Leishmaniasis: Introduction

“Change yours thoughts and you change your world”

-Norman Vincent Peale
LEISHMANIASIS: INTRODUCTION

1.1 An Overview

Leishmania spp. are digenetic obligatory intracellular parasitic protozoans that survive in hostile environments – the midgut of the insect vector and the phagolysosome of the mammalian macrophage. More than 20 species of Leishmania cause leishmaniasis and it is transmitted to humans by ~30 different species of phlebotomine sandflies (Pearson et al., 1996). This disease is a severe public health problem in tropical and subtropical regions of the world. Major characteristic of this disease is its diversity and complexity (Herwaldt, 1999) and threatens about 350 million men, women and children in 88 countries around the world. As many as 12 million people are believed to be currently infected, with about 1–2 million new cases occurring every year (www.who.int/leishmaniasis). Leishmaniasis is classified as one of the “most neglected diseases” (Yamey & Torreele, 2002) based on the limited resources invested in diagnosis, treatment, and control and its strong association with poverty (Alvar et al., 2006a). The disease is second in mortality and fourth in morbidity among all tropical diseases (Bern et al., 2008).

Leishmaniases has several diverse clinical manifestations: Cutaneous Leishmaniasis (CL) - ulcerative skin lesions, Mucocutaneous Leishmaniasis (MCL) - destructive mucosal inflammation and Visceral Leishmaniasis (VL) - disseminated visceral infection, each presenting distinct diagnostic challenges, most requiring prolonged, expensive drug therapy and each contributing differently to disease burden. Post Kala-azar Dermal Leishmaniasis (PKDL) is characterized by macular, maculopapular or nodular rash and is a complication of VL that is frequently observed after treatment. Interactions with malnutrition and HIV alter the clinical course and complicate therapeutic strategies. In the absence of Highly Active Antiretroviral Therapy (HAART), the relapse rate after treatment approaches 100%. Other complicated forms include disseminated cutaneous leishmaniasis (DCL) recognized by diffuse nodular non-ulcerating disease, and leishmaniasis recidivans (LB) characterized by localized and slowly progressive non-healing lesions. Both are rare,
difficult to treat, and can be severe. Among these VL is the most severe. It is caused by *Leishmania donovani* in the Indian subcontinent, Asia, and Africa and by *L. infantum* or *L. chagasi* in the Mediterranean region, Southwest and Central Asia, and South America (Murray, 2005). It is characterized by progressive fever, weight loss, splenomegaly, hepatomegaly, hypergammaglobulinemia and pancytopenia. Complications include immunosuppression and secondary bacterial infections, hemorrhage, anemia and during pregnancy, it causes foetal wastage or congenital leishmaniasis (Pagliano *et al.*, 2005). In short Kala-azar is 100% fatal if, left untreated (Desjeux, 1996). Even in treated patients, case-fatality rates are often 10% or higher; jaundice, wasting, severe anemia, and HIV co-infection are associated with increased risk of mortality (Collin *et al.*, 2004; Bern *et al.*, 2005; Rey *et al.*, 2005).

The recommended drugs for VL and CL were the antimonials, first introduced 75 years ago (Dep* et al.*, 2000) however, lack of response to pentavalent antimonials actively widespread in India and Sudan led to the use of amphotericin B or pentamidine. It is unlikely that one single drug or drug formulation will be effective against all forms of leishmaniasis since (a) the visceral and cutaneous sites of infections impose varying pharmacokinetic requirements on the drugs to be used and (b) there is an intrinsic variation in drug sensitivity of the 20 Leishmania species known to infect humans. In addition, there are other new problems to be surmounted by novel treatments, namely: (i) the need for drugs for treatment of VL in Bihar State, India, where there is acquired resistance to the pentavalent antimonials and (ii) the need for treatment for VL and CL in immunocompromised patients, in particular due to HIV co-infection, where there is exacerbation of disease or emergence from latent infection due to the depleted immune response. In the latter case standard chemotherapy is frequently unsuccessful (Alvar *et al.*, 2006b). Among the new drugs discovered miltefosine and paromomycin are at top priorities. Miltefosine, a hexadecylphosphocholine, is the first promising oral drug which can be used against leishmaniasis and a major milestone in chemotherapy of VL. It was initially developed as an anticancer agent, quickly and effectively eliminated Leishmania promastigotes from culture. Attention to this compound led to preclinical and clinical studies conducted for leishmaniasis (Croft *et al.*, 1987). As a result, miltefosine has been
registered for the treatment of VL in Germany and India in 2004, as well as for CL and VL in Colombia. Miltefosine, is although an effective oral drug but its use in women of child-bearing age is restricted due to teratogenicity. In addition, it has a long half-life, which might encourage the emergence of resistance once its use becomes widespread (Bryceson, 2001). Second strong candidate, Paromomycin (formerly known as aminosidine) is an aminoglycoside active against Gram-negative and many Gram-positive bacteria as well as some protozoa and cestodes. The antileishmanial properties of paromomycin were recognized by Kellina in 1961 and were confirmed by Neal et al (1968 & 1995). It was registered in India in August, 2007 for treatment of VL (den Boer & Davidson, 2006). The results from initial studies in India were promising (Chunge et al., 1990; Jha et al., 1998). Currently, the non-profit group Drugs for Neglected Diseases Initiative (DNDi) is conducting studies on paromomycin (as monotherapy and in combination) in VL in Africa, and the Institute for One World Health (iOWH) is conducting a Phase IV study in India (Davidson et al., 2009). Other drugs such as sitamaquine, azoles and azythromycin have been reported as having variable cure rates. Consequently, there is still a real need for new active compounds that can provide therapeutic benefits but with fewer side effects (Pape, 2008).

1.2 History of Visceral Leishmaniasis

Descriptions of conspicuous lesions similar to CL have been discovered on tablets from King Ashurbanipal from the 7th century BC, some of which may have been derived from even earlier texts from 1500 to 2500 BC. Muslim physicians including Avicenna in the 10th century gave detailed descriptions of what was called Balkh sore (Cox, 1996). In 1756, Alexander Russell, after examining a Turkish patient, gave one of the most detailed clinical descriptions of the disease. As for the new world, evidence of the cutaneous form of the disease was found in Ecuador and Peru in pre-Inca potteries depicting skin lesions and deformed faces dating back to the first century AD. 15th and 16th century texts from the Inca period and from Spanish colonials mention ”valley sickness”, ”Andean sickness” or ”white leprosy” which are likely to be CL (WHO, 2007). Physicians in the Indian subcontinent would describe it as Kala-azar. Kala-azar first came to the attention of
Western doctors in 1824 in Jessore, India, where it was initially thought to be a form of malaria. India gave kala-azar its common name, which is the Hindi for “black fever”, so called for the darkening of the skin on the extremities and abdomen that is a symptom of the Indian form of the disease. The agent of the disease was also first isolated in India by Scottish doctor William Leishman and Irish physician Charles Donovan, working independently. As they published their discovery almost simultaneously, the species was named for both of them – Leishmania donovani. Today, the name Kala-azar is used interchangeably with the scientific name visceral leishmaniasis for the most acute form of the disease caused by L. donovani. Along with India this disease is seen at its most deadly in north and east Africa. It can also be found throughout the Arab world and southern Europe. But, while the disease’s geographical range is broad, it is not continuous. The disease clusters around areas of drought, famine, and high population density. In Africa common infection centres are in Sudan, Kenya, and Somalia (Francois, 1995).

