Diagnosis and Treatment of Indian Visceral Leishmaniasis

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Resurgence of visceral leishmaniasis (VL; kala-azar) in India was reported in early seventies, and since then it continues to affect millions of people especially in Bihar, and to a lesser extent in Eastern UP and West Bengal. Kala-azar is characterised by prolonged fever, splenomegaly, hepatomegaly, weight loss, pancytopenia, hypergammaglobulinemia etc. and is complicated by secondary infections. It is the most severe form of leishmaniasis and if untreated is usually fatal. Currently prevailing modes of diagnosis and treatment are highly unsatisfactory, and in recent years important progress has been made in both these aspects of the disease. In this article, we have dealt with the state of the art in the diagnosis and treatment of Indian VL.

**DIAGNOSIS OF KALA-AZAR**

Diagnosis of visceral leishmaniasis (VL) is complex as its clinical features are shared by a host of other commonly occurring diseases like malaria, typhoid, tuberculosis etc. Many of these conditions can be present as co-infection with VL. Laboratory diagnosis of leishmaniasis can be made by

I. Demonstration of parasite in the tissue of relevance by
   a) Light microscopy of the stained specimen
   b) In vitro culture
   c) Animal inoculation

II. Detection of parasite DNA in tissue sample using PCR.

III. Immunodiagnosis
   a) Detection of immunoglobulins
      i) Nonspecific or,
      ii) Specific anti-leishmania antibodies
   b) Detection of parasite antigen

I. Demonstration and isolation of parasites

This is the most reliable and conventional method for diagnosing VL. The parasite is commonly demonstrated in the splenic or bone marrow aspirate and in the buffy coat of peripheral blood (in HIV co-infection). The amastigotes or LD bodies are identified by light microscopy of the specimen obtained after staining with Giemsa or Leishman stain. The sensitivity of splenic aspirate smear is more than 95% and is regarded as the gold standard for the diagnosis of Kala-azar.

But the sensitivity of bone marrow smear is only about 60-85% and the procedure is painful whereas splenic aspiration carries the risk of severe hemorrhage. Even though this fatal complication is rare in experienced hands, splenic puncture should be avoided in patients with a platelet count of less than 40,000/µL and a prothrombin time of more than five seconds over the control.

Leishmania can be grown as promastigotes in artificial culture medium, Novy-McNeal Nicolle Medium (NNN), M199 or Grace’s medium with 20% fetal calf serum. The parasite can also be demonstrated after inoculating in laboratory animals such as hamster, mice or guinea pig. Golden hamster is the animal of choice for maintaining Leishmania donovani complex. Culture of parasite and animal inoculation can improve the sensitivity of detection of parasite, but these are rarely needed in routine clinical practice.

II. DNA detection method

In recent years PCR based diagnostic methods have been studied for leishmaniasis. Several primer sets have been described, but genus specific 13A and 13B primers are used most commonly. Recently species specific LDI-primers have been developed which is capable of amplifying kinetoplast DNA of Leishmania donovani. The PCR assay developed using this primer is sensitive enough to detect even a single parasite in the sample. In an Indian study, the sensitivity of this test using whole blood in VL patient was 96%. There are limitations of using PCR in the diagnosis of VL as it may pick-up parasite in infected but non-diseased persons and when used for assessment of cure, PCR might amplify dead parasitic DNA leading to misdiagnosis. Further, independent evaluation of several primers developed in various Indian laboratories is needed before these can be employed for routine diagnosis. For field applicability cheap and simple versions need to be developed.

III. Immunodiagnosis
   a) Antibody detection
      i) Nonspecific test: For several decades, certain nonspecific methods, which depend upon the raised
immunoglobulin levels have been used in the diagnosis of VL. Some of these tests are Napiers formol gel and Chopra’s antimony test. Since globulins do not increase early in the course of the disease, they are of no value in patients presenting early. As they indicate raised globulin only, they can be positive in host of other conditions. This lack of specificity and varying sensitivity renders them highly unreliable.

ii) **Specific serodiagnostic tools**: Several immunodiagnostic methods have been developed which are more sensitive and specific. The sensitivity may depend upon the antigen rather than the serological procedure used. The initial methods used for antibody detection include gel-diffusion, complement fixation test, indirect haemagglutination test, indirect fluorescent antibody (IFA) test and countercurrent immunoelctrophoresis (CCIEP). But sensitivity and specificity of most of these tests have been the limiting factor.

