Introduction
1.0. Leishmaniasis

Parasitic diseases are of immense global significance as around 30% of the world's population experiences parasitic infections and impose a substantial burden of mortality and morbidity. Among human protozoan diseases, leishmaniasis alone comes second in public health importance (after malaria) as measured by mortality and Disability Adjusted Life Years (DALYs, Edwards and Krishna 2000, Table 1.1).

Leishmaniasis is a disease complex caused by a protozoan parasite of the genus *Leishmania*, which is transmitted to humans by the bite of female sandflies of the genus *Phlebotomus* in the Old World, and of the genus *Lutzomyia* in the New World. Over 20 species and subspecies of this parasite infect humans, each causing a different spectrum of symptoms (Fig. 1.2.), ranging from self-healing skin ulcers (Cutaneous leishmaniasis), disfiguring (Mucocutaneous leishmaniasis) to severe, life-threatening disease (Visceral leishmaniasis (Herwaldt, 1999).

<table>
<thead>
<tr>
<th>Disease</th>
<th>Mortality</th>
<th>DALYs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malaria</td>
<td>12,72,000</td>
<td>46,486,000</td>
</tr>
<tr>
<td><strong>Leishmaniasis</strong></td>
<td><strong>51,000</strong></td>
<td><strong>20,900,000</strong></td>
</tr>
<tr>
<td>African trypanosomiasis</td>
<td>48,000</td>
<td>15,25,000</td>
</tr>
<tr>
<td>South American trypanosomiasis (Chagas disease)</td>
<td>14,000</td>
<td>6,67,000</td>
</tr>
<tr>
<td>Schistosomiasis</td>
<td>15,000</td>
<td>17,02,000</td>
</tr>
<tr>
<td>Lymphatic filariasis</td>
<td>0</td>
<td>57,77,000</td>
</tr>
<tr>
<td>Onchocerciasis</td>
<td>0</td>
<td>4,84,000</td>
</tr>
</tbody>
</table>

Table 1.1. Global burden of parasitic diseases (source: www.who/int/tdr/disease/default.html).

1.1. History of leishmaniasis

The designs on pre-Colombian pottery and the evidence of the disease on thousand-year-old sculls prove its presence in the Americans for a long time (Manson-Bahr, 1996). The visceral leishmaniasis first came to the attention of Western doctors in 1824 in Jessore, India (now part of Bangladesh), but considered to be a form of malaria. Cunningham first noticed the *Leishmania* parasites in 1885 which were identified subsequently by William Boog Leishman in 1901 (Peters, 1988), but were considered to be trypanosomes initially. Simultaneously, in 1903 Captain Donovan described them as being new genus. Major Ross discovered the link between these organisms and kala-azar (Black fever) and named the organisms as *Leishmania donovani*. 
1.2. Epidemiology and geographical distribution of leishmaniasis

Leishmaniasis is prevalent in all tropical and subtropical zones of the world (Fig. 1.1.). It founds in parts of about 88 countries (22 in the New World and 66 in the Old World) affecting 12 million people worldwide with an estimated 1.5-2 million new cases occurring annually. Approximately 50,000 deaths due to visceral leishmaniasis (VL) occur annually, a death toll that is surpassed among the parasitic diseases only by malaria (www.who.int/emc/diseases-leish/leishmaniasis, 2001 report, Desjeux et al. 2004).

More than 90% of the world’s cases of visceral leishmaniasis occur in Bangladesh, Brazil, India, Nepal, and Sudan. While that of cutaneous leishmaniasis come from Afghanistan, Algeria, Brazil, Iran, Iraq, Peru, Saudi Arabia, and Syria. South America, Bolivia, Brazil, and Peru account for >90% of Mucocutaneous leishmaniasis cases (Herwaldt, 1999).

