

## Review Article

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# New developments in diagnosis of leishmaniasis

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**In this review recent advances made in the field of human leishmaniasis have been discussed with special emphasis on the parasite, and various serological and molecular methods of diagnosing the infection. The article also reviews various modes of parasite transmission including vector borne, blood transfusion, needle sharing, sexual and person-to-person. Microbiological methods including the bone marrow, spleen, liver, lymph node aspirations and various staining methods used to demonstrate the amastigotes of the parasites and various *in vitro* promastigote culture methods are discussed in detail with their comparative sensitivity rates.**

**Key words** Amastigotes - cutaneous leishmaniasis - *Leishmania*-HIV co-infection - mucocutaneous leishmaniasis - promastigotes - transmission - visceral leishmaniasis, rKE16

Leishmaniasis, a vector-borne disease caused by obligate intramacrophage protozoa, is characterized by diversity and complexity<sup>1,2</sup>. A total of about 21 *Leishmania* species have been identified to be pathogenic to human. *Leishmania* are one of several genera within the family *Trypanosomatidae*, and are characterized by the possession of a kinetoplast, a unique form of mitochondrial DNA<sup>3</sup>. In most instances they cause disease in animals, and humans become infected incidentally when they enter in an area of endemicity.

Leishmaniasis have been considered tropical afflictions that together constitute one of the six entities on the World Health Organization/Tropical Disease Research (WHO/TDR) list of most important diseases<sup>1,2</sup>. The disease is endemic in 88 countries of

5 continents with a total of 350 million people at risk and 12 million cases. Of the 88 endemic countries, 22 are in the New World and 66 in the Old World with an estimated incidence of 1-1.5 million cases of cutaneous leishmaniasis (CL) and 500,000 cases of visceral leishmaniasis (VL). Of all the VL cases, more than 90 per cent are from India, Bangladesh, southern Sudan, Nepal and northeast Brazil. Despite this widespread geographic distribution, human leishmaniasis is often very focal within an endemic area, leading to 'hotspots' of disease transmission<sup>2</sup>.

### **Aetiological agent**

Leishmaniasis is caused by different species of *Leishmania*, under the kingdom Protista and phylum

Euglenozoa. This phylum is characterized by the presence of an associated cytostome (mouth) supported by one of three microtubule groups that arise from the flagellar bases. A number of other ultrastructural peculiarities also distinguish the group, most notably the presence of a paraxial rod in each flagellum, which respectively has tubular and latticed structures<sup>4</sup>. These flagellated protozoa known as kinetoplastids, include a number of pathogens responsible for serious diseases in humans and other animals. They are characterized by the presence of a kinetoplast, a DNA-containing granule located within the single mitochondrion and associated with the flagellar bases. The members of family Trypanosomatidae have reduced or absent cytostomes and feed entirely through absorption. They have complex life-cycle involving more than one host, and go through various morphological stages. All members are exclusively parasitic. There are nine genera under this family: *Blastocrithidia*, *Crithidia*, *Endotrypanum*, *Herpetomonas*, *Leptomonas*, *Phytomonas*, *Wallaceina*, *Trypanosoma* and *Leishmania*. This review pertains only to the parasite *Leishmania* which causes leishmaniasis in humans.

There are mainly three clinical form of leishmaniases, caused by various species of *Leishmania* (Table I). The visceral leishmaniasis (VL) is caused by species of *L. donovani* complex that consists mainly of *L. (d) infantum*, *L. (d) donovani* and *L. (d) chagasi*<sup>5</sup>. Mucocutaneous leishmaniasis (MCL), or espundia, produces lesions, which can lead to extensive and disfiguring destruction of mucous membranes of the nose, mouth and throat cavities. The causative species of MCL are *L. (viannia) braziliensis* and *L. (viannia) guyanensis*. The cutaneous leishmaniasis (CL) can produce large numbers of skin ulcers, as many as 200 in some cases, on the exposed parts of the body<sup>6,7</sup>. The causative species of CL are, *L. major*, *L. tropica*, *L. mexicana* and *L. amazonensis*. The fourth form is diffuse cutaneous leishmaniasis (DCL). It is an anergic variant of localized cutaneous leishmaniasis in which lesions are disseminated, resembling lepromatous leprosy. The disease is caused by *L. (mexicana) amazonensis* and *L. aethiopica*<sup>5,7,8</sup>.

### Morphology of the parasite

The parasite *Leishmania* exists at least in two forms:

*Amastigote form:* Amastigotes are ovoid and non-flagellated form of *Leishmania*, measuring 3-5  $\mu\text{m}$  in length<sup>9</sup>. On simple light microscopy, a central round or oval nucleus and adjacent but smaller round or rod shaped kinetoplast can be discovered. An infolding of the surface membrane creates an internal space, termed as 'flagellar pocket'. The flagellum is not functional in amastigotes and does not extend beyond the cell body. In addition to anchoring the flagellum the main function of the pocket is to function as a site of endocytosis and exocytosis<sup>10</sup>. Immediately below the origin of the flagellum lies a dense mass of mitochondrial DNA known as kinetoplast. The kinetoplast DNA is composed of several thousand circular DNA molecules linked together in a catenated network<sup>11</sup>. These DNA networks are of two types: each kinetoplast contains 25-250 maxicircles of approximately 30kb, and 5000-10,000 minicircles of about 2kb size each. Together these constitute the mitochondrial genome. The cytoplasm contains both rough and smooth endoplasmic reticulum. The Golgi complex is typically found in the vicinity of the flagellar pocket, which probably reflects the role of this organelle in the endocytic and exocytic pathways. Lysosomes are also found in the cytoplasm together with an organelle unique to kinetoplastids, the glysosome<sup>12</sup>.

The developmental cycle is initiated by the interaction of metacyclic promastigotes with skin macrophages. After uptake and internalization of metacyclic promastigotes in a phagosome, fusion with lysosomes proceeds as normal and the parasite inhabits a secondary lysosome or phagolysosome. During this process the metacyclic promastigote transforms into an amastigote within 12-24 h and continues to grow and divide within the phagolysosomal compartment. The amastigotes have to overcome two environmental challenges: the battery of lysosomal enzymes and low pH (4.5-5.5). Low pH is not a problem as amastigotes seem to be acidophiles: they are metabolically more active at low pH<sup>13,14</sup>.

**Table I.** Human pathogenic species of *Leishmania* in New World, their clinical manifestations and geographical distribution

