gtacgaccgc ggcgtgaaca cctt
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      121 cgtacgcccg agggcgtcgt tcttgccgcg gagaaacgcg tgcc
      181 ccgtccagca tgagtaagat tatggagatc gacagccaca ttgc
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      361 atccagtttg gtgagggcgg tggcaagcgc aagctgatgt cacg
      421 ctgctcatcg ccggcgtaga tgaaaagggc ccgcagctgt ggca
      481 acgcacacgc gctacgacgc ccaagccatt ggtggcggtg ccga
      541 ttcacggagc gctaccaccg caacatgacg ctggaggagg gcga
      601 attctgaagc aggtgatgga ggaccagctg tcgccggaga acat
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      721 agcatgacgg agtaa
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1: EF141073. Reports Leishmania donova...[gi:119656394] Links

- Features
- Sequence

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LOCUS       EF141073                 1593 bp    DNA     lin
DEFINITION  Leishmania donovani isolate 2039 proteophospho
            partial cds.
ACCESSION   EF141073
VERSION     EF141073.1  GI:119656394
KEYWORDS    .
SOURCE      Leishmania donovani
  ORGANISM  Leishmania donovani
            Eukaryota; Euglenozoa; Kinetoplastida; Trypano
            Leishmania.
REFERENCE   1  (bases 1 to 1593)
  AUTHORS   Samant,M., Sahasrabuddhe,A.A., Kumar,A. and Du
  TITLE     Proteophosphoglycans of Leishmania donovani
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1593)
  AUTHORS   Samant,M., Sahasrabuddhe,A.A., Kumar,A. and Du
  TITLE     Direct Submission
  JOURNAL   Submitted (27-NOV-2006) Parasitology, Central
            Institute, M G Road, Lucknow, Uttar Pradesh 22
FEATURES             Location/Qualifiers
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     CDS             1..>1593
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/db_xref="GI:119656395"