1.3. Clinical manifestations/types of leishmaniasis

Leishmaniasis consists of four main clinical syndromes: cutaneous leishmaniasis, mucocutaneous leishmaniasis, diffused cutaneous leishmaniasis, visceral leishmaniasis and post kala-azar leishmaniasis.

1.3.1. Cutaneous leishmaniasis (CL)

It is the most common form of leishmaniasis, produce large numbers of skin ulcers as many as 200 on the face, arms and legs (Fig. 1.2.A). Although this form is often self-healing, it can create serious disability and permanent scars which cause serious social prejudice.

1.3.2. Diffuse cutaneous leishmaniasis (DCL)

It is difficult to treat due to disseminated lesions that resemble leprosy and do not heal spontaneously (Fig. 1.2.B). This form is related to a defective immune system and is often characterised by the relapse after treatment.
1.3.3. Mucocutaneous leishmaniasis (MCL)

It is the most feared form, produces destructive and disfiguring lesions on the face. The lesions can lead to partial or total destruction of the mucous membranes of the nose, mouth, throat cavities and surrounding tissues (Fig. 1.2.C). The disease attacks cartilaginous areas but usually spares bony structures. Mucosal lesions are very painful and sometimes leads to sepsis.

1.3.4. Visceral leishmaniasis (VL) or Kala-azar

It is the most severe form, also known as kala-azar (Sanskrit word, meaning "black disease," or “black fever”) in India. It is a disseminated protozoal infection; the parasite migrates to the visceral organs such as liver, spleen and bone marrow. The clinical symptoms include irregular bouts of fever, substantial weight loss, swelling of the spleen and liver (hepatosplenomegaly), hypergammaglobulinemia, lymphadenopathy and anaemia (Fig. 1.2.D). Darkening of the skin is the characteristic feature of the disease (thus, the name kala-azar or black fever). The patient becomes immunocompromized and several secondary infections like pneumonia, tuberculosis, septicemia, measles and dysentery are omnipresent. Patient may die of hemorrhage (secondary to infiltration of the hematopoietic system), severe anemia or due to secondary infections. If left untreated, the fatality rate in developing countries can be as high as 100% within 2 years.

1.3.5. Post kala-azar dermal leishmaniasis (PKDL)

Even after full and adequate treatment of VL, after few months to years, a secondary form of the disease often sets in, called post kala-azar dermal leishmaniasis, or PKDL. First, small, measles-like skin lesions on the face appear which gradually increase in size and spread over the body (Fig. 1.2.E). Eventually the lesions may coalesce to form disfiguring, swollen structures resembling leprosy. 50% in Sudan, 10% in India and 2% in Africa of the VL patients develop PKDL. People with chronic PKDL can also serve as reservoir hosts for infection.

Fig.1.2. Symptoms and types of leishmaniasis: (A) cutaneous, (B) diffuse-cutaneous, (C) mucocutaneous, (D) visceral and (E) post kala-azar dermal leishmaniasis.
1.4. The extent of problem of Kala-azar

VL is prevalent in more than 80 countries in Asia, Africa, Southern Europe and South America. An increased number of worldwide travellers, U.S. Gulf War, AIDS patients, and environmental changes have led to the increased incidence of the disease (CDC, 2004; www.who.int/inf-fs/en/fact.html.factsheet116, 2000 report).

Of the estimated 500,000 people in the world infected each year, nearly 100,000 are estimated to occur in the WHO’s South East Asia Region (Bangladesh, India and Nepal) and India alone contributes more than 80% of the cases in the SEA Region. Approximately 200 million people in this Region are “at risk” from the disease. The disease is now being reported in 45 districts in Bangladesh, 52 in India and 12 from Nepal (www.searo.who.int).

In India the disease is endemic in eastern States namely Bihar, Jharkhand, Uttar Pradesh and West Bengal (Fig. 1.3.A) with an estimated 165.4 million population at risk (www.nvbdcp.gov.in). Bihar state has witnessed two major epidemic outbreak of Kala-azar in the years 1978 and 1992 (Fig. 1.3.B) with an official estimate of 430,000 cases over the past 11 years, although the actual number is believed to be at least 5 times as great (www.nvbdcp.gov.in). The number of cases has increased furiously after 2002 (Fig. 1.3.C; www.oneworldhealth.org).