Country	Species	Disease forms
Argentina	<i>L. chagasi</i>	VL
	<i>L. (V.) braziliensis s.l.</i>	CL
Belize	<i>L. mexicana</i>	CL
	<i>L. (V.) braziliensis s.l.</i>	CL
Bolivia	<i>L. amazonensis</i>	CL, DCL
	<i>L. chagasi</i>	VL
	<i>Leishmania</i> sp.	CL
	<i>L. (V.) braziliensis s.l.</i>	CL, MCL
Brazil	<i>L. amazonensis</i>	CL, DCL, MCL,
	<i>L. chagasi</i>	VL, (CL)
	<i>Leishmania</i> sp.	CL
	<i>L. (V.) braziliensis</i>	CL, MCL
	<i>L. (V.) guyanensis</i>	CL, MCL
	<i>L. (V.) lainsoni</i>	CL
	<i>L. (V.) naiffi</i>	CL
	<i>L. (V.) shawi</i>	CL
Colombia	<i>L. amazonensis</i>	CL, DCL
	<i>L. chagasi</i>	VL
	<i>L. mexicana</i>	CL, DCL
	<i>L. (V.) braziliensis s.l.</i>	CL, MCL
	<i>L. (V.) colombiensis</i>	CL
	<i>L. (V.) guyanensis</i>	CL
	<i>L. (V.) panamensis</i>	CL, MCL
Costa Rica	<i>L. mexicana</i>	CL
	<i>L. (V.) braziliensis s.l.</i>	CL, MCL
	<i>L. (V.) panamensis</i>	CL
Dominican Republic	<i>L. mexicana-like</i>	DCL
Ecuador	<i>Leishmania</i> sp.	CL
	<i>L. mexicana</i>	CL
	<i>L. (V.) braziliensis s.l.</i>	CL, MCL
El Salvador	<i>L. chagasi</i>	VL
	<i>L. mexicana</i>	CL
French Guyana	<i>L. amazonensis</i>	CL, DCL
	<i>L. (V.) braziliensis s.l.</i>	CL, MCL
	<i>L. (V.) guyanensis</i>	CL
	<i>L. (V.) naiffi</i> <sup>c</sup>	CL
Guadeloupe	<i>L. chagasi</i>	VL
	<i>Leishmania</i> sp.	CL
Guatemala	<i>L. chagasi</i>	VL
	<i>L. mexicana</i>	CL
	<i>L. (V.) braziliensis s.l.</i>	CL
Guyana	<i>L. (V.) guyanensis</i>	CL
	<i>Leishmania</i> sp.	MCL
Honduras	<i>L. chagasi</i>	VL, CL
	<i>L. mexicana</i>	CL, DCL
	<i>L. (V.) braziliensis s.l.</i>	CL, MCL
	<i>L. (V.) panamensis</i>	CL, MCL
Martinique	<i>Leishmania</i> sp.	CL
Mexico	<i>L. chagasi</i>	VL
	<i>L. mexicana</i>	CL, DCL
Nicaragua	<i>Leishmania</i> sp.	CL, DCL
	<i>L. chagasi</i>	VL
	<i>L. (V.) braziliensis s.l.</i>	CL, MCL
	<i>L. (V.) panamensis</i>	CL, MCL
	<i>L. (V.) braziliensis/panamensis</i>	CL
Panama	<i>L. aristedesi</i>	CL
	<i>L. (V.) braziliensis s.l.</i>	CL
	<i>L. (V.) panamensis</i>	CL
	<i>Leishmania</i> sp.	MCL
Paraguay	<i>L. amazonensis</i>	CL, DCL
	<i>L. chagasi</i>	VL
Peru	<i>L. (V.) braziliensis s.l.</i>	CL, MCL
	<i>L. (V.) peruviana</i>	CL
	<i>L. (V.) braziliensis/peruviana</i>	CL, MCL
Surinam	<i>Leishmania</i> sp.	CL
USA	<i>L. mexicana</i>	CL, DCL
Venezuela	<i>L. chagasi</i>	VL
	<i>L. garnhami</i>	CL
	<i>L. pifanoi</i>	CL, DCL
	<i>L. venezuelensis</i>	CL
	<i>L. braziliensis s.l.</i>	CL, MCL
	<i>L. colombienensis</i>	VL
	<i>L. braziliensis/</i>	CL
	<i>L. guyanensis</i>	

VL, Visceral leishmaniasis; CL, cutaneous leishmaniasis; MCL, mucocutaneous leishmaniasis; DCL, diffuse cutaneous leishmaniasis

Source: Ref. 7

**Promastigote form:** In the sandfly host the parasite is found in the promastigote form. The transformation of amastigotes to promastigotes starts within hours of ingestion of the amastigotes (either free or intracellular) and occurs exclusively in the gut. The amastigotes are completely transformed into motile promastigotes within 24-48 h and keep on dividing by binary division. The mature metacyclic promastigotes are accumulated in the midgut and foregut<sup>1,4,5</sup>. The main difference from amastigotes is that the cell body is elongated, in the range of 8-15  $\mu\text{m}$ , the flagellum emerges from the cell body, and is functional, making these cells motile. The promastigote flagellum has a paraxial rod, a paracrystalline structure running parallel to the microtubules of the axoneme. There is a variety of different promastigote forms that can be separated on morphological grounds but functional distinction is less complete (e.g., procyclic promastigotes, paramastigotes, nectomonad promastigotes, haptomonad promastigotes, paramastigotes and metacyclic promastigotes). The first developmental event in the sandfly is probably the transformation of amastigotes to procyclic promastigotes. These events occur in the posterior midgut of the sandfly<sup>5,15</sup>. Multiplication of procyclic promastigotes occurs, they elongate and transform to nectomonad forms of 15-20  $\mu\text{m}$  body length. Approximately 3 days after bloodfeeding the peritrophic membrane (a secretory sheath) which contains these parasites usually begins to breakdown and promastigotes begin to set free and they forward to the anterior midgut<sup>5,16-18</sup>.

Lipophosphoglycan (LPG) plays important role in attachment and maturation of infection<sup>19,20</sup>. Within five days the infection reaches to the anterior midgut. Here haptomonad promastigotes are attached to the stomodeal valve<sup>21</sup>. From day 5 onwards, increasing numbers of small (5-8  $\mu\text{m}$ ), narrow, highly motile, metacyclic promastigotes can be observed in the lumen of the anterior midgut or foregut, or both. The role of a fall in gut pH in inducing metacyclogenesis is more speculative, but promastigotes are known to acidify their culture media during growth *in vitro*<sup>22</sup>. In a suitable culture medium and at an appropriate temperature (usually 26°C) within 24-28 h at pH 7.0-7.5, these promastigotes are obtained.

## Modes of transmission

Worldwide, vector-borne transmission is the most common mode of transmission<sup>1,4,5,23</sup>. Other modes of transmission such as parenteral, congenital, sexual, occupational (needle stick) exposures, and person-to-person transmission could also theoretically occur<sup>24</sup>. Because in endemic areas it is generally presumed that leishmaniasis is transmitted through sandfly bites, transmission via other modes might be underestimated<sup>1,4,5</sup>.

**Vector-borne transmission:** When sandflies bite an infected host, they swallow *Leishmania* amastigotes, which circulate freely in the host's blood or inside peripheral blood mononuclear cells (PBMCs). These amastigotes migrate to the sandfly's proboscis where they develop into stationary, infective-stage organisms that could be qualified as "metacyclic" promastigotes. When this infected sandfly bites a second host, e.g., a human being, these promastigotes are released and deposited on the site of bite or injected along with potent vasodilators (*i.e.*, maxadilan) that produce long-lasting erythema<sup>25-30</sup>. Macrophages phagocytize these promastigotes and, enable them to survive inside the phagolysosome, and again transform into amastigotes. There, they proliferate by binary fission, ultimately causing lysis of the host cells and infection of the surrounding macrophages<sup>25,30</sup>. This ecologic cycle ends when another sandfly comes to feed on this host, carrying the infection to another host.

**Transfusion-transmitted leishmaniasis:** Transfusion-transmitted leishmaniasis has been reported widely from many countries including India<sup>31</sup>. It requires the parasites to be present in the peripheral blood of the donor, preferably asymptomatic, survive processing and storage in the blood bank, and infect the recipient. Though, the incidence of parasitaemia in infected asymptomatic patients should be exceedingly low in those who satisfy the screening criteria of blood banks, but the strict screening of volunteers may not be done always that leads to transfusion-transmitted leishmaniasis.