In 1988, a modified direct agglutination test (DAT) was reported to be very useful. In this test, the trypsinised whole promastigotes are formalin fixed and stained with a vital dye. Patient’s serum is incubated with the antigen and agglutination is observed next day. The tests performed in India and other countries were found to be 91-100% sensitive and 72-100% specific. In other study on Somalian patients, the sensitivity and specificity could be improved to 100% by the use of 0.8% of 0.1 M 2-mercaptoethanol in the sample diluent. Since the test remains positive for long period after complete cure, it is not of much prognostic value. Further, batch to batch variations, instability of aqueous antigen, fragility during transport, multiple steps, prolonged incubation period etc., have been the limiting factors. Thus, DAT does not meet the requirements for being an ideal tool for the field diagnosis of kala-azar.

Enzyme linked immunosorbent assay (ELISA) has been used as a potential serodiagnostic tool in almost all infectious diseases including leishmaniasis. The technique is highly sensitive, but specificity depends upon the antigen used. The commonly used antigen is a crude soluble antigen (CSA). The sensitivity is reported to be 100%, but cross-reaction with sera from trypanosomiasis, tuberculosis and toxoplasmosis patients have been recorded. Several other antigens have also been studied, but the most promising one has been the recombinant antigen rK39. This antigen has been shown to be specific for antibodies caused by members of L. donovani complex. This antigen is a member of kinesin family containing 39 amino acids. Several studies with this antigen have shown 100% sensitivity and 100% specificity in the diagnosis of VL by ELISA. The antibody titre directly correlates with the activity of the disease. At diagnosis of VL, the anti-rK39 antibody titre was 59-fold higher than that against CSA, and with successful therapy it fell sharply at the end of treatment and during follow up. In patients who relapsed, the titre rose again. These observations suggest its utility in serodiagnosis, monitoring the treatment and in detecting relapse of the disease.

However, because of the adverse conditions prevailing in the endemic areas, sophisticated methods like ELISA cannot be employed. In these conditions, there is a need for a simple, cheap, rapid and accurate test with good sensitivity and specificity, which can be used without any specific expertise. Using rK39 a promising ready-to-use immunochromatographic strip test has been developed as a rapid test for use in difficult field conditions. In this, the antigen is immobilized on a small rectangular piece of nitrocellulose membrane in a band form, and goat anti-protein A is attached to the membrane above the antigen band. After finger prick, less than a drop of whole blood/serum is smeared at the tip of the strip, 4-5 drops of PBS is placed on a clean glass slide or tube and the lower end of the strip is allowed to soak in this solution. If the antibody in patient’s serum is present it will react with the conjugate (protein A colloidal gold) which is predried on the assay strip. The mixture moves along the strip by capillary action and reacts with rK39 antigen band and gives a pink band. In positive patients two pinkish lines appear in the middle of nitrocellulose membrane, the upper pinkish band serves as procedural control (Fig. 1). In several studies from Indian subcontinent, the strip test was 100% sensitive and 93-98% specific. But due to prevailing low titres, the sensitivity fell to only 67% in Sudan. In a similar study done in Southern Europe, the strip test was positive in only 71.4% cases of VL. This difference may be due to differences in antibody response observed in different ethnic groups. A high level of the specificity of the test (97-100%) has been uniformly reported. As anti K39 IgG are present in the serum for an extended period after successful treatment for VL, the strip test should not be used for the diagnosis of relapse or reinfection by Leishmania. Though with few limitations, rK39 strip test is a very useful diagnostic modality in the field conditions in India and Nepal.

![Fig. 1: rK39 strip test.](image-url)
b) Antigen detection

Antigen detection is more specific than antibody based immunodiagnostic tests. The method is also useful where the antibody production is low as in AIDS. De Colmenares et al from Spain have reported two polypeptide fractions of 72-75 kDa and 123 kDa in the urine of kala-azar patients. The sensitivity and specificity of 72-75 kDa fraction was 96% and 100%, respectively. Moreover, after three weeks of antileishmanial treatment, these antigens were not detectable in the urine suggesting a good prognostic value of the test.

Recently, a new latex agglutination test (KATEX) has been developed to detect leishmanial antigen in urine of patients with kala-azar. In preliminary trials, the test had 100% specificity and a sensitivity between 68-100%. In laboratory animals the test became positive one week after inoculation and became negative rapidly after chemotherapy. Compared to microscopy, KATEX performed better than any single serological test in predicting positivity and a particularly good result was obtained by combining KATEX and direct agglutination test (DAT). A multicentre evaluation of this test is currently underway in India, Sudan, Nepal and Brazil.