![Fig. 1.3. (A) Geographical distribution of VL in India, (B) Kala-azar situation in India since 2002, (C) The increasing number of VL cases in India. Source: www.nvbdcp.gov.in/kala-azar.html](image-url)
1.5. VL/HIV co-infection

Coexistence of leishmaniasis with HIV adds a serious dimension to the problem and recently declared as a major emerging public health problem by WHO (WHO, 2000). So far, the co-infection has been reported in 34 countries in Africa, Asia, Europe, and South America (Fig. 1.4.). However, most of the cases have been notified in south-western Europe (France, Italy, Portugal and Spain) where, up to 70% of adult cases of VL are associated with HIV infection.

![Fig. 1.4. Country wise distribution of VL-HIV.](image)

1.6. Etiological agent

The causative agent of leishmaniasis is an obligate intracellular protozoan of the genus *Leishmania* (order Kinetoplastida). About 21 species of the parasite causes infection in human with various form of the disease (Table 1.2., Shaw, 1994; Ashford, 1997).

<table>
<thead>
<tr>
<th>Type of leishmaniasis</th>
<th>Region</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffuse-Cutaneous leishmaniasis</td>
<td>America</td>
<td><em>L.</em> mexicana</td>
</tr>
<tr>
<td>Old World</td>
<td></td>
<td><em>L.</em> aethiopica</td>
</tr>
<tr>
<td>Mucocutaneous leishmaniasis</td>
<td>Americas</td>
<td><em>L.</em> braziliensis</td>
</tr>
<tr>
<td>Old World</td>
<td></td>
<td><em>L.</em> aethiopica</td>
</tr>
<tr>
<td>Visceral leishmaniasis</td>
<td>Americas</td>
<td><em>L.</em> chagasi</td>
</tr>
<tr>
<td>S. Europe, N. Africa</td>
<td></td>
<td><em>L.</em> infantum</td>
</tr>
<tr>
<td>India, Kenya</td>
<td></td>
<td><em>L.</em> donovani</td>
</tr>
</tbody>
</table>

Table 1.2. Etiology of leishmaniasis.
1.7. Transmission of the disease

Predominantly, the parasite is transmitted by the bite of an infected female sandfly. These are primarily infected by animal reservoir hosts or infected humans. The reservoir of infection in Indian kala-azar is humans, whereas it is the rodents in African kala-azar, foxes in Brazil and Central Asia, and canines in the Mediterranean and Chinese kala-azar (Vidyashankar and Agrawal, 2002). Other uncommon modes of transmission are through congenital transmission, through blood transfusion and sharing needle, and rarely through inoculation of cultures.

1.8. Life cycle of Leishmania parasite

The Leishmania leads digenetic life cycle that completes in sandfly and vertebrate host (Fig. 1.5).

![Life cycle and life stages of the Leishmania parasite.](image)

During blood meal, infected sandfly expels promastigote metacyclic form (infective form) of the parasite (elongated, motile, and flagellated) into blood stream of the host. The parasites are phagocytosed by reticuloendothelial cells, where they metamorphose into amastigote stage (round, non-motile, aflagellated) and reproduce profusely by binary fission inside the phagolysosome and eventually burst free from the macrophages. The daughter
amastigotes then infect other reticuloendothelial cells such as macrophages, bone marrow cells, spleen cells and the kupfer cells of the liver spreading the disease within the mammal host macrophages which causes a systemic infection. As an insect bites an infected vertebrate host, it swallows infected macrophages, and the amastigotes differentiate into promastigotes, multiply and migrate into the midgut, transformed into metacyclic forms during the next four to seven days, and migrate to the cardial valve ready to be re-inoculated into a vertebrate host and the life-cycle continues (Killick-Kendrick and Molineux 1981).