1.3 Taxonomy

The classification of Leishmania was initially based on ecobiological criteria such as vectors, geographical distribution, tropism, antigenic properties and clinical manifestations (Marsden & Lumsden, 1971; Bray, 1974; Pratt & David, 1981; Ryan et al., 1990). However, biochemical and molecular analysis showed that pathological and geographical criteria were often inadequate and thus, other criteria such as the patterns of polymorphism exhibited by kinetoplastic DNA (k-DNA) markers, proteins or antigens came to be used to classify Leishmania (Arnot & Barker, 1981; Miles et al., 1981; de Ibarra et al., 1982; Handman & Curtis, 1982; Wirth & Pratt, 1982; Brainard et al., 1986; Travi et al., 2002). A modern scheme of classification of Leishmania is shown in Fig.1.1. All members of the genus Leishmania are parasites of mammals. The two subgenera, Leishmania and Viannia, are separated on the basis of their location in the vector’s intestine (Ryan et al., 1990). Rioux et al (1990) used iso-enzyme analysis to define species complexes within the subgenera. Initially, species classification was based on various extrinsic criteria such as clinical, geographical and biological characteristics- for example, L. guyanensis (isolated
in Guyana), *L. peruviana* (isolated in Peru), *L. infantum* (isolated from a child in Tunisia) and *L. gerbilli* (isolated from gerbils). Since the 1970s, intrinsic criteria such as immunological, biochemical and genetic data have been used to define species of *Leishmania*. Use of these molecular techniques led to the publication of a taxonomic scheme by the World Health Organization (WHO, 1990).

![Fig.1.1 Taxonomy of Leishmania](image)

*Fig.1.1 Taxonomy of Leishmania*

Source: Based on the scheme published by the WHO, 1990

New methods of detection, isolation and genetic identification resulted in a massive increase in the number of species described. Today, 30 species are known and approximately 20 are pathogenic for humans. These species generally present different epidemiological and clinical characteristics related to different genetic and phenotypic profiles. The validity of the classification scheme, considered by some workers as too arbitrary, has been questioned several times. Debate has centered on *L. panamensis, L. peruviana, L. chagasi, L. infantum, L. archibaldi, L. garnhami, L. pifanoi, L. venezuelensis and L. forattinii* (Mauricio *et al.*, 2000; Cupolillo *et al.*, 2001; Sharma *et al.*, 2005).
Different studies have already clarified the status of some of these species; for example, *L. chagasi* is accepted as a synonym of *L. infantum* (Mauricio et al., 2000) and *L. peruviana* has been validated as an independent species (Banuls et al., 2000). The other species listed above are still under discussion.

### 1.4 Geographical Distribution

Leishmaniases has been reported in 88 countries in five continents - Africa, Asia, Europe, North America and South America (22 in the New World and 66 in the Old World) (Desjeux, 2001), 16 are developed countries, 72 are developing, and 13 of them are among the least developed (WHO, 2005). Approximately, 350 million individuals are at risk of this disease and 20 million people are infected worldwide, and an estimated 2.0 million new cases occur each year (Leishmaniasis control, [www.who.int/health-topics/leishmaniasis.htm](http://www.who.int/health-topics/leishmaniasis.htm), update 2007) with an incidence of 1.5 million cases per annum of the disfiguring CL and 0.5 million cases per annum of the potentially fatal VL (Ashford et al., 1992). However, with increasing travel to and from endemic regions more and more patients with leishmaniasis are seen by physicians in western countries (Herwaldt, 1999; Murray et al., 2000; Guerin et al., 2002). The relevance of this parasitic disease is further stressed out by the rise of Leishmania/HIV co-infection in many parts of the world including European countries such as Spain, Italy, France and Portugal where up to 9% of the AIDS patients suffer from visceral leishmaniasis (Berhe et al., 1999).

Over 90% of the global total of VL cases occur in India, Bangladesh, Nepal, Sudan, Ethiopia, and Brazil, while 90% of CL occurs in Afghanistan, Algeria, Iran, Saudi Arabia, Syria, Brazil, Colombia, Peru, and Bolivia (Desjeux, 2004; Modabber et al., 2007). The distribution is dynamic: Colombia and Ethiopia have recently joined this list, and Pakistan currently faces a large epidemic of CL in Baluchistan and Sindh (Bern et al., 2008). Climate change and other environmental changes have the potential to expand the geographic range of the vectors and leishmaniasis transmission in the future (Patz et al., 2000).
1.4.1 Current Status in India

In the Indian subcontinent (Bangladesh, Nepal and India), the most common endemic form of the disease is VL or kala-azar or Dum-Dum fever. Kala-azar is present in India for more than 100 years. The first appearance of kala-azar in India was recorded in 1862, when about 75,000 cases were reported from Mohammadapur in Jessor district of East Bengal (now in Bangladesh) (Sen Gupta, 1944; Peters & Prasad, 1983).

![Areas effected with VL in India](www.indg.gov.in)

All the districts north of the river Ganges were affected with Kala-azar. Now the disease has spread southwards up to Darjeeling, Malda, West Dinajpur and Burdwan districts of West Bengal bordering Bihar state. A sample survey in Bihar carried out in 1977 on the epidemic of kala-azar showed an estimated number of 100,000 cases in the state with 4500 deaths (Sanyal et al., 1979) and in 1989, 30,000 cases with 450 deaths. In 1990 infected cases reached to 54,000 with 590 deaths and by 1991 the number of cases increased to 250,000 with 75,000 deaths (Thakur et al., 1993). It is obvious that the number of reported cases largely underestimated. Some local surveys revealed that the real prevalence of disease was five times more than what was reported. The situation is particularly grave in the state of Bihar, India, known as the “heartland of kala-azar”. It has been posed a serious threat involving 38 out of 42 districts of Bihar state, 8 districts of West Bengal and 2
districts of eastern Uttar Pradesh (Guerin et al., 2002). In a recent survey at least 75% of the VL cases in Bihar live below the poverty threshold of less than one dollar a day and this is similar in other endemic countries although exact data are scarce. At present the disease is present in almost all districts of Bihar, four districts of Jharkhand, five districts of Uttar Pradesh and 10 districts of West Bengal, 40 out of total 54 districts in Bihar are badly affected with VL (Fig. 1.2). The known endemic districts of kala-azar are located north of the river Ganges namely Muzaffarpur, Vaishali, Darbhanga, Samastipur, Madhubani, East Champaran, Sitamarhi, Begusarai, Saran, Saharsa and Purnea. Sporadic cases have also been reported from Gujarat (Gajwani et al., 1967), Kashmir (Jacob & Kalra, 1951) Himachal Pradesh (Gupta & Bhatia, 1975). In a recent study, total of 68,358 VL cases were reported in Muzaffarpur district from 1990 to 2008, ranging from 1248 in 1992 to 1161 in 2001. The blocks with the highest number of cases shifted from East (1990–98) to West (1999–2008). Monthly averages of cases ranged from 149 to 309, highest peak in March–April and another one in July. Monthly VL incidence was associated positively to rainfall and negatively to relative humidity and the numbers of VL cases in the previous month (Malaviya et al., 2011)