After HIV epidemic, most of the countries have adopted a policy of rational use of blood and safe

**Table II.** Human pathogenic species of *Leishmania* in Old World, their clinical manifestations and geographical distribution

Geographical distribution	Causative species	Disease form
North Africa, Central & West Asia	<i>L. major</i>	rural, zoonotic, cutaneous leishmaniasis, oriental sore
Central & West Asia, Western India	<i>L. tropica</i>	urban, anthroponotic cutaneous leishmaniasis, oriental sore
Ethiopia, Kenya	<i>L. aethiopica</i>	cutaneous leishmaniasis, diffuse cutaneous leishmaniasis
Indian subcontinent, (India, Nepal, Bangladesh) East Africa	<i>L. donovani</i>	visceral leishmaniasis, kala azar, post kala-azar dermal leishmaniasis (PKDL)
Mediterranean basin, Central & West Asia	<i>L. infantum</i>	infantile visceral leishmaniasis

Source: Ref. 5

blood transfusion. Despite this, a number of pathogens including some viruses and parasites including *Leishmania* continue to get transmitted through transfusion<sup>32-35</sup>, because except screening for HIV, hepatitis B and C, majority of blood banks are not screening their donor blood samples for other potential pathogenic organisms, such as *Leishmania*. The *L. donovani* are expected to remain present in the blood for an undefined period between the bite of sandfly and their final localization to the target organs. By the time the clinical symptoms appear in the patient, the parasites may have already been circulating in the peripheral blood<sup>36</sup>. Such asymptomatic but parasitaemic blood donors may serve as a source of transfusion-transmitted leishmaniasis. Circulation of *L. donovani*, *L. tropica*, and *L. braziliensis* in the peripheral blood has been reported by various workers<sup>37-40</sup>. Usually asymptomatic infection does not persist for more than one year, but rarely asymptomatic infection may last for decades<sup>39</sup>. However, with the advances in diagnostic techniques such asymptomatic cases can be diagnosed easily<sup>41</sup>.

*In vitro* studies have clearly shown that viscerotropic *L. tropica* survived as intracellular parasites in monocytes for 30 days at 4°C, and for at least five days at 24°C<sup>38</sup>. Intracellular parasites survived longer than did stationary phase extracellular promastigotes or free amastigotes. The parasites survived as intracellular forms in monocytes

for 25 days in the red blood cell fraction kept at 4°C, five days in the platelet fraction kept at 24°C, 35 days in the red blood cell fraction frozen with glycerol, and for 30 days in unprocessed whole blood left at 4°C. It was reported that one detectable viscerotropic *L. tropica* parasite survived for 15 days when whole blood was kept under blood bank conditions, but an inoculum of 256 organisms was required in culture to have viable parasite(s) up to 35 days<sup>38</sup>. Animal studies were carried out to determine the presence of infected monocytes in the blood of cutaneously infected animals and the possibility of transmitting the disease by blood transfusion from both infected donor animals and seeded CPDA-1 bag of human whole blood kept for 30 days at 4°C under blood bank conditions<sup>38</sup>. It was observed that *Leishmania* not only survived under blood bank storage conditions, but the parasites retained their infectivity to healthy experimental animals<sup>38</sup>. The parasite may remain latent inside the body also for up to 30 yr after clinical cure<sup>39,40</sup>.

Of the 11 cases of transfusion transmitted leishmaniasis reported so far in the literature<sup>42-46</sup>, 10 were individual case reports and in one study 32 cases of kala-azar from Brazil were reported out of 82 patients undergoing haemodialysis<sup>47</sup>. All 10 individual case reports were from Asia and Europe<sup>42,45</sup>. Of these five were infants and four were children of less than 6 yr age. Only one adult case of transfusion-transmitted leishmaniasis was reported

by us in a 30 yr old female from Haryana, India<sup>31</sup>. The first report of transfusion-transmitted kala-azar came from China in 1948<sup>42</sup>. Other reports of transfusion-transmitted kala-azar followed these two reports and have been published from France<sup>43</sup>, Sweden<sup>44</sup>, Belgium<sup>45</sup>, United Kingdom<sup>46</sup>, India<sup>31</sup> and Brazil<sup>47</sup>. The time between the transfusion of the *Leishmania* infected blood and first clinical manifestation was available in 10 reports; with a mean incubation period of  $7.4 \pm 5$  months. Recently one case of kala-azar transmission through platelet transfusion has also been reported from India<sup>48</sup>.

**Needle-sharing:** By March 1993, 18,347 cases of AIDS and about 200 cases of HIV-associated leishmaniasis were detected in Spain, of which more than 85 per cent occurred among intravenous drug users (IVDUs)<sup>49</sup>. The infection is so common that 17 per cent of 111 bone marrow aspirates (BMAs) in HIV-positive subjects with fever had amastigote. Alvar *et al*<sup>49</sup> described a high variability of *L. infantum* zymodemes circulate among drug users who share syringes and, therefore, act as reservoirs to a degree that is as yet unknown. In another study Molina *et al*<sup>50</sup> tested the indirect xenodiagnosis of VL in 10 HIV-infected patients, of whom nine were IVDUs; they found that minute volumes of blood (0.3-0.5  $\mu$ l) proved infective to *Phlebotomus perniciosus*, thereby concluding that the possibility of needle-mediated transmission cannot be ruled out. Other studies have been conducted with similar findings<sup>50-55</sup>.

**Congenital transmission:** At least 10 cases of congenital transmission have been reported in the literature<sup>56-66</sup>. Most of these cases have been reported from India. The first case of congenital leishmaniasis was reported in 1926 by Low & Cooke<sup>57</sup>. *L. donovani* has been found to traverse the placenta of the Syrian hamster and mice but no parasite could be demonstrated in the organs of an aborted 5 months foetus while the placenta had numerous amastigotes<sup>60</sup>. This indicated that the infection might have occurred in most of these cases during the exchange of mother's blood at the time of passage of foetus through birth canal. Congenital VL manifests within three months of life and manifestations are by and large similar to that

*Leishmania* acquired through sandfly bite, but the course is usually rapid<sup>58-66</sup>. Pregnant women become more susceptible to leishmaniasis due to shift of cell mediated immunity to humoral immunity<sup>67</sup>.

**Sexual transmission:** Urine and prostatic fluid cultures from patients with VL have yielded promastigotes. Reports of sexual transmission<sup>51,52,67-70</sup> include transmission from a man to his wife, as well as probable transmission in a homosexual man with AIDS who had rectal lesion and to have admitted frequent receptive anal intercourse while vacationing in endemic areas of Spain<sup>51,52</sup>.

**Laboratory-acquired transmission:** Laboratory-acquired infections caused by *L. tropica*, *L. braziliensis* and *L. donovani* had been reported<sup>51,52,71</sup>. Many of them were from needle-stick injuries, which led to ulcers at the inoculation site; few were related to handling of contaminated specimens; and some to oral exposure, which led to visceral involvement. It is recommended that individuals with a significant exposure history should be followed with monthly serology for 6 months and again at 12 months. Aggressive attempts at parasitologic diagnosis should be pursued in individuals who have an unexplained systemic illness or develop a skin lesion at or near a site of accidental contamination.

**Person-to-person transmission:** Some data, mainly from animal models, suggest that this mode of transmission is theoretically possible via contact with infected fluids (nasal and oral secretions, tonsillopharyngeal mucosa, and urine) of patients with VL<sup>72-74</sup>. Transmission to the suckling offspring of infected female hamsters by direct contact with lesions has been reported, as well as infection in BALB/c mice after prolonged contact with infected animals<sup>73,74</sup>. However, current clinical experience shows that person-to-person transmission is extremely rare.