1.9. Diagnosis

The routine diagnosis of leishmaniasis relies on either the microscopical demonstration of *Leishmania* amastigotes (Leishman Donovan, bodies) in aspirates from lymphoid tissue/liver, in slit skin smears and in peripheral blood. However, the retrieval of samples is uncomfortable for the patient and the isolation of parasites by culturing is time-consuming, difficult and expensive. A number of indirect immunological methods, such as enzyme-linked immunosorbent assay (ELISA), dipsticks and direct agglutination test (DAT), have been developed (Rijal et al. 2004; Guerin et al. 2002; Desjeux et al. 2004), however, there is still no gold standard diagnostic test. This is in part because of the fact that none of the tests is 100% sensitive and specific. Moreover, the spread of Leishmania/HIV co-infection complicates the use of the serological techniques as a result of low or lack of antibody responses of these patients (WHO, 2000).

Over the years, a number of different PCR assays have been developed for the detection of Leishmania DNA in a variety of clinical samples using sequence from ribosomal RNA genes, kinetoplast DNA, mini-exon-derived RNA genes and genomic repeats (Osman, 1998). PCR may also be useful for the confirmation of the diagnosis in HIV/*Leishmania* co-infected patients (Pizzuto et al. 2001).

1.10. Prevention of the disease

1.10.1. Vector control

Infection can be prevented by avoidance of sandfly bites through use of repellents or insecticides. Repellent with N, N-diethylmetatoluamide (DEET) should be applied to exposed skin. Impregnation of clothing with permethrin can provide additional protection. Impregnating bed nets and window screens with permethrin can provide some protection, as can spray dwellings with insecticide.
1.10.2. Vaccine

Several vaccination strategies have been tested in experimental CL and a number of vaccine trials have been initiated however, other than previous infection-simulated by inoculation of live *Leishmania* as a vaccine (leishmanization), none of the preparations have shown significant prophylactic efficacy. Relatively fewer efforts have been focused on VL because the immune response defining disease vs. protection is not so well established in VL (reviewed by Reed & Scott, 1993; Miralles et al., 1994). Various antigenic preparations which include the use of attenuated or killed parasites with/without adjuvants, crude antigen fractions, purified *L. donovani* membrane proteins and DNA vaccines has been tested for VL (reviewed by Ghosh and Bandyopadhyay, 2003; Tripathi et al. 2007). These strategies exhibited various degree of success in experimental model. Currently no effective vaccine against *Leishmania* is available anywhere in the world.

1.11. Chemotherapy

Due to non-existence of effective vaccine to date, chemotherapy is the only effective way to control *Leishmania* infections.

1.11.1. Pentavalent antimonial

Organic salts of pentavalent antimony [also abbreviated pentavalent Sb or Sb(V)] have been the cornerstone of treatment for all forms of leishmaniasis for more than 60 years (Herwaldt et al 1992). Two major pentavalent antimonials are currently used: Sodium stibogluconate (Pentostam®; manufactured by GlaxoSmithKline; available in US and UK) and meglumine antimoniate (Glucantime®; manufactured by Aventis; available in France and Italy). Sodium stibogluconate contains antimony 100mg/ml while; meglumine antimoniate contains antimony 85mg/ml. The drugs are given intravenously or intramuscularly, and they are equal in efficacy when used in equivalent doses. The recommended regimen consists of once-daily injection of full-dose drug (20mg/kg) for 30 days. Disadvantages of antimonials include the parenteral mode of administration, the long duration of therapy and the adverse reactions. Systemic toxicity normally relates to total dose administrated. Secondary effects (such as fatigue, body ache, electro-cardiographic abnormalities, raised aminotransferase levels and chemical pancreatitis) are frequent, albeit usually reversible. A novel liposome-based meglumine antimoniate formulation appears to be promising as a pharmaceutical product for the treatment of VL (Frezard et al., 2000). While active elsewhere in world, in India, antimonials are no longer
in use in Bihar, where as many as 65% of the previously untreated patients fail to respond to or promptly relapse after therapy with antimonial compounds (Sundar et al., 2000).