In Uttar Pradesh occurrence of sporadic cases of kala-azar started in the year 1987 with most of the cases reported so far from this state are imported cases (Thakur et al., 1999) and in West Bengal 9 districts are affected including Malda, Dinajpur and Darjeeling districts. It is one of the major health problems in Bihar and adjoining areas of West Bengal, Jharkhand, and Uttar Pradesh in India; there are focal and sporadic cases in 50–52 districts for many decades (Ranjan & Bhattacharya, 2001). An average of more than 90% of VL cases in India is reported from Bihar alone (Bora, 1999)

There is no current active surveillance mechanism for proper reporting of VL in the affected areas, which leads to serious under-reporting of VL cases. It has been reported that the total number of estimated cases could be 2–2.5 times higher than the actual incidence and may be even 5 times higher than the officially reported figures (Bora, 1999; Thakur, 2000). In the absence of accurate statistics, it is difficult for health planners and policy makers to evolve a suitable control strategy for elimination of VL by the year 2015 from
Many studies have been conducted for estimating the extent of under-reporting of VL cases in different areas in Bihar in which VL is endemic (Singh et al., 2006). These studies were based on the total cases reported in the source population or observed in the study population, without stratifying for age and sex variables. Thus, these studies estimated only the crude incidence proportion (risk) or rate. Taking these facts into account, we conducted a study with the objective to estimate the level of under-reporting of VL cases in the total population and stratified by age and sex.

Two public health centers in an area in which VL is endemic (Lalganj and Goraul in India) were selected on the basis of passive VL case reporting in past five years. These two public health centers had estimated populations of 412,035 and 235,730, respectively, in 2006 determined by an exponential growth rate equation and a population growth rate of 2.98% (Census of India, 2001). The age-sex specific initial population and exponential growth rate were taken from Census 2001 of India.

In 2005 the health ministers of three Member States of WHO South-East Asia Region, India, Nepal and Bangladesh, had signed a Memorandum of Understanding pledging to collaborate to eliminate VL from their countries. Geographical distribution of kala-azar closely coincides with the distribution of insect vector, *Phlebotomus argentipes* and ecological factors (Napier & Smith, 1926; Shivaramakrishnamaiah & Ramanathan, 1967) such as:

a) An altitude less than 2000 feet

b) Abundant rainfall more than 80 cm. annually and mean humidity of about 70% to 80%

c) Alluvial soil

d) Temperature below 38 °C and above 4 °C with diurnal variation less than 10 °C

e) Abundant vegetation with subsoil water

f) Rural setting.

All these conditions prevail in Assam valley, West Bengal, Tamil Nadu and Bihar.
1.5 Disease and its Types
Leishmaniases is not a single disease but a variety of syndromes that differ remarkably with one another. The WHO considers leishmaniasis as one of the most important parasitic diseases (WHO, 1990). Governed by parasite and host factors and immunoinflammatory responses, the clinical spectrum of leishmaniasis encompasses subclinical (unapparent), localised (skin lesions), and disseminated infection (cutaneous, mucosal, or visceral). These wide-ranging differences of clinical manifestations define *Leishmania* virulence (degree of pathogenicity) in human infection. According to the form of the disease, site of infection and species involved, the leishmaniases can be divided into following general clinical patterns.

1.5.1 Cutaneous Leishmaniasis (CL)
CL is commonly known as oriental sore. Its causative agents are *L. major*, *L. tropica*, *L. aethiopica*, in old World and *L. mexicana*, *L. venezuelensis*, *L.amazonensis*, *L. braziliensis*, *L. panamensis*, *L. guyanensis*, *L. peruviana* and *L. chagasi* are in New world. It produces skin lesions mainly on the face, arms and legs (Fig.1.3).

![Fig.1.3 Lesions in Cutaneous Leishmaniasis](source: www.pudsandlosers.blogspot.com)

It is frequently self-healing but when the lesions are multiple and disabling with disfiguring scars, it creates a lifelong aesthetic stigma. After recovery or successful treatment, cutaneous leishmaniasis induces immunity to re-infection by the species of Leishmania that cause the disease. It is prevalent in Mediterranean Basin, Syria, Arabia, and Mesopotamia, Persia to Central Asia, Central Africa and some parts of Western India. There are two chronic forms.
1.5.1.1 Diffuse Cutaneous Leishmaniasis (DCL)

It is difficult to treat DCL due to disseminated lesions that resemble leprosy and do not heal spontaneously (Fig. 1.4). This form is especially related to a defective immune system and it is often characterized by relapses after treatment. It is rare and disfiguring. Widespread plaques containing huge numbers of amastigotes persist for decades. It is caused mainly by *L. aethiopica* in Africa and *L. amazonensis* in South and Central America. DCL due to *L. amazonensis* can be treated with antimonials associated with isoniazid and rifampicin due to the synergistic effect, or immunotherapy combining killed Leishmania promastigotes and BCG (Convit *et al.*, 1989). DCL due to *L. aethiopica* normally does not respond to meglumine antimoniate but to SSG (3–4 mg/kg once a week). Without this healing is not ensured and relapses can be seen months later. It has been suggested to combine sodium stibogluconate with PM (Teklemariam *et al.*, 1994).

![Fig.1.4 Facial lesion in Diffuse Cutaneous Leishmaniasis](http://pathmicro.med.sc.edu/parasitology/blood-proto)

1.5.1.2 Leishmaniasis recidivans

It is a chronic, non-healing or relapsing cutaneous infection caused mainly by *L. tropica* in the Middle East. It needs antimonial treatment for long periods and relapses are common leading to the use of combined treatments (Momeni & Aminjavaheri, 1995) or surgical interventions; thermotherapy is effective only in primary lesions of reduced size.
(Reithinger et al., 2005). It is very difficult to treat, long lasting, destructive and disfiguring (Fig.1.5).

![Image](http://pathmicro.med.sc.edu/parasitology/blood-proto)

**Fig.1.5** Facial lesion in leishmaniasis recidivans  
Source: [http://pathmicro.med.sc.edu/parasitology/blood-proto](http://pathmicro.med.sc.edu/parasitology/blood-proto)

1.5.2 Mucocutaneous Leishmaniasis (MCL)

It is also called 'espundia' in South America. Causative agents of MCL in Old World are *Leishmania aethiopica* (rare), *L. major* and in New World are *L. mexicana, L.amazonensis, L. braziliensis, L. guyanensis* and *L. panamensis*. The parasite invades the mucocutaneous region of the body and spread to the oronasal/pharyngeal mucosa (Fig.1.6).

![Image](www.stanford.edu.com)

**Fig.1.6** Deformity in nasal mucosa due to Mucocutaneous Leishmaniasis  
Source: [www.stanford.edu.com](http://www.stanford.edu.com)
The soft tissues and cartilage of the oronasal/pharyngeal cavity undergo progressive erosion. Contrast to cutaneous leishmaniasis, these lesions does not heal spontaneously. Suffering and mutilation are severe and death occurs as a result of bronchopneumonia or malnutrition. There is always a large danger of bacteria infecting the already open sores. Reconstructive surgery of deformities is an important part of therapy. MCL is normally treated with meglumine antimoniate (20mg/kg/d for 28 days); non-healing lesions are susceptible, and have to be treated for 2 months instead or, eventually, with Amphotericin B. Immunotherapy are a promising method to treat MCL although more experience is needed (Convit et al., 2003).