### **Leishmaniasis in AIDS patients**

*Leishmania*-HIV co-infection has emerged as a major complication of leishmaniasis. Most of the *Leishmania* endemic countries are also facing HIV

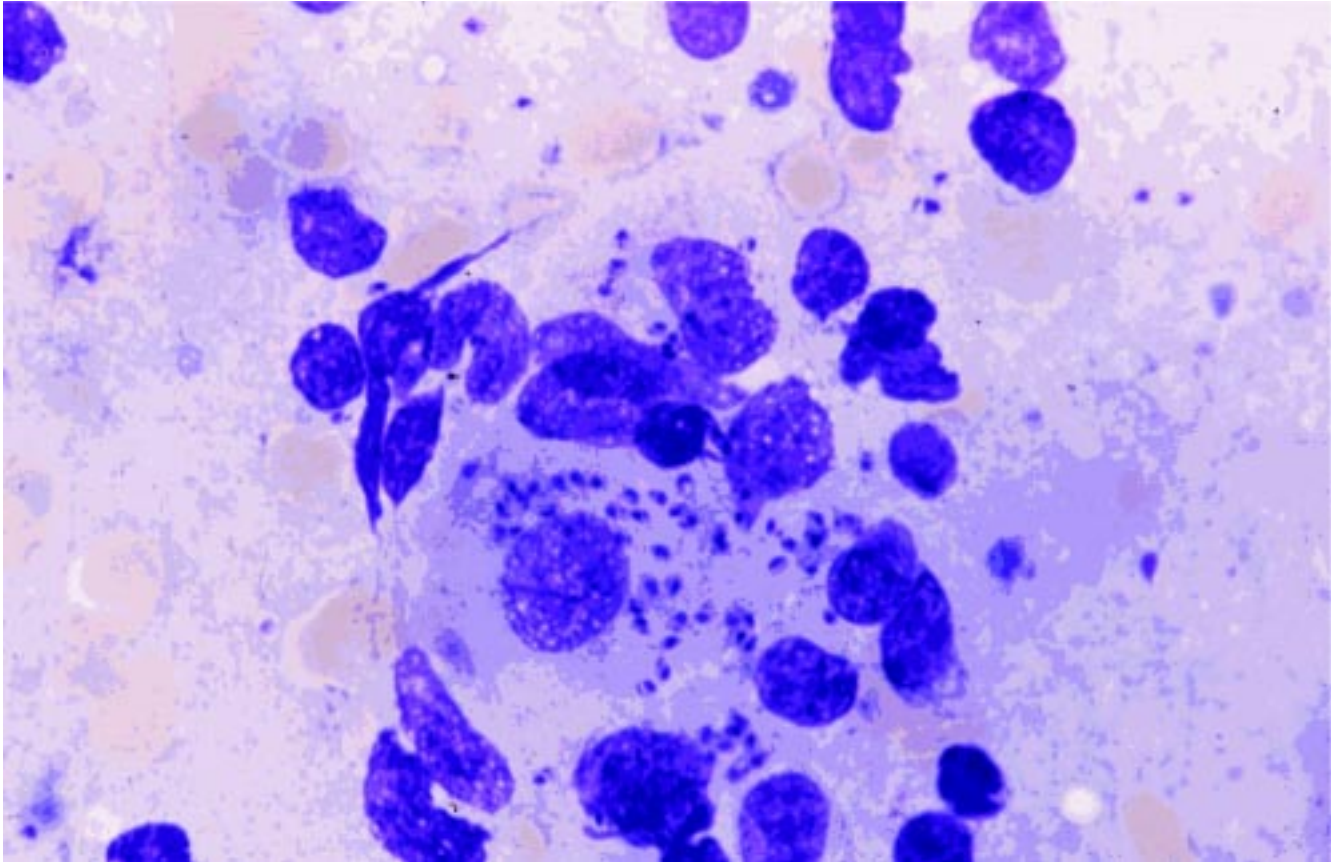
epidemic and resulting into high rate of co-infection. Of the first 1700 cases of *Leishmania*-HIV co-infection reported to the World Health Organization from 33 countries up to 1998, 1440 cases were from the Mediterranean region: Spain (835); Italy (229); France (259); and Portugal (117). It is very important to know that HIV modifies the clinical presentation of leishmaniasis in the co-infected patients<sup>75</sup>. Several atypical aetiologic strains and species have been described in HIV-infected subjects. In HIV-associated leishmaniasis caused by non-visceralizing species the parasite may disseminate to reticuloendothelial system and various other organs, and conversely the visceralizing species can manifest in atypical manner<sup>75</sup>. Fulminant presentation of VL is possible in patients with AIDS, and relapses are usual. VL is now the fourth most common opportunistic parasitic disease in HIV-positive individuals in Spain and 20-40 per cent cases had absence of splenomegaly<sup>52</sup>. In Africa particularly Ethiopia and Sudan and Southern Europe, HIV-*Leishmania* co-infection is regarded as emerging disease, and as many as 70 per cent adults with VL also have HIV infection<sup>2</sup>. Recently this co-infection has been noticed in Asia also. At least 10 cases of HIV-*L. donovani* have been reported from India<sup>76</sup>. Dissemination is common and gastrointestinal involvement is commonest. The *Leishmania* amastigotes can be seen in gastrointestinal mucosal biopsy specimen and are commonly found in Kaposi's sarcoma cutaneous lesions concomitant with VL. *Leishmania* parasites were recently found in herpes zoster lesions in an HIV-positive patient<sup>75</sup>. Leishmaniasis has also been reported presenting as a dermatomyositis-like eruption in three patients with AIDS<sup>52,53</sup>.

## Diagnosis

The clinical signs and symptoms are not pathognomic of VL or CL. The kala-azar may be confused with other similar conditions such as malaria, tropical splenomegaly, schistosomiasis or cirrhosis with portal hypertension, African trypanosomiasis, millitary tuberculosis, brucellosis, typhoid fever, bacterial endocarditis, histoplasmosis, malnutrition, lymphoma, and leukaemia<sup>77</sup>. Similarly, numerous primary and secondary skin conditions are frequently

overdiagnosed as early lesions of cutaneous leishmaniasis in endemic countries and in non endemic countries CL is misdiagnosed as other diseases. Some of the common conditions that should be differentiated from cutaneous leishmaniasis are tropical ulcers, impetigo, infected insect bites, leprosy, lupus vulgaris, tertiary syphilis, yaws, blastomycosis, skin cancer, etc<sup>1,4,5,77,78</sup>. Whenever, there is suspicion of leishmaniasis, only the laboratory diagnosis can give final answer. There are several methods of laboratory diagnosis of leishmaniasis including parasitological, immunological, molecular and by using experimental animals.

*Microscopic examination:* The confirmatory diagnosis of leishmaniasis relies on either the microscopical demonstration of *Leishmania* amastigotes in the relevant tissues aspirates or biopsies such as bone marrow, spleen, lymph nodes or liver, skin slit smears or biopsies<sup>77,78</sup> or in the peripheral blood buffy coat<sup>79</sup>. The smears can be stained with Romnowsky's, hemotoxyline eosine (H & E) or immunoperoxidase stains. The amastigotes are readily seen in smears or touch preparations of infected tissue stained with Giemsa's stain, preferably at pH 7.2 rather than the pH 6.8 normally used in haematology. Sections of tissue stained more conventionally, with H & E, are much more difficult to interpret. To ensure that the visualized structures are amastigotes, rather than other "dot"-like structures (e.g., *Histoplasma* spp, platelets), an experienced observer should look for the characteristic size (2-4 mm in diameter), shape (round to oval), and internal organelles, the nucleus and kinetoplast. The amastigote stage seen in clinical samples is commonly known as Leishman-Donovan (LD) bodies (Fig. 1). This name was given by Sir Ronald Ross to commemorate discovery of these bodies in patients of kala-azar by WB Leishman and C Donovan in 1903<sup>9</sup>. It is important to discern the kinetoplast. With Giemsa staining, the cytoplasm typically takes pale blue and the nucleus and kinetoplast take purple-pink colour<sup>1</sup>. The immunoperoxidase stain provides improved sensitivity in cases of cutaneous and mucocutaneous leishmaniasis<sup>1,5,7</sup>. Various clinical samples can be used depending on the clinical forms of diseases and aimed sensitivity.