1.1.2. Pentamidine

Pentamidine isothionate (4mg/kg im., thrice-weekly for six weeks) was used in the treatment of antimonial-resistant VL. However, side effects such as myalgia, nausea, headache and hypoglycaemia were common at this dose, with an exceptional risk of developing irreversible diabetes. Besides, the drug achieves poor response rates (around 75%) when used as a second-line drug in antimonial-resistant areas that limited the interest of clinicians in pentamidine (Mishra et al., 1992; Das et al., 2000).

1.1.3. Amphotericin B and its formulations

Originally developed as a systemic antifungal agent, amphotericin B deoxycholate (Fungizone®) is also an efficient antileishmanial. However, its wider use in VL endemic regions is limited by its high cost and toxicity. The toxic measures varied from fever, shaking chills, hypotension, anorexia, nausea, vomiting, headache, dyspnea, and tachypnea, nephrotoxicity (kidney damage) and hepatotoxicity. The liposomal amphotericin B formulation, AmBisome® is registered treatment for VL (Meyerhoff, 1999) and a single-dose therapy of 5mg/kg has been shown to cure 90% of patients in India (Sundar et al. 2006) but its use in is limited by its high cost. Amphotericin B lipid complex (Abelcet®) and an amphotericin B colloidal dispersion (Amphocil™) have also been manufactured (Robinson and Nahata 1999), but their use against VL has not been as extensive as AmBisome® and they too are unaffordable to the patients (Murray, 2000).

1.1.4. Miltefosine

Miltefosine, initially developed as an anticancer drug, is the first effective oral treatment for VL and the latest antileishmanial drug to enter the market. It has been registered in India in March 2002 for oral treatment of VL and in Colombia for CL in 2005 (Sundar et al., 2002; Croft et al., 2006). It has been used successfully to treat cases resistant to conventional antimony therapy. However, the drug does have some serious side effects such as vomiting, diarrhea and teratogenicity; therefore, it can not be given to pregnant women. The long half-life of the drug might encourage the emergence of resistance (Bryceson, 2001). Variation in species sensitivity is also a concern as it could contribute to different clinical outcomes in different regions as observed in a recent trial against CL in Colombia and Guatemala (Soto et al., 2004).
1.11.5. Paromomycin

Paromomycin (PM), an aminoglycoside antibiotic, was originally identified as an antileishmanial in the 1960s and has been used in clinical trials for both VL and CL. Development of the parenteral formulation of PM, a drug with poor oral bioavailability, for VL has been slow, but phase III clinical trials are currently ongoing in India under the aegis of the Institute of One World Health (www.iowh.org) and in East Africa managed by DNDi and partner institutes (www.dndi.org).

This drug also has limitations such as resistance to paromomycin could be induced in *L. donovani* promastigotes experimentally *in vitro*. The resistance was specific to PM, stable in nature and its mechanism seems to be due to decreased drug uptake (Maarouf et al., 1998). A combination of PM and sodium stibogluconate has been the subject of various clinical trials in Sudan and India (Seaman et al., 1994; Thakur et al., 2000), but further studies to optimize the combination and define drug-drug interactions are required.

1.11.6. Sitamaquine

Another oral drug that might have an impact on VL is the 8-aminoquinoline derivative sitamaquine, currently in development with GlaxoSmithKline (http://www.gsk.com). The antileishmanial activity of this compound was first identified in the 1970s at the Walter Reed Army Institute of Research (http://www.wrair.army.mil). Limited Phase I/II clinical trials have been completed with varying levels of success (67-92% cure rate) (Dietze et al., 2001; Wasunna et al., 2005; Jha et al., 2005). Sitamaquine is rapidly metabolized, forming diethyl and 4-CH₂OH derivatives, which might be responsible for its activity. Toxicity appears to be relatively mild, it causes mild methemoglobinemia and further studies are underway on this drug.