**1.5.3 Visceral Leishmaniasis (VL)**

The second largest parasitic cause of death (after malaria), VL is prevalent in 47 countries, with approximately 200 million at risk, and an annual estimated incidence of almost 500,000 cases and > nearly 50,000 deaths. Overall, 90% of cases occur in India, Bangladesh, Nepal, Sudan and Brazil, and 60% in the Indian subcontinent alone. Eastern Africa (Sudan, Ethiopia, Kenya, Uganda and Somalia) has the second largest number of cases. The real burden of VL may be far higher than the number of reported cases. In Bihar, India, only one in eight cases is reported through official channels and approximately 20% of cases end in death undiagnosed. Retrospective mortality surveys in southern Sudan estimated that in an epidemic in Western Upper Nile province in the 1980s and 1990s, VL caused > 100,000 deaths in a population of 280,000. A later study estimated that only 50% of cases in Sudan can access treatment, resulting in nearly 90% of VL deaths going unreported (den Boer et al., 2009). It is also known as ‘Kala-azar’ (in India). It is caused by *L. donovani* complex i.e. *L. donovani donovani* (India, Africa), *L. d. infantum* (Middle East and some parts of Asia) and *L. d. chagasi* (South America). These species are morphologically indistinguishable but have been identified by molecular methods, predominantly multilocus enzyme electrophoresis. The disease can present an acute, sub-acute or chronic evolution, but most infected individuals remain completely asymptomatic (Bittencourt et al., 1995). The asymptomatic individual is characterized by positive serology to Leishmania and, possibly, a positive intradermal test. Infected individuals can evolve to a subclinical form of VL or directly to an overt form of disease
(classical VL). Initially, the disease is characterized by high fever, headache, chill, malaise, dizziness, anorexia, and vomiting and weight loss. In chronic stage the disease is followed by hepatomegaly, splenomegaly, lymphadenopathy, occasional acute abdominal pain, emaciation, anemia, leukopenia, and blackness of skin, hence the name given Kala-azar or black-fever. As the disease advances, splenomegaly can increase, causing abdominal distension and pain, which is sometimes increased by concomitant hepatomegaly followed by severe anemia and cachexia (Fig.1.7). Symptoms and signs of bacterial co-infections such as pneumonia, diarrhoea or tuberculosis can confuse the clinical picture at the time of initial diagnosis (Chappuis et al., 2007).

Fig.1.7 Enlargement of liver and spleen in Visceral Leishmaniasis
Source: www.pathmicro.med.sc.edu

It is the most severe form of leishmaniasis and is usually fatal (100% deaths) if, left unattended. The incubation period can be months or years and, unlike the cutaneous forms of leishmaniasis, in this disease, the parasite uses the bloodstream to travel and it involves the internal organs such as liver, spleen, lymph nodes, and bone-marrow. After treatment and recovery, the patients may develop chronic cutaneous leishmaniasis that requires long and expensive treatment.
1.5.4 Post Kala-azar Dermal Leishmaniasis (PKDL)

Post kala-azar dermal leishmaniasis is a sequel to the infection with *L. donovani*. Its causative agents in Old World are *L. infantum*, *L. donovani*, and *L. tropica* (rare; also may produce the atypical viscerotropic disease) and in New World *L. chagasi* is responsible for this. This syndrome, characterized as skin lesions, nodules or papules, frequently on the face, has been well characterized in India and Sudan (Fig.1.8).

![Fig.1.8 Nodular lesions on face and other body parts in PKDL](www.icp.ucl.ac.be.com)

PKDL often appears in patients 2–7 years after apparently successful antimonial treatment of VL. Treatment of PKDL has long been a problem and formal recommendations for this treatment, based upon studies in India and Sudan, have been using SSG at 20 mg/kg dose for at least 120 days (Thakur & Kumar, 1990; Zijlstra & El-Hassan, 2001). PKDL in India resembles lepromatous leprosy with verrucous papilomatous, xanthomathous and gigantic nodular forms; while in East Africa it resembles more to sarcoidosis and tuberculosis with popular rash over face or well defined rounded papules (Rashid *et al.*, 1986). A reconsideration of the recommended treatment is required due to antimonial resistance in India, making Amphotericin B the preferred drug (Thakur *et al.*, 1997). AmBisome, very effective in treatment of VL, has also proved to be an effective treatment for PKDL at 2.5 mg/kg for 20 days (Musa *et al.*, 2005), with the same caveat of high cost restricting its use.
1.6 Epidemiology and Ecology

Leishmaniasis is one of the most neglected tropical diseases, with a major burden among the poorest segments of impoverished populations in Asia, Africa, South America and, in less degree, Europe (Yamey & Torreele, 2002). It is associated with malnutrition, displacement, poor housing, illiteracy, gender discrimination, weakness of the immune system and lack of resources. Leishmaniasis is also linked to environmental changes,* such as deforestation, building of dams, new irrigation schemes and urbanization, and the accompanying migration of non-immune people to endemic areas. Transmission of leishmaniasis to humans occurs through sylvatic, domestic, and peridomestic cycles. Sylvatic cycles involve an animal host which act as indefinite reservoir for the disease and maintain enzootic transmission without human disease. Such hosts are common in New World rain forests and the deserts of Central Asia. Disease gets transmitted to humans only when they enter the sylvatic habitat for economic or military purposes or when human habitation encroaches on the sylvatic setting. In domestic cycles, humans or dogs form the predominant or sole infection reservoir. Female sand fly of genus Phlebotomus in the Old World and Lutzomyia in the New World are the only proven vector responsible for transmission of the disease (Berman, 1997) (Fig. 1.9). In India, Phlebotomus argentipes is the only proven vector for the disease. Female sandflies (Phlebotomus and Lutzomyia spp) get infected by taking a blood meal from infected human beings (anthroponoses) or terrestrial mammals (zoonoses). Imbibed amastigotes transform in the sandfly gut and replicate as promastigotes; at a subsequent bloodmeal, metacyclic promastigotes are regurgitated and injected into the skin to complete the cycle (Rogers & Titus, 2004). About 70 of around 1000 known sandfly species transmit leishmaniasis. Vector competence in most species seems to be controlled by parasite ability to resist proteolytic enzymes during bloodmeal digestion and avoid excretion by binding to midgut epithelium. Binding is mediated by promastigote surface lipophosphoglycan and the phosphoglycan domains differ between species (Sacks, 2001). Sandfly saliva affects local host immune responses, promoting experimental cutaneous infection (Sacks & Noben-Trauth, 2002). 500,000 new cases for VL and more than 50,000 deaths from the disease every year have been reported (Desjeux, 2004). Such a death toll is surpassed among the parasitic diseases only by
malaria (WHO, 2002). Both figures are approximations as VL is frequently not recognized or not reported (Collin et al., 2006; Singh et al., 2006a). The majority (>90%) of cases occur in just six countries: Bangladesh, India, Nepal, Sudan, Ethiopia, and Brazil. Severe VL epidemics have been reported in the past in southern Sudan, in context of civil war and famine, VL killed approx 100,000 people out of a population of 280,000 between 1984 to 1998 (Jacquet et al., 2006). As India, Nepal and Bangladesh harbour an estimated 67% of the global VL disease burden (Hotez et al., 2004), the commitment of the government of these countries to launch regional VL elimination programme is welcome. The target of this programme is to eliminate VL as a public health problem by 2015, by using a local approach to reduce the annual incidence of VL to less than one case per ten thousand individuals. Leishmaniasis cause considerable morbidity and mortality. It is a typical example of an anthropozoonosis. The majority of infections are originally zoonotic, although some cases are known of transmission of *L. donovani* from human to human. In a primitive or sylvatic cycle human infection is accidental, transmission occurring in wild foci, e.g. *L. braziliensis*; in a secondary or peridomestic cycle the reservoir is a peridomestic or domestic animal, the parasite being transmitted to humans by anthropophilic sand flies, e.g. *L. infantum*; and in a tertiary, strictly anthroponotic cycle the animal reservoir disappear and disease transmit from infected human to healthy human by sandflies, e.g. *L. donovani*. The epidemiology of leishmaniasis in a given area is directly dependent on the behaviour of the human and/or animal population in relation to the cycle of transmission. The foci that account for the largest number of human cases, for example, VL in South Asia and CL in Afghanistan, usually reflect anthroponotic transmission (Reithinger et al., 2003; Jeronimo et al., 2006). In anthroponotic VL foci, the reservoir includes humans with untreated kala-azar (Bern et al., 2005) but PKDL patients may maintain the infection between kala-azar epidemics (Addy & Nandy, 1992). Up to half the population in highly affected foci may have asymptomatic leishmanial infection; the contribution of such individuals to transmission is presumed to be less than for active kala-azar, but has never been quantified (Costa et al., 2002; Bern et al., 2007). During the past decade there have been epidemics of VL in Sudan (Ashford et al., 1992; Jacquet et al., 2006), northeast Brazil (Costa et al., 1990), Bangladesh and the states of Patna and Bengal.
in India (Bolognesi et al., 1999). Leishmaniasis is now an emerging zoonosis in the United States (Enserink, 2000; McHugh et al., 2003; Rosypal et al., 2003) and US soldiers and peace keeping corps currently in the Middle East are experiencing a large outbreak of leishmaniasis with more than 500 parasitological confirmed cases (CDC, 2004). There are a variety of factors that influence the transmission of the disease (Lane, 1993; Kettle, 1995). They are as follows:

- Proximity of residence to sand fly breeding and resting sites
- Type of housing
- Occupation
- Extent of exposure to sand fly bites
- Natural resistance, genetic or acquired
- Virulence of the parasite species
- Zoonotic or anthroponotic reservoirs.
- The vectorial capacity, which is defined as the number of infective bites delivered per human per annum (Dye, 1992)
- Density, seasonality, longevity and flight range of sandfly populations
- Anthropophilia or zoophilia of sandflies and degree of it.

1.7 Vector (Sandfly)

![Sandfly transferring parasite during blood meal](www.albaeco.com)

Fig.1.9 Sandfly transferring parasite during blood meal
Source: www.albaeco.com

1.7.1 Entomology
The insect vectors of Leishmania parasites are sandflies belonging to the family Psychodidae, sub - family Phlebotominae and genera Phlebotomus (Old World) and
*Lutzomyia* (New World) with hundreds of species spread all over the world (Volf *et al.*, 1994). Of the 500 known phlebotomine species, only 30 of them have been positively identified as vectors of the disease. Only the female sandfly transmits the protozoa, infecting it with the *Leishmania* parasites contained in the blood, during the blood meal from human or mammalian host in order to obtain the protein necessary to develop its eggs. In the Old World (Europe, Asia, and Africa) sandfly vectors belong to the genus *Phlebotomus* and in the New World (America), to the genera *Lutzomyia* and *Psychodopygus*. There are different vectors in different regions for a single spp, for example, vectors of *Leishmania donovani* are *Phlebotomus argentipes* in India, *P. chinensis* in China, *P. perniciosus* in North Africa, Italy, France and Portugal, *P. perfiliewi* in Greece, *P. orientalis* in Sudan, and Ethiopia, *P. martini* in Kenya (Le Blancq & Peters, 1986), and vectors *L. infantum* are *P. erniciosus*, *P. ariasi*, *P. perfiliewi* and *P. neglectus*. Sometimes a single species is transmitted by a single vector, e.g. *L. chagasi* is transmitted by sandflies belonging to the genus *Lutzomyia* (*L. longipalps*) (WHO, 1990). Transmission of parasite may be anthroponotic (from one human to another) or zoonotic (from animal to human). In India, the disease is completely anthroponotic where as in certain parts of the world, there are one or more reservoirs (zoonotic host) e.g. dogs in the Mediterranean region and rodents in South Africa.

1.7.2 Distribution of Vector in India

Fauna of Indian sub-zone is represented by 46 species out of these 11 species belong to *Phlebotomine* species and 35 to *Sergentomyia* species. *P. argentipes* is the proved vector of kala-azar (VL) in India (Bhattacharya *et al.*, 2006). It prefers to hot and humid climates in all the VL abundant endemic areas of Bihar, West Bengal, Assam and Eastern Uttar-Pradesh. High densities have also been recorded in Southern peninsula and Central India. Vertical distribution has been reported to up to 1300 m above sea level in Garhwal (Uttaranchal) and 1100 m in Nilgiri Hills (Tamil Nadu).

1.7.3 Habit and Habitats

The vector is crepuscular in its habit, inactive during daytime, and seeks shelter in cracks and crevices in the dark corners of houses on cattlesheds (Palit *et al.*, 1996). In outdoor situations, it is found in caves, crevices, animal burrows, termite hills, tree holes etc. The
sandflies are incapable of flying long distances and move by characteristic hopping movement. They have been detected up to a height of 2.7m from the ground. It is found throughout the World inter tropical and temperate regions.

1.7.4 Seasonal Prevalence
Studies conducted in endemic areas revealed that the vector density starts increasing from February onwards, with some decrease in May to June, followed by an increase with the advent of the monsoon. In Southern and Eastern India, with very mild cold season, *P. argentipes* is common throughout the year.

1.7.5 Vector Control Strategies
Control of VL mainly depends on its epidemiological features. In India, Bangladesh and Nepal where visceral leishmaniasis is anthroponotic, vector is controlled by chemical and environmental control measures.

1.7.5.1 Environmental Control
Sandflies breed in dark corners in the crevices of the walls having rich humus and moisture. The principle behind the environmental control is to manage the environment to make it unsuitable for breeding. Vyokov in USSR successfully controlled leishmaniasis by destroying rodent burrows (Vyokov, 1980). In a study on technological control of sandflies, the walls of the resting sites were plastered filling all the cracks and crevices by mud and lime, and the breeding of sandflies could be stopped successfully (Kumar *et al.*, 1995). Lime has a powerful water absorbing capacity which makes it unsuitable for the sandfly breeding. In another experiment 70% population of *P. papatasi* successfully controlled by constructing cement skirting of 9” vertically on the wall and 9” horizontally on the floor (Dhiman, 1995).

1.7.5.2 Chemical Control
Under National Vector Borne Disease Control Programmes, DDT was extensively used for Indoor residual spraying, which is a simple and cost-effective method of controlling vector. DDT remains the insecticide of choice because of its low cost, high efficacy, long residual action and relative safety when used for indoor residual spray. Dosage schedule of 1 or 2 g/m² or 100-200 mg/ft² has been found to be quite effective; 5 per cent emulsified suspension of DDT is the choice. Unfortunately, the disease quickly re-emerged when
these spraying campaigns were discontinued. Resistance of *P. argentipes* to DDT remains limited, but has been reported in Bihar (Singh *et al*., 2001).