**Fig. 1.** Geimsa stained bone marrow aspirate smear from a kala-azar patient, showing numerous extracellular as well as intramacrophage amastigote forms of the *Leishmania donovani*. X 1000.

**Spleen aspirate and biopsy:** For VL in the immunocompetent patients, best samples are those obtained from spleen aspirations<sup>1,80,81</sup>. The splenic aspirate is the best with sensitivity > 94 per cent than other tissue aspirations and in experienced hands the iatrogenic splenic bleeding can be minimized. Although many practitioners are reluctant to take spleen aspirates, others have no hesitation, and even use this method to monitor treatment. It is vital to use the correct technique and equipment with confidence, so that the capsule of the spleen is penetrated by a fine needle for only a fraction of a second<sup>80</sup>. The thinnest needle possible, preferably, 21-gauge (0.8 mm) should be used to minimize the risk of complications such as haemorrhage of the spleen<sup>81</sup>. However, even in experienced hands, the risk cannot be zeroed and fatal bleeding can occur in 2/10,000 patients, in spite of prior precautions in the form of >40000/ ml platelet count and a good prothrombin time control

measures are adopted<sup>80</sup>. Part of the splenic aspirate can be used to make smears for direct microscopic examination and the rest should be cultured. In splenic aspirate smears the amastigotes of *Leishmania* appeared ovoid in shape and measure about 3 x 5  $\mu\text{m}$  in size. We have observed that amastigotes which are short and more stout measuring as big as 4.5 x 5  $\mu\text{m}$  are resistant to sodium antimony gluconate while patients who show elongated amastigotes in their specimen are sensitive to standard doses of SAG<sup>78</sup>. This morphological observation needs confirmation. The parasites appear purple blue with central nucleus and a rod shaped structure at the right angle of nucleus, both pink in colour. This rod shaped structure is an extrachromosomal DNA mass known as kinetoplast. The splenic aspirates can also be used for determining the disease prognosis and therapeutic response by estimating parasite load by counting the number of amastigotes in the smears

in relation to the white blood cell counts<sup>78,81</sup>. A logarithmic scale from 0 (no parasites in 1,000 microscopic fields) to 6+ (greater than 100 parasites per microscopic field) can be applied<sup>81</sup>.

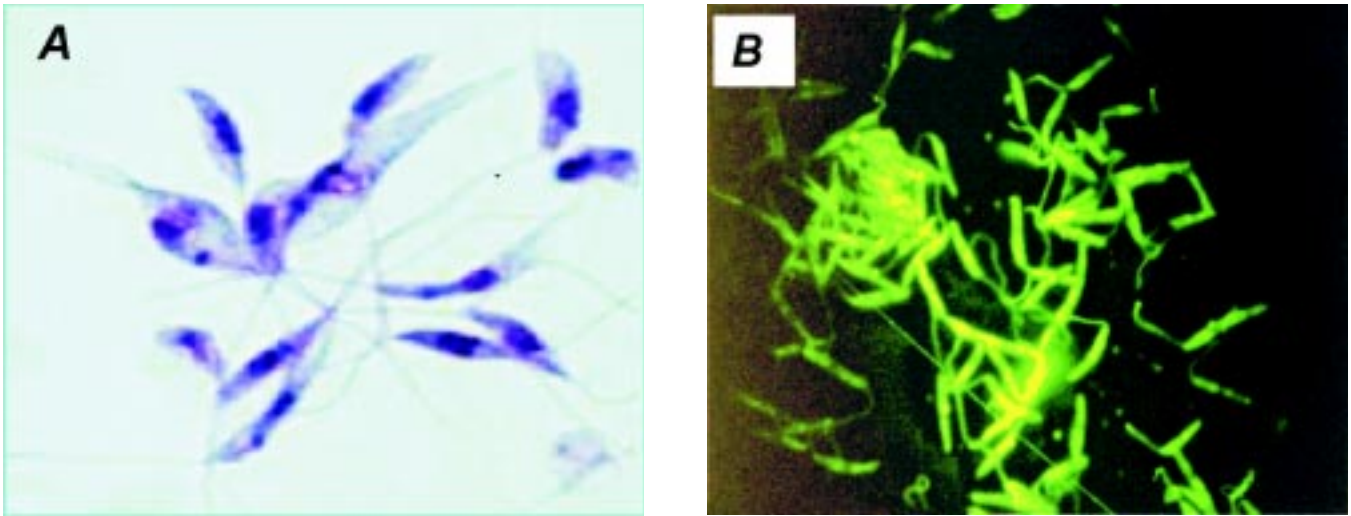
*Liver biopsy:* Demonstration of the parasites in the liver aspirates and biopsies is another option. The sensitivity of liver biopsies has been reported by various authors as low as 40 per cent, when most of the amastigotes are colonized within the Kuffer cells<sup>82</sup> to as high as 90 per cent. Liver aspiration should also be attempted with utmost care as in the case of spleen puncture not to tear the capsule.

*Bone marrow aspiration:* Marrow obtained from sternal or iliac crest puncture is a much safer but a painful method. It is less likely to demonstrate parasites in direct stained films<sup>78,79,81</sup> and in most studies the sensitivity ranges from 76-85 per cent. However, on culture it can give positive results in up to 80 per cent of the cases<sup>78,81</sup>.

*Lymphnode fine needle aspiration cytology (FNAC) and biopsy:* Lymph gland puncture may give positive results in up to 40-50 per cent of the kala-azar cases but its sensitivity has been found much higher (58.6%) in cases of cutaneous leishmaniasis in a study carried out in Brazil<sup>83</sup>. The aspirate is extracted from any enlarged lymph gland after injecting sterile normal saline and the aspirate is subjected to both direct examination and culture to give the best chance of diagnosis. In CL, the lymph nodes draining from the lesion sites are most yielding. Parasites may be scanty and are mostly extracellular in slide preparations, so these may have to be examined for at least 15 min using oil immersion before the diagnosis can be confirmed.

*Blood buffy coat:* Rarely the amastigotes can be demonstrated in the buffy coat of peripheral blood. Such a parasitaemia is common in severely immunocompromised patients such as AIDS<sup>84</sup> and patients on immunosuppressive therapy<sup>85</sup>. While some authors have found sensitivity up to 53 per cent<sup>86</sup> other have found this method very poorly (7.6%) sensitive<sup>87</sup>. In our laboratory we have also not found this method very sensitive (unpublished observation).