**TREATMENT**

In general, treatment of leishmaniasis is far from satisfactory. Most antileishmanial drugs are toxic and have to be given parenterally for prolonged periods. Once successful therapy is instituted there is a prompt return of temperature to normal, regression of spleen and recovery of blood counts occur towards normal. In tissue aspirates the parasite density is graded on a log scale of 0 to 6+. An apparent or initial cure can be declared if there is clinical improvement and no parasites are seen at the end of treatment. Complete regression of splenomegaly may take several months, best indicator of definite or final cure is freedom from a clinical relapse at six months follow up.

**PENTAVALENT ANTIMONIALS (SB\textsuperscript{3})**

Since early 1940s, this drug has been the sheet anchor of treatment for VL and has been used as the first line drug. In most part of the world these compounds are still used to treat all forms of leishmaniasis.

There are two pentavalent antimony compounds: sodium antimony gluconate and meglumine antimoniate. These drugs can be administered either IV or IM, and inhibit glycolytic enzymes and fatty acid oxidation in the amastigotes of leishmania and there is a dose-dependent inhibition in the net formation of ATP and GTP. Side effects are quite common and include arthralgia, myalgia, nausea, vomiting, metallic taste, local pain and stiffness of injected muscles, increase in AST/ALT and ECG changes (decreased height of T wave or T wave inversion). Occasionally severe cardiotoxicity, manifested by prolongation of QT\textsubscript{T} to > 0.5 ms, ventricular premature complexes, ventricular tachycardia, torsades de pointes, ventricular fibrillation and cardiac arrest can occur. In India, SB\textsuperscript{3} induced cardiotoxicities have been reported in about 10% patients, but mortality attributed to SB\textsuperscript{3} related cardiotoxicity is 5.9%. There is a steady decline in the response rate to treatment with SB\textsuperscript{3}. In early fifties, SB\textsuperscript{3} in a dose of 10 MKD (max 600 mg) could cure most patients with Indian kala-azar, but it became apparent in late seventies that this traditional dose left a large proportion of patients unresponsive. This led to successive recommendations to increase the dose and duration of the drug. In 1984, WHO recommended the dose to be increased to 20 MKD and duration to 20 days which was further increased to 40 days in 1990. The increased dose led to the recovery of cure rate to > 90% initially. However, after a decline was noted by most workers and now up to 60% of patients fail to respond to SB\textsuperscript{3} treatment in Bihar, though in other areas (Eastern UP, West Bengal) SB\textsuperscript{3} continues to be effective.

Emergence of SB\textsuperscript{3} resistance in Bihar has been demonstrated in *in vitro* studies using isolates from SB\textsuperscript{3} unresponsive patients which require 3-5 times greater amount of the drug for similar effects as opposed to those from SB\textsuperscript{3} responsive patients.

**PENTAMIDINE ISETHIONATE**

It is a polyamine and acts by disrupting kinetoplast DNA. When used at 4 mg/kg parenterally for 10-15 injections, it cured most (99%) patients with kala-azar in early 80s. It is quite toxic and hypoglycemia, hyperglycemia, insulin dependent diabetes mellitus (IDDM), shock, myocarditis and death have been reported with its use. Like SB\textsuperscript{3}, its efficacy has also waned and in a study from Bihar, up to 33 injections were needed to achieve a cure rate of 78%. Due to its potential toxicities especially IDDM, declining efficacy and high cost, its use has been abandoned in India.

**AMPHOTERICIN B**

Amphotericin B is a polyene antibiotic that has high affinity to ergosterol like sterols in the membrane, and it inhibits its biosynthesis forming micropores, leading to increased membrane permeability and ultimate killing of leishmania.

In visceral leishmaniasis, amphotericin B should be used in doses of 0.75-1 mg/kg body weight for at least 15 injections or till the cure is achieved. The drug, which is available as dry powder, is first suspended in 10 ml of water and then diluted in 500 ml of dextrose solution. Initially a test dose of 5 mg is given and then full dose can be administered 6-8 hours later. Infusions are given on alternate days and daily administration is recommended only if intensive monitoring of cardiac status, electrolyte abnormalities, hepatic and renal functions is possible. This is because amphotericin B infusions can cause renal dysfunction, hypokalemia, hepatic dysfunction, bone marrow suppression and myocarditis, all of these can be fatal. Fever with chills, aches and pains all over the body,
nausea and vomiting are common and can occur acutely during each infusion. Thrombophlebitis of the injected vein is also common. Response to the treatment is excellent with long term cure rate almost 100%. Major limitation of this treatment is the need for prolonged hospital stay for 4-6 weeks, need for IV infusion, serious toxicities and high cost.\textsuperscript{35,36}

With the increasing failure of Sb\textsuperscript{v} and pentamidine, many workers advocate amphotericin B to be used as a first line drug in visceral leishmaniasis.\textsuperscript{37,38} Government of India Expert Committee on Treatment of Kala-azar has also recommended amphotericin B to be used as first line agent in the regions with > 10% prevalence of Sb\textsuperscript{v} resistance.\textsuperscript{39} In endemic regions lack of skilled manpower, hospital beds and resources are major limiting factor to treat all kala-azar patients with amphotericin B as the first line drug. Alternative safe and easily administrable treatment regimen are sorely needed.