A recent study showed that 4% DDT and 0.05% deltamethrin seem to be acceptable discriminating concentrations to separate resistant from susceptible populations. Resistance to DDT was confirmed in Bihar and in a border village of Nepal, but the sand flies were still susceptible in villages more inside Nepal where only synthetic pyrethroids are used for indoor spraying. The low effectiveness of indoor spraying with DDT in Bihar to control VL can be partially explained by this resistance hence other classes of insecticides should be tested. In both countries *P. argentipes* sand flies were susceptible to deltamethrin (Dinesh *et al*., 2010).

1.7.5.3 Biological Control

Very scanty information is available on the biological control of sandfly. As the application of biolarvicides in the field condition is difficult due to diverse breeding habitat of sandfly, their practical application appears to be of limited use in the control of visceral leishmaniasis. In the laboratory experiment De Barjac *et al* (1981) first time demonstrated the role of *Bacillus thuringiensis* var. israelensis in the control of larvae of *P. papatasi* and *Lutzomyia longipalpis*. Robert *et al* (1998) successfully used *B. sphaericus* in the control of *P. martini* in Kenya based on the work of Schlein (1987) and Yuval & Warburg (1989). This approach requires further evaluation.

1.7.5.4 Remote Sensing

Satellite remote sensing has been successfully used in the identification of high risk areas. A pilot study has been concluded at RMRIMS, Patna, for identifying and mapping of *P. argentipes* distribution for early prediction of disease with the help of satellite remote sensing in integration with geographical information system (GIS) (Palit *et al*., 2001).

1.7.5.5 Use of Insecticide Impregnated Bed Nets in the Control of Leishmaniasis

Insecticide impregnated bed nets (ITN) were shown as one of the most effective methods of reducing man-vector contact and intra and peridomiciliary transmission of vector-borne diseases. In most studies the insecticides used were synthetic pyrethroids (permethrin, deltamethrin, Lambda-cyhalothrin), which combine the properties of low to moderate mammalian toxicity, low volatility and high insecticidal activity (WHO tech report, 1990).
ITNs act as ‘baited traps’ but have also important deterrent and repellent effect. Compared with house spraying ITNs have theoretically the following advantages: \( (i) \) their effectiveness is independent of the endophilic and exophilic behaviour of the vectors; \( (ii) \) less insecticide is used; and \( (iii) \) the household exerts control over its application and thus depends less on the performance of a knock-down planned disease control programme. Mass distribution of ITNs in Sudan was accompanied by a 27% reduction in the incidence of VL in an observational study (Ritmeijer et al., 2007). The principle of an ITN combines the effect of individual protection and insect-killing activity while a strong repellent effect could possibly enlarge its efficacy by reducing indoor and peri-domestic vector density. ITNs therefore have the potential to achieve individual protection for VL and users are not dependent on a top-down, government led intervention. The new long-lasting impregnated bed nets make yearly re-impregnation no longer necessary (Ostyn et al., 2008).

The current control strategies for VL rely on reservoir and vector control, the use of insecticide- impregnated materials and active case detection and treatment (Boelaert et al., 2000; Davies et al., 2003).

1.7.5.6 Reservoir Control

Dogs are the main reservoir of \( L. \) infantum in zoonotic VL. Despite evidence from experimental studies showing a decreased incidence of VL in both dogs and children following serological screening of dogs and killing of sero-positive animals (Ashford et al., 1998; Palatnik-de-Sousa et al., 2001), the efficiency and acceptability of this control strategy is increasingly being debated (Alvar et al., 1994; Tesh, 1995; Reithinger & Davies, 2002). Treating infected dogs is not an effective control strategy as relapses are frequent and dogs can regain infectivity weeks after treatment, despite being clinically cured (Alvar et al., 1994). Moreover, the widespread veterinary use of VL drugs might lead to resistance in parasites. A new control approach is the use of deltamethrine-treated collars, which reduced the risk of infection in dogs (by 54%) and children (by 43%) in a study conducted in Iran (Gavgani et al., 2002).
1.8 Transmission

1.8.1 Life Cycle

During their complex life cycle, the single cell parasites of the genus *Leishmania* are exposed to different extra and intracellular environments. These organisms are digenetic parasites with two basic life cycle stages: one extracellular stage within an invertebrate host (phlebotomine sand fly) and one intracellular stage within a vertebrate host. Thus, the parasites exist in two main morphological forms, promastigotes and amastigotes, which are found in vertebrate hosts and invertebrate hosts, respectively (Fig.1.10).

![Life cycle of Leishmania](Fig.1.10 Life cycle of Leishmaniasis)

Leishmaniasis can manifest as sores on the skin (the cutaneous form), or it can lead to multiple organ failure and death (the visceral form). The infective forms of the parasite, promastigotes, enter the skin (vertebrate hosts) through a sand fly (invertebrate hosts) bite and then are engulfed by host macrophages. Inside the parasitophorous vacuole they transform into amastigotes, multiply and are eventually ingested by a sand fly feeding on blood of the infected host. In the sand fly the amastigotes transform into promastigotes, completing the parasite’s life cycle.
1.8.2 Stage in the Invertebrate Host (Promastigote)

Like female mosquitoes, the female sandfly needs a blood meal for egg development and it is haematophagous. Some phlebotomine species can support the growth of only those species of *Leishmania* with which they are infected in nature, such as *P. papatasii* and *P. sergentii*; these species are considered to be restricted vectors (Kamhawi et al., 2000). By contrast, other phlebotomine species such as *Lutzomyia longipalpis* and *P. argentipes* are permissive vectors since they are able to develop mature transmissible infections when infected with several *Leishmania* species (Kamhawi et al., 2000; Sadlova et al., 2003; Warner et al., 2004). Within the intermediate host, *Leishmania* develops as promastigote forms with a cell body measuring 5-20 x 1-4 µm, elongated motile extracellular stages possessing a prominent free flagellum up to 20 µm long (Fig. 1.11). Nevertheless, a variety of different promastigote forms have been distinguished on morphological grounds (Bates & Rogers, 2004).

![Fig.1.11 Promastigotes (100X under oil immersion lens)](source: www.msu.edu)

1.8.3 Stage in the Vertebrate Host (Amastigote)

In the vertebrate host, the parasite evolves into an amastigote form. Amastigotes are ovoid (2.5–5 µm diameter), non-motile, intracellular stages. They do not have a free flagellum and are located in the parasitophorous vacuoles of the host’s macrophages (Fig. 1.12). In both developmental forms, the flagellum emerges from a flagellar pocket and in the amastigote form, it is almost completely restricted to it, so it is only observed by electron microscopy. Infection begins when an infected female sand fly takes a blood meal from a
healthy human host. Following inoculation into the skin by the sand fly bite, the infective flagellated metacyclic promastigotes are ultimately ingested by macrophages via receptor mediated endocytosis (Chang et al., 1990), transforms into amastigotes, and multiplies by binary fission. The infected macrophage eventually bursts and the released parasites are able to infect new phagocytic cells. When the infected host is bitten by another female sand fly, at the time of infective blood meal, the amastigotes in the gut of sandfly, due to change in temperature and other conditions, develop in to flagellated promastigotes and the life cycle continues.