*Tegumentary leishmaniasis:* The routine diagnosis of CL patients depends on examination of skin lesions using smears and cultures of dermal scrapings or examination of sections obtained from a skin biopsy. Conventionally 3-5 aspirates from different lesions or portions of lesions are obtained. This is best done by injecting 0.1 ml sterile normal saline into the lesion site so that it inflates a bit. For ulcerative lesions, needle (23-27 gauge) is inserted through intact skin into dermis of active border. Small-gauge needles are appropriate for facial lesions. The needle is repeatedly moved back and forth under skin, tangentially to ulcer, simultaneously rotating the syringe and applying suction, until pink-tinged tissue fluid is noted in hub of needle. However, others<sup>88</sup> have not found significant difference in the diagnostic outcome whether smears or culture samples are taken from the center or the border of the ulcer or from an incision made tangential from the ulcer. Navin *et al*<sup>87</sup> found no difference when they compared smears obtained with scalpels, capillary tubes, or dental broaches. The use of scrub brushes soaked in iodine neither decreased the rate of culturing parasites nor the contamination rates. The most sensitive method was a combination of thin smears made from superficial scrapings of the ulcers and inoculation of culture medium with either aspirates or scrapings. The diagnosis was confirmed in 70 per cent patients<sup>88</sup>. Ability to cultivate *Leishmania* was correlated with the concentration of amastigotes seen on thin smears. *Leishmania* were cultured in 42 (27%) of 153 patients with no amastigotes found in 400 oil-immersion fields and in 174 (83%) of 209 patients with at least 1 amastigote<sup>88</sup>. Whatsoever sample collection method is used, each aspirate should be collected into separate tubes of Novy-MacNeal-Nicolle (NNN) culture medium to make separate slides for microscopy. If punch-biopsy samples are intended, one to two full thickness of skin at active border of lesion including some non ulcerated tissue should be obtained and used for culture and histopathology. For dermal scraping, 3-5 dermal scrapings from different lesions or portions of lesions should be taken. The first collections should be used for microscopy and last for culture to minimize the risk of contamination. For slit skin smear technique, first an incision is made then skin is pinched to exclude blood and scalpel blade is used to incise several mm



**Fig. 2.** Promastigotes *in vitro* culture conditions can be demonstrated either using Romnowsky's stains (panel A x 1000) or by direct fluorescent staining (Panel B x 450). In Romnowsky's stain the nucleus and mitochondria take light to dark pink-purple colour while in Fluorescent stains these fluoresce more intensely than the rest of the parasite body and flagella.

long and deep slit through intact skin into dermis. For ulcerative lesions, incision should be started from active border and proceed radially out across several mm of intact skin. Cotton swabs can be used for collecting 'samples from open ulcers'<sup>88</sup>. The sensitivity of direct microscopic identification of *Leishmania* amastigotes from tegumentary diseases forms can be achieved upto 60-65 per cent while the sensitivity of culture remains less than 42 per cent. It is mainly due to high chances of culture contamination of these samples<sup>77</sup>.

**Culture examination:** Isolation of the causative agent is most specific diagnostic criterion and also to characterize the organisms up to species or genotype level. The promastigote form can be culture isolated from these specimens on solid NNN medium having 20-30 per cent rabbit blood or liquid Schneider's insect medium. Up to 90 per cent of the active kala-azar cases will grow promastigotes in their splenic and liver aspirates<sup>89</sup>. Various other liquid media such as M199, Tobies medium supplemented with foetal calf serum can be used. Human urine has been successfully used in place of foetal calf serum in *in vitro* culture of *L. donovani*<sup>90</sup>. The promastigotes *in vitro* transformation in the NNN medium usually starts after 3 days of incubation at 22-26 °C in a BOD incubator. The wet mounts prepared from the liquid part of this diphasic medium will show several motile

organisms, the details of which can be delineated after staining the smear with Giemsa or any other Romnowsky's stain ( Fig. 2 panel A) or by using fluorescent antibody staining<sup>78</sup> (Fig. 2, panel B). Culture based diagnosis of MCL has low sensitivity as the organisms are often scant. The biggest handicap is culture contamination at early stages, even in best laboratory setups<sup>78,91</sup>.

**Isolation in experimental animals:** Alternative methods to isolate the parasite can be used. Inoculation of the clinical material obtained either into a susceptible BALB/c mouse or into a hamster footpad or nose may improve the yield. Histopathologic evaluation of biopsy samples of animal lesions may be characteristic but is rarely specific enough to make a diagnosis without identification of the amastigote. Weigle *et al*<sup>91</sup> from Columbia, compared seven methods of diagnosing leishmaniasis in 177 patients presenting with lesions of the skin or mucosa. Microscopic methods of visualizing amastigotes in tissue samples were less sensitive than the *Leishmania* isolation methods. The aspirate-culture and biopsy-hamster methods employed in this study proved most sensitive of the four methods for the recovery of parasites.

All methods were less sensitive in lesions of greater than 6 months duration than in lesions of more

recent onset. Mucosal lesions were best diagnosed by the culture or hamster inoculation of a macerated mucosal biopsy. The diagnosis by inoculation of hamsters was achieved within 2 to 12 wk, a mean of 34.5 days<sup>91</sup>. In another study from Kenya<sup>92</sup> on VL, portions of splenic or subcutaneous saline aspirates from suspected visceral or cutaneous leishmaniasis patients were inoculated into NNN medium with an overlay of Schneider's medium or Schneider's medium alone for routine parasitological diagnosis. The remaining portions of the aspirates were used for preparing Giemsa-stained smears and for subcutaneous inoculation into hind foot-pads of BALB/c mice. Saline aspirates obtained from the foot-pads 2-14 days after inoculation were inoculated into Schneider's medium and examined for promastigotes. Parasite isolation was achieved from 90 per cent of confirmed leishmaniasis patients by culture method alone. Mouse foot-pad aspiration demonstrated parasites in 95 per cent of all patients, and in over 80 per cent of the confirmed cases of leishmaniasis. Combined culturing and aspirate smear examination was more efficient than foot-pad inoculation alone for the demonstration of leishmanial infection. Foot-pad aspiration does not entail killing animals and was sensitive for parasite isolation; it may be a useful short-term adjunct to existing parasite isolation methods, especially under field conditions where the risks of culture contamination may be high<sup>92</sup>.

### Immunological methods of diagnosis

The hallmark of visceral leishmaniasis is hyperimmunoglobulinaemia, while in case of cutaneous and mucocutaneous leishmaniasis, the humoral immune response is extremely poor. Exploiting this host-parasite interaction, for the diagnosis of visceral leishmaniasis a number of antibody detection methods have been developed from time to time. Some of these tests include indirect haemagglutination (IHA), counter current immunoelectrophoresis (CCIEP), immunodiffusion (ID) and several others<sup>78,93-95</sup>. These tests are cumbersome and lack sensitivity and specificity and hence not commonly used but the interested readers can find more information from some recent reviews<sup>77,78,93</sup>.

*Fluorescent antibody test:* The indirect fluorescent antibody (IFA) test is one of the commonly used tests for anti-leishmanial antibody detection using fixed promastigotes. The test is based on detecting antibodies, which are demonstrated in the very early stages of infection and are undetectable six to nine months after cure. Titres above 1:20 are significant and above 1:128 are diagnostic. However, there is a possibility of a cross-reaction with trypanosomal sera<sup>78,93</sup>. The sensitivity of these tests varies extremely from as low as 28.4<sup>93</sup> to 86.6 per cent<sup>95</sup>. This can be overcome by using *Leishmania* amastigotes as the antigen instead of the promastigotes. To detect the antigen (amastigotes) in the tissue sections or smears, fluorescent dye conjugated antibodies can be used as tracers. This test is known as direct fluorescent test. The direct fluorescence test is more useful in the diagnosis of CL, MCL and PKDL. In place of fluorescence, horse radish peroxidase (HRP) can be used to tag the antibody. This will not require fluorescence microscope and the stained slides can be stored for long time.