1.11.7. Allopurinol

At the beginning of the 1980s, non-randomized trials showed that a combination of antimonials and allopurinol (15mg/day) was efficacious in treating VL (Chunge et al., 1985; Jha, 1983; Ragusa et al., 1993). At the present, allopurinol is not in use as monotherapy in India.

1.11.8. Combination therapies

Drug combinations have proven to be an essential feature of antimicrobial treatment. Previous studies on drug combinations for VL, for example, allopurinol plus sodium stibogluconate (Chunge et al., 1985) and paromomycin plus sodium stibogluconate (Neal et al., 1995; Thakur et al., 2000) were aimed to improve efficacy. The Kala-azar patients usually fail to initiate Th1 type of immune response, which is initiated by IL-12 and mediated through IL-2
and IFN-γ (Ghalib et al., 1993; Kenney et al., 1998; Sundar et al., 1994). Addition of IFN-γ as adjunct to Sb(V) might improve the treatment outcome (Badaro et al., 1993; Sundar, 1994), but it would also be a costly affair.

Eventually, there is no cheap, effective and safe drug available for the treatment of VL.

1.12. Clinical resistance to antimonial drugs and its implications

In general, resistance is defined as a decline in the efficacy of a drug against a population of parasites previously susceptible to that compound (Alicia Ponte-Sucre, 2003). Pentavalent antimonial drugs were used worldwide for the treatment of VL and CL for over six decades as a first-line treatment. But from past 15 years acquired resistance has become a major clinical threat (Table in Fig. 1.6.). Until the late 1970s, a small daily dose (10mg/kg; 600 mg maximum) for short duration (6 to 10 day) was considered adequate. 13% treatment failure in 1980 was reported with this regimen from most severely affected areas viz Muzaffarpur, Samastipur, Vaishali, and Sitamarhi (Jha, 1980).

The dose was revised to 20mg/kg/day up to a maximum of 850mg for 20 days in 1984 by WHO which was found to cure only 81% of patients (Thakur et al., 1988) and cure rate was further decreased year after year even after extended duration of treatment (30 days) in a hyperendemic district of Bihar (Fig. 1.6.). In villages of Darbhanga and Sitamarhi districts in Bihar, 100% treatment failure has been observed (Jha 1992, 1998). Therefore, it became clear that Sb(V) refractoriness has been increasing. There are reports of antimony resistance spreading to the Terai regions of Nepal also, where up to 24% of patients seem to be unresponsive (Rijal et al., 2003). Obviously, possibility of host failure in this subset population is unlikely. The widespread misuse of the drug, irregular use and incomplete treatments are the possible reason of emergence of antimony resistance (Sundar et al., 1994).

Because of very few drugs in the pipeline, resistance to first line drug(s) has a very big impact on the treatment of leishmaniasis. Therefore, understandings of resistance mechanisms operating in Leishmania parasite are very important. Such studies will help to design the strategy to deal with parasite by developing tools to recognize resistance early in infection and prevent useless and often toxic chemotherapy; by suggesting more rational use of drugs and drug combinations to minimize development of resistance; by pinpointing intracellular drug targets and defense mechanisms allowing the development of drug analogues that evade the most common defenses.
Introduction

A multiplicity of the resistance mechanism has been described in vitro developed *Leishmania* drug resistant mutants. With the availability of resistant field isolates, it has now become possible to elucidate mechanisms of clinical resistance. Limited studies on field isolates revealed that the mechanism of natural antimony resistance is multi-factorial and may differ from laboratory resistance. This study will help in exploration of mechanism contributing towards the clinical ineffectiveness of pentavalent antimony and also in rational drug design for the treatment of resistant *Leishmania* spp.