**Fig.1.12** Stained infected macrophages bearing amastigotes (100X under oil immersion lens)
Source: [www.upload.wikimedia.org](http://www.upload.wikimedia.org)

1.8.4 Survival Strategy of Parasite

1.8.4.1 In Sandfly

Female sand flies (*Phlebotomus* species in the Old World, *Lutzomyia* species in the New World) acquire Leishmania parasites when they feed on an infected mammalian host in search of a blood meal. Sand flies are pool feeders, meaning they insert their saw-like mouthparts into the skin, and agitate them to produce a small wound into which the blood flows from superficial capillaries (Lane, 1993). It is this tissue damage associated with the creation of the wound that releases skin macrophages and/or freed amastigotes into the pool of blood, and enables their subsequent uptake into the abdomen of the sand fly. The change in conditions moving from the mammalian host to the sand fly midgut (decrease in temperature, increase in pH) triggers development of the parasite in the vector (Bates &
Rogers, 2004; Kamhawi, 2006). The amastigotes transform into motile promastigotes with flagella beating at the anterior end (Fig.1.13a). This first stage in the vector is called a procyclic promastigote – it is a weakly motile, replicative form that multiplies in the blood meal (Fig.1.13b). This initial “blood meal phase” is confined by the peritrophic matrix, a chitin and protein mesh secreted by the midgut epithelium that encloses the blood being digested within (Secundino et al., 2005). After a few days, the parasites begin to slow their replication and differentiate into elongate, strongly motile nectomonad promastigotes. These are migratory forms that accumulate at the anterior end of the peritrophic matrix and break out of the blood meal. This escape is facilitated by the action of a parasite secretory chitinase (Schlein et al., 1991; Shakarian & Dwyer, 2000) and probably by the action of endogenous sand fly chitinase (Ramalho-Ortigao et al., 2005). They move towards the anterior midgut, some of them attaching to the microvilli of the midgut epithelium, until they reach the stomodeal valve (cardia) that guards the junction between foregut and midgut. These nectomonad promastigotes mediate the establishment phase of the infection that marks a true vector i.e. persistence beyond the blood meal and avoidance of expulsion during defecation.

Thus, the ability to attach is an important property of Leishmania (Leishmania) promastigotes (Sacks & Kamhawi, 2001). Glycoconjugate lipophosphoglycan (LPG) present on parasite surface is responsible for binding to a galectin on the sand fly gut epithelium in certain species e.g. *L. major* in *Phlebotomus papatasii* (Pimenta et al., 1992; Kamhawi et al., 2004). Once they reach the stomodeal valve the nectomonad promastigotes transform into leptomonad promastigotes, shorter forms that resume replication (Gossage et al., 2003). These are responsible for the secretion of promastigote secretory gel (PSG) (Rogers et al., 2002), which plays a key role in transmission. Some of the nectomonad/leptomonad promastigotes also attach themselves to the cuticle-lined surface of the valve and differentiate into haptomonad promastigotes (Killick-Kendrick, 1999). This form of attachment is mechanistically different to that seen with the midgut epithelium and is mediated by expansion of the flagellar tip into hemi-desmosome-like structures (Vickerman & Tetley, 1990; Wakid & Bates, 2004). Finally, some of the leptomonads differentiate into metacyclic promastigotes (Rogers et al., 2002), which are
the mammal-infective stages. These are deposited in the skin of a new mammalian host when the fly takes another blood meal, leading to the transmission of disease.

![Diagram](image)

**Fig.1.13 Development of Leishmania parasite in the sand fly vector (Bates, 2007).**
(a) The morphology of amastigotes and promastigotes. Each form has a nucleus (N), kinetoplast (K) and flagellum (F). The kinetoplast is the mitochondrial genome. The flagellum in amastigotes is internal and non-functional; in promastigotes the flagellum extends from the cell body, beats and pulls the organism in the direction shown, emerging from the anterior end of the cell.
(b) The developmental sequence of the five major promastigote forms: procyclic promastigotes, nectomonad promastigotes, leptomonad promastigotes, haptomonad promastigotes and metacyclic promastigotes. The exact position of haptomonad promastigotes in the developmental sequence is uncertain.

### 1.8.4.2 In Macrophages
Survival of intracellular infectious pathogens in the host organism is best achieved when the pathogen resides in a cell type that *per se* is not able to exert antimicrobial activities. In Leishmania infections a number of cell types were postulated to function as ‘safe targets’ during long-term persistence. These include immature myeloid precursor cells and
monocytes (Mirkovich et al., 1986), sialoadhesin-positive stromal macrophages of the bone marrow (Leclercq et al., 1996; Cotterell et al., 2000), hepatocytes (Gangneux et al., 2005) and fibroblasts (Bogdan et al., 2000). Promastigotes are phagocytosed by macrophages, either directly or after infection of neutrophils initially recruited to the sandfly bite (van Zandbergen et al., 2004). Promastigotes are targeted to vacuolar compartments in the macrophage that have the characteristics of mature phagolysosomes, where they differentiate to the smaller aflagellated amastigote stage. Amastigotes proliferate by binary cell division and can spread to other macrophages as well as some other phagocytic (i.e. dendritic cells) and no-professional phagocytic (i.e. fibroblasts) cells. The capacity of these pathogens to target and replicate within the mature phagolysosome compartment is remarkable. LPG is the primary ligand for multiple macrophage opsonic and pattern recognition receptors (Naderer et al., 2004). LPG plays a critical role in macrophage infection it protects promastigotes from a transient rise in reactive oxygen species (ROS) generated during phagocytosis (Spath et al., 2003). LPG may scavenge ROS directly and/or inhibit macrophage signaling pathways and the recruitment of the NADPH oxidase to the phagosome membrane (Lodge et al., 2006). Some researchers have suggested uptake via the host cell apoptotic receptors prevents the activation of microbicidal responses (as well as pro-inflammatory cytokine release) and may be critical for promastigote survival in neutrophils (Gueirard et al., 2007; van Zandbergen et al., 2007). In contrast to promastigote stages, intracellular amastigotes downregulate the expression of LPG (and other surface macromolecules) and lack a conspicuous surface coat (Naderer et al., 2004), although they retain a glycoalyx of parasite-synthesized GIPLs and host derived glycosphingolipids (McConville & Blackwell, 1991; Winter et al., 1994; Naderer et al., 2004). Leishmania amastigotes also synthesize inositol phosphoceramide (IPC), using sphingolipid bases salvaged from the host (Zhang et al., 2005), and this abundant phospholipid may also complement the functions of the GIPLs. The plasma membrane of Leishmania amastigotes is unusual in several other respects. It contains relatively high levels of externally exposed phosphatidylserine that may provide a mechanism for entering host cells via apoptotic cell receptors without activating microbicidal processes or proinflammatory responses (Wanderley et al., 2006).
Studies have been done by many researchers on different signaling processes like Protein Kinase C, the JAK2/STAT1 cascade, and the MAP Kinase pathway against which parasite has evolved strategies (Dey et al., 2007, Chandra & Naik, 2008, Kar et al., 2010, Matte & Descoteaux, 2010, Shadab & Ali, 2011)

Most species of *Leishmania* proliferate within individual, tight-fitting vacuoles, which have the characteristics of mature phagolysosomes and can fuse with late endocytic vesicles, phagosomes and autophagosomes, suggesting that there is a continuous flux of low molecular weight metabolites and macromolecules into the lumen of this compartment (Antoine et al., 1998; Burchmore & Barrett, 2001). Phagolysosome lumen contains a variety of carbon sources and essential nutrients, but is hexose poor (McConville et al., 2007; Opperdoes & Coombs, 2007). This requirement for hexose synthesis/uptake may be linked to the need for the pentose phosphate pathway [required for regeneration of NADPH and precursors for RNA and DNA synthesis (Maugeri et al., 2003) and/or the production of intracellular mannan (linear oligomers of b1–2mannose), the major short-term energy reserve of these parasites (Sernee et al., 2006). *Leishmania* are auxotrophic for many amino acids and intracellular stages must scavenge their essential amino acid needs from the phagolysosome in order to use them in protein and polyamine biosynthesis as well as a major carbon source (McConville et al., 2007). Amastigotes upregulate a large family of amino acid permeases for continuous supply of free amino acids. They also upregulate the expression of lysosomal cysteine proteases and endocytose host proteins from the lumen or limiting membrane of the phagolysosome, providing an alternative source of amino acids (Besteiro et al., 2007).