*Direct agglutination test:* The direct agglutination test (DAT) is a highly specific and sensitive test. It is cheap and simple to perform making it ideal for both field and laboratory use. DAT in various studies has been found to be 91-100 per cent sensitive and 72- 100 per cent specific<sup>79,96</sup>. The test can be carried out on plasma and serum. For long time DAT remained first line diagnostic tool in resource poor countries. The method uses whole, stained promastigotes either as a suspension or in a freeze-dried form. The freeze-dried form is heat stable and facilitates the use of DAT in the field<sup>97</sup>. However, the major disadvantage of DAT is the long incubation time of 18 h and the need for serial dilutions of blood or serum. Also the DAT has no prognostic value for evaluating the parasitological cure of the disease, as the test may remain positive for several years after cure. Recently, Schoone *et al*<sup>98</sup> have developed a fast agglutination-screening test (FAST) for the rapid detection (<3 h) of anti-*Leishmania* antibodies in serum samples and on blood collected on filter paper. The FAST utilizes only one serum dilution leading to qualitative results. The FAST offers advantages over the DAT as it uses freeze-dried antigen, which gives more antigen stability, reproducibility, specificity and sensitivity.

*Enzyme linked immunosorbent assay (ELISA):* ELISA is a valuable tool and one of the most sensitive tests for the serodiagnosis of visceral leishmaniasis. The test is useful for laboratory analysis or field applications and to screen a large number of samples at a rapid pace. With the advances in automation, ELISA can be performed easily and is adaptable for use with various antigens such as whole cytoplasmic (soluble antigen, SA), purified antigens such as fucose-mannose<sup>99</sup>, defined, synthetic peptides and recombinant proteins as antigen<sup>100</sup>. The sensitivity and specificity of ELISA is greatly influenced by the antigen used. Beside the most commonly used soluble promastigote antigen, several antigenic molecules have been reported and their negative and positive predictive values (NPV & PPV) compared<sup>100</sup>. An excretory, secretory and metabolic antigens released by *L. donovani* promastigotes (Ld-ESM) into a protein-free medium was used for the serodiagnosis of VL by ELISA<sup>101</sup>. This antigen has been reported to be 100 per cent specific and sensitive, the PPV was 99.99 per cent and NPV was 95.45 per cent. However, further retrospective and prospective multisite evaluation is required to validate these findings. Lately, a variety of recombinant antigens have been developed. A *L. major* gene B encoding a hydrophilic protein (gene B protein, rGBP) expressed on the surface of both promastigotes and amastigotes and characterized by an amino acid repeating motif of 5.5 copies of a 14-amino acid sequence has been identified and shown to be expressed in *L. donovani* also. The protein encoded by *L. donovani* gene B homologue (Ld-rGBP) contains up to 22 copies of a repetitive element in which 9 out of 14 residues are completely conserved between the two species. An ELISA using this antigen is reported to be specific for *L. donovani* infections only<sup>102</sup>. Another recombinant protein rORFF of *L. infantum* origin has been developed by Raj *et al*<sup>103</sup> for diagnosis of VL in India. The ORFF protein is encoded in the LD1 locus of chromosome 35 of *L. infantum*. An ELISA based test using this antigen was found to be highly sensitive and specific. Its sensitivity and specificity was compared with DAT and soluble antigen (SA) ELISA. The sensitivity of rORFF ELISA was found to be significantly more. However, it also showed mild cross-reaction in 40 per cent cases of confirmed CL from Turkey caused by *L. major* or *L. tropica*. Further, the antigen is in infant

stage; needs to be evaluated widely and its utility for the field diagnosis is yet to be studied. Recombinant gp63, a major surface antigen of *Leishmania* was cloned as early as in 1988 but failed in early evaluation<sup>78</sup>.

A recombinant antigen, rK39 has been shown to be specific for antibodies arising during VL caused by members of the *L. donovani* complex. It is highly sensitive and predictive for onset of acute disease and evokes high antibody titres in VL patients<sup>41</sup>. In addition, rK39 ELISA, has a high predictive value for detecting VL in immunocompromised persons, like AIDS patients<sup>78</sup>. This antigen is now commercially available in the form of antigen-impregnated nitrocellulose paper strips adapted for use under field conditions. However, reports from Sudan and other countries revealed that this antigen showed decreased sensitivity and specificity. In Sudan the rK39 ELISA test is reported to miss 7 per cent parasitologically proven cases<sup>104</sup>. In its strip test format the sensitivity is further compromised to only 67 per cent in Sudan, 71.4 per cent in Southern Europe and 60 to 90 per cent in Brazil<sup>104-107</sup>. Besides rK39, two more recombinant proteins (rK26, and rK9) have been cloned from *L. chagasi* kinesin gene<sup>108</sup>. A significant difference between K9 and K26 is the presence of 11 copies of a 14 amino acid repeats in the open reading frame of K26. The region flanking the repeats of K26 shares a 69 per cent identity with the open reading frame of K9. However, none of these antigens were found useful on Indian sera (Singh *et al*, unpublished data). Therefore, a need was felt to clone the kinesin antigen from any Old World species of *Leishmania*. Very recently, we have cloned and characterized a recombinant antigen from an Indian isolate of *L. donovani* strain KE16. The antigen (rKE16) is found to be 100 per cent sensitive and specific. In fact it has better sensitivity than rK39 which showed 98 per cent sensitivity for the diagnosis of Indian kala-azar and PKDL<sup>109</sup>. It also showed 100 per cent concordance with rK39 in sera from leishmaniasis patients from China, Pakistan, and Turkey<sup>109</sup>. This antigen has now been commercialized and has got tremendous potential for the serological diagnosis of VL worldwide.

*Immunoblotting:* Serodiagnosis using immunoblotting of soluble antigens has been

attempted and reported highly sensitive and specific. The band pattern can correlate with disease stages<sup>110</sup>. Using cytoplasmic, soluble antigens from 5 Indian strains of *L. donovani* and three *L. major* strains from Pakistan separated by SDS-PAGE and electrotransferred on nylon membrane followed by Western blotting with Indian PKDL patients, a ~72-74 kDA antigen band was found to be most predominant<sup>77</sup>. The commercially available electrochemiluminescent kit (ECL, Amersham, UK) enhances its sensitivity, several-folds. It also has an added advantage of permanent documentation.

**Rapid antibody detection methods:** In most of the *Leishmania* endemic areas resources are limited in terms of poor or non-availability of electricity, poor laboratory set up and lack of equipment. Therefore, need of rapid, simple and easy to perform tests has always been felt. With this objective two rapid tests have been developed, one by InBios (USA) which uses Lc-rK39 antigen and the other one is by Sa pn Diagnostic Limited (India) which uses Ld-rKE-16 antigen. Both are commercially available and are based on membrane filtration technology.

**Antigen detection:** Antigen detection test would, in principle provide better means of diagnosis of active leishmaniasis. Since antigen levels are expected to theoretically correlate with the parasite load, the antigen detection may be an ideal test in immunocompromised patients, where antibody response is very poor. The detection of antigen in the patient's serum is complicated by the presence of high level of antibodies, circulating immunocomplexes, serum amyloid, rheumatoid factor and autoantibodies, all of which may mask immunologically important antigenic determinants or competitively inhibit the binding of free antigen. Recently, a latex agglutination test (KATEX) for the detection of leishmanial antigens in the VL patients' urine has been developed<sup>111</sup>. The results obtained with KATEX using samples collected from different foci of VL indicated that the test worked well regardless of the geographical origin of samples. The test had 100 per cent specificity and sensitivity between 68-100 per cent. Whether the test has applications for the detection of

asymptomatic cases of VL and monitoring therapy is yet to be confirmed.

**Diagnosis using amastigote specific antigen:** The *in vivo* parasitic stage of *Leishmania* in humans is amastigote form and it is not difficult to appreciate that antigens specific or prepared from this stage of *L. donovani*, would be more ideal. However, due to difficulty in maintaining the culture of the amastigote stages in bulk quantity not many studies are available in the literature. Otherwise also the sensitivity and specificity of crude antigens prepared from amastigotes have not been found superior to the recombinant antigen rK39<sup>112</sup>.

**Leishmanin skin test (LST):** Delayed hypersensitivity is an important feature of cutaneous forms of human leishmaniasis and can be measured by the Leishmanin test, also known as the Montenegro reaction. No cross-reaction occurs with Chagas' disease, but some cross-reactions are found with cases of glandular tuberculosis and lepromatous leprosy. LST is used as an indicator of the prevalence of cutaneous and mucocutaneous leishmaniasis in human and animal populations and successful cure of the visceral leishmaniasis. During active kala-azar, there will be no or negligible cell mediated immune response<sup>79</sup>. However, the Leishmanin antigen is not commercially available and no field study has been carried out in Indian subcontinent.

Despite the availability of large number of serological tests, no serological method is helpful for cutaneous and mucocutaneous leishmaniasis because antibodies tend to be undetectable or present in low titre due to poor humoral response<sup>113,114</sup>.

### **Molecular methods**

Molecular biology is increasingly becoming relevant to the diagnosis and control of infectious diseases. Information on DNA sequences has been extensively exploited for the development of polymerase chain reaction-based assays for various applications in the understanding of the parasite and the diseases. With the advent of nucleic acid engineering and recombinant technology, a number of strategies have been developed to produce

recombinant proteins for diagnostic purposes<sup>78,79,96</sup>. A variety of nucleic acid detection methods targeting DNA and RNA genes have been developed. However, amongst all the molecular advances gene amplification techniques have been most rewarding as far as diagnosis and disease management is concerned.

*Polymerase chain reaction (PCR):* Amongst the molecular methods used for clinical diagnosis, PCR has been proved to be most sensitive and specific technique, albeit limited to tertiary care hospitals and research laboratories. The specificity of the PCR can be adapted to specific needs by targeting conserved region of the gene. Gene amplification through the PCR has several advantages compared to traditional techniques, because of its extremely high sensitivity, rapidity and the ability to be performed with a broad range of clinical specimens<sup>96</sup>. Also the detection or identification of the causative agent is possible directly from the clinical specimens. Unlike the ELISA based antibody detection methods, no host species specific reagents are required and the same reagents can be used for specimens from humans, dogs, or any other animal host<sup>115</sup>. Various gene targets and nucleic acids can be used in PCR. The important gene targets are 18S-rRNA, small subunit rRNA (SSU rRNA), a repetitive genomic sequence of DNA, the miniexon (spliced ladder) gene repeat, the  $\beta$ -tubulin gene region, gp63 gene locus, internal transcribed spacer (ITS) regions; micro-satellite DNAs such as maxi- and minicircles of kinetoplast DNA<sup>116-119</sup>.

Several studies have reported that PCR assay could detect parasitaemia a few weeks before the appearance of any clinical signs or symptoms. Recently Martin-Sanchez *et al*<sup>36</sup> using PCR-ELISA found 24 per cent asymptomatic individuals carrying *Leishmania* kDNA in their blood. It was pointed out that these individual could be potential source of transmitting the infections in the community. These authors also found a good correlation between the antibody titres, skin test positivity and PCR positivity. The PCR methods could also be useful where transfusion transmitted kala-azar is a potential threat in multiply transfused patients in whom serology has limited role<sup>120</sup>.

Bone marrow, lymph node aspirates, skin biopsy, skin scrape/exudates and blood samples have been used for PCR in several studies<sup>36,79,116-122</sup>. The specificity of PCR on bone marrow aspirates has been reported up to 100 per cent<sup>36</sup> and sensitivity 80-93.3 per cent as compared to 50-60 per cent sensitivity of smear and culture examination<sup>96</sup>. Also a modified form of PCR such as nested PCR has proved its predictive values in diagnosis of PKDL. In a study, nested PCR was positive in 27 of 29 (93%) samples while only 20 of 29 (69%) samples were positive in the primary PCR assay<sup>121</sup>. Using PCR methodology, it is no more essential to undergo invasive methods such as bone marrow, splenic punctures, lymph node biopsy, liver biopsy, *etc.*, or collect large volumes of blood samples. Even a few drops of blood on filter paper may be sufficient<sup>116</sup>.

The chronic CL patients are greatest diagnostic challenge and are easily misdiagnosed by clinical criteria because they are often atypical. These patients often have low or no *Leishmania* antibodies, and thus serological tests are not rewarding. In such cases, PCR has been proved to be the most important tool for diagnosis. The sensitivity of PCR in CL has been reported 100 per cent. In a study de Oliveira *et al*<sup>122</sup> could confirm the diagnosis in all 50 dogs using PCR. Various types of specimens may be used such as skin biopsies, dermal scrapings from the bottom of the ulcer as well as exudates and syringe-sucked fluid taken from ulcerative lesions. Recently, the sampling method has been further improved using cotton swab for diagnosis of CL<sup>123</sup>. Collection of the exudates material with swabs is easy, painless and convenient for both the patients as well as the collectors. The cotton swabs had no inhibitory effect on PCR. Exudates collected by cotton swab is recommended to be a better alternative to biopsy samples, especially in field conditions<sup>123</sup>. In MCL, PCR was capable of detecting parasites in 17 of 24 (71%) patients, whereas by conventional techniques only 4 (17%) patients could be diagnosed<sup>123</sup>. ELISA based detection of PCR products further increased sensitivity and specificity. Using this technique the percentage of detection was found to be 83.3 per cent with blood samples<sup>124</sup>.

A recent development in PCR technology (fluorogenic probes and automation) takes care of non-specific amplification, speed and inter-operator variability. In this technique, two fluorogenic dyes, a reporter dye, and a quencher dye, are attached to 5' and 3' ends of the probes. The real-time PCR is used qualitatively and quantitatively, as the fluorescence is directly proportional to the number of amplicons, or in other words, the parasite load in the given specimen<sup>118,125</sup>. The automatization of real-time PCR with comprehensive portable units has made these tools field friendly. The multiplex PCR can be used whenever, double or mixed infections are suspected as in AIDS patients<sup>118</sup>.

### Conclusions

Leishmaniasis is caused by a kinetoplastid protozoan parasite. The parasite is transmitted from one host to another through the bites of female sandfly, or occasionally through non-vector routes including blood transfusion, congenital, sexual, laboratory acquired and person-to-person. The disease may manifest in 3 main clinical forms, of which visceral form known as kala-azar is most severe and may be fatal if not treated. The causative species of the parasite is specifically identified but in immunosuppressed patients the manifestation may be atypical. The diagnosis is made by the demonstration of the amastigote stage of the parasite in the bone marrow, spleen, liver or lymph node aspirates or using the histopathological and touch smear preparations. Rarely the parasite can be demonstrated in the peripheral blood. The parasite is culture isolated *in vitro* in enriched media or in experimental animals for taxonomic and other biological studies on the isolates. However, with the advances in the non invasive serological and molecular methods, direct methods are rarely used in clinical practice except for research purposes. The serological methods are sensitive, specific and cost-effective, while molecular tools are extremely sensitive and useful in molecular epidemiological studies beside diagnosis. Also, these can be performed on clinical as well as archival samples.

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