*Leishmania* must scavenge other essential nutrients (purines, haeme, vitamins) and cations (iron, magnesium) from the phagolysosome (Burchmore & Barrett, 2001; McConville et al., 2007). Most of the metabolite transporters are proton-symporters that utilize the low pH of the phagolysosome to drive high affinity uptake (Burchmore & Barrett, 2001). Leishmania amastigotes express a high affinity ferrous (Fe$^{2+}$) transporter, LIT1, which scavenges iron from the phagolysosome in competition to the host transporters (Huynh et al., 2006).
1.9 Scope of the Study

Visceral leishmaniasis (VL) caused by the parasite *L. donovani* is a potentially fatal disease. It is a major cause of morbidity and mortality in East Africa and the Indian subcontinent (Tripathi *et al.*, 2007) which is characterized by fever, weight loss, hepatosplenomegaly, anemia and depression of the immune system (den Boer *et al.*, 2009). Treatment options for VL are limited. The available drugs, pentavalent antimonials (Sb⁺), paromomycin and amphotericin B (AmB) are toxic, require parenteral administration, prolonged treatment duration & hospitalization and are thus inevitably very costly. The development of resistance to Sb⁺ in patients with VL in North Bihar, India, has increased the problems of treatment. Lipid formulations of AmB have lower toxicity and a shorter duration of therapy, but at prohibitive cost. Paromomycin also has some disadvantages, such as a 3-week treatment regimen, injection site pain, hepatic and ototoxicities (Banerjee *et al.*, 2011). The recent introduction of an oral treatment for VL in India, miltefosine, has raised hopes for improved treatment, but at the same time also raised concerns about safety, patient compliance and possible sub-optimal use leading rapidly to the development of resistance (den Boer *et al.*, 2009).

Cure of leishmaniasis, even during chemotherapy, appears to be dependent upon the development of an effective immune response that activates macrophages to produce toxic nitrogen and oxygen metabolites to kill the intracellular amastigotes (Banerjee *et al.*, 2011). The efficacy of treatment is usually associated with a depression of Th1 cells and preferential expansion of Th2 cells and, accordingly, skewing of T helper cells towards a Th1 response is considered a promising therapeutic strategy. Keeping current status of leishmania treatment in mind, use of low-dose or short course of an effective drug in combination with an immunomodulator is also an approach for effective treatment of leishmaniasis (Musa *et al.*, 2010). It is evident that drug combination therapy is an essential feature of antimicrobial treatment and is able to prevent/delay drug resistance, resulting in lesser toxic side effects, increase the spectrum of activity, higher compliance, fewer burdens on the health system, reduce treatment duration and cost as has been successfully employed for malaria, tuberculosis and HIV (Griensven & Boelaert, 2009).
In this study, we used three immunomodulators: Picroliv, $p$-tuftsin, and Pam3Cys in combination with antileishmanial drug, miltefosine.

Picroliv is a standardized fraction from the alcoholic extract of root and rhizome of *Picrorhiza kurrooa*, which contains 55–60% of picroside-I and kutkoside in a ratio of 1:1.5. Constituents of picrorhiza with hepatoprotective, immunomodulatory, and related activities have been described in detail recently by Verma et al. (2009). The immunostimulant activity and hepatoprotective potential of picroliv was demonstrated in mice by Puri et al. (1992) and Rajeshkumar & Kuttan (2000) respectively. It is under Phase III trial and is expected to be in market very soon for human use.

Tuftsin is capable of stimulating white blood cells (monocytes, macrophages, and neutrophils) and exhibits a wide spectrum of biological activities; notably enhances phagocytosis, immune response, bactericidal, tumoricidal, and antifungal activities (Wardowska et al., 2007). The immunostimulatory activity of tuftsin and its effect against microorganisms is further improved upon its incorporation in liposomes, after attaching a sufficiently long hydrophobic anchor of fatty acyl residue (palmitoyl) with the help of a spacer at its C-terminus (Gupta & Haq, 2005). This lipopeptide is commonly referred as palmitoyl tuftsin ($p$-tuftsin) and has been shown to express potent immunostimulatory activity both *in vitro* as well as *in vivo* models.

Pam3Cys, a synthetic bacterial lipopeptide (bLP) and TLR-2/1 ligand (Aliprantis et al., 1999; Buskas et al., 2006) have been reported to possess immunomodulatory properties (Sieling et al., 2003; Deetz et al., 2006; Imanishi et al., 2007; Brull et al., 2009). Pam3Cys as an in-built immunoadjuvant (Buskas et al., 2006) was also used in synthetic carbohydrate-based vaccines against anticancer therapy (Buskas et al., 2009; Renaudet et al., 2010). Hernández-Ruiz et al. (2010) reported that functional capacity of CD8 cells was restored when pre-incubated with Pam3Cys in diffused cutaneous leishmaniasis (DCL) patients during clinical trials. In another study, the synthetic cap tetrasaccharide was conjugated to the Pam3Cys to create fully synthetic carbohydrate vaccine against Leishmania (Hewitt & Seeberger, 2001).

Therapeutic switching or “piggy-back chemotherapy” is another potential source for new antileishmanial compounds (Croft et al., 2006). It involves the use of drugs already
marketed in other therapeutic areas against new targets. The approach of ‘therapeutic switching’ or drug repositioning can deliver new drugs more quickly and at lower cost, as much of the development work has already been done (Croft, 2005). This has been also well rehearsed in antimalarials e.g. primaquine, an antimalarial drug possesses antileishmanial activity (Jain et al., 2005). If a target enzyme has a mutation rate of $10^7$, the chance of resistance to a single agent developing is high, but the likelihood of developing resistance to two compounds with different targets is very low. Studies to identify such combinations are new for leishmaniasis; limited studies are under way to examine interactions between miltefosine with other antileishmanials to identify suitable combinations (Siefert & Croft, 2006). Bryceson (2001) advocated the examination of combinations of strong antileishmanials with “weak” drugs (for example, azoles) and expressed the need that combination therapy should be evaluated for safety and optimization for either concomitant or sequential administration of component drugs. Azoles originally developed as antifungal drugs are also the examples of this area. Ketoconazole, fluconazole, itraconazole, are essentially sterol biosynthesis inhibitors and Berman first reported their efficacy against $L. tropica$ in 1981. In the present study experiments were carried out using azoles in combination with antileishmanial drug, miltefosine and immunomodulator, picroliv individually and in different combinations in $in vitro$ and $in vivo$ conditions. Thus, the present work comprises the proposed objectives as under:

1. Effect of immunomodulators alone and their formulations on the efficacy of antileishmanial drugs.
2. Assessment of combined effect of azoles with existing antileishmanial drugs.