**LIPID ASSOCIATED AMPHOTERICIN B**

To minimize the adverse reaction associated with amphotericin B, several lipid/liposomal formulations of this drug have been developed in recent years. In these deoxycholate is replaced by other lipids which results in remarkably decreased uptake of the drug by organs like kidney, liver etc. leading to markedly decreased or no toxicity. It has been possible to deliver large amount of amphotericin B in a short time, decreasing the duration of treatment without any significant adverse events or loss of efficacy.

Three lipid formulations of amphotericin B are commercially available: 1) Liposomal amphotericin B (AmBisome; Gilead Sciences, Foster City, Ca), 2) Amphotericin B lipid complex (Abelcet [ABLC]; Liposome Company, Princeton, NJ, Ampholip; Bharat Serums and Vaccines Ltd., Thane, India) and 3) Amphotericin B colloidal dispersion (Amphocil [ABCD; Amphote); Sequus Pharmaceuticals, Menlo Park, Calif). All these three are commercially available and have been tested successfully in visceral leishmaniasis.

In our four clinical trials with ABLC,\textsuperscript{25,40-42} the drug was found to be very safe and infusion reactions and other toxicities were minimal. A total dose of 10-15 mg/kg could cure 90-100% of patients, and the duration of therapy could be compressed to five days. No organ specific toxicity was observed.

Liposomal amphotericin B (AmBisome) is approved in several European countries for primary treatment of kala-azar. The US FDA approved it recently\textsuperscript{43} and recommended a total dose of 21 mg/kg (3 mg/kg once daily on days 1-5, 14 and 21), whereas in Europe, Africa and South America, 18 mg/kg or more is considered adequate. AmBisome 0.75 mg/kg for five days (Total dose 3.75 mg/kg) cured 89% of Indian visceral leishmaniasis patients in long term follow-up.\textsuperscript{44-46} Mild infusion related fever and rigor was seen in one-third of patients, and no other systemic toxicity occurred. In a recent study we observed that low dose liposomal amphotericin B (5 mg/kg) given either as a five day course or as a single infusion, seems to be effective without any significant difference in the response rate.\textsuperscript{45}

Amphotericin B, cholesterol dispersion consists of cholesterol sulphate and amphotericin B (1:1 molar ratio) in disc shaped particles. Amphootec, when used in 2 mg/kg for seven and ten doses cured 90% and 100% of patients respectively.\textsuperscript{45} But the experience is limited because, it produced severe side effects in the form of respiratory distress and cyanosis along with fever and chills in children younger than three years. This drug is being evaluated in India.

Results in one lipid associated amphotericin B cannot be extrapolated even in the same region as these different drugs do not share the same structure and composition and it should not be assumed that they will have the same spectrum of activity and toxicities.

Most important drawback of lipid formulations of Amphotericin B is their high cost. Short duration of therapy leading to reduced hospitalization cost might partially offset the high cost of lipid associated amphotericin B. Still these excellent drugs are unaffordable for most patients in the developing countries.

**AMPHOTERICIN B-FAT EMULSION**

In Europe, several workers have used amphotericin B mixed in 100 ml of commercially available 20% fat emulsion for treatment of fungal infections in patients with AIDS, malignancy or those critically ill, in an effort to reduce the infusion-related toxicities. When tried in Indian patients with alternate day infusions of amphotericin B (2 mg/kg) mixed with 100 ml of 20% fat emulsion for a total of five doses, 93% responded completely after six months of follow up.\textsuperscript{46} The final cost of this regimen was 45% less than re-treatment with either Sb\textsuperscript{v} or conventional amphotericin B deoxycholate alone. Another major advantage was a considerably shortened amphotericin B deoxycholate alone. Another major advantage was a considerably shortened duration of treatment, i.e. 10 versus 20-40 days. Further studies are needed before this combination can be employed.

**AMINOSIDINE (PAROMOMYCIN)**

It is an aminoglycoside antibiotic which has good leishmanicidal activity in *in vitro* and animal models.\textsuperscript{49} Trials have been conducted all over the world using aminosidine alone or in combination with Sb\textsuperscript{v}. In India, Thakur et al\textsuperscript{50,51} used it in 12 MKD with Sb\textsuperscript{v} 20 MKD for 20 days and of the 24 patients, 18 had permanent cure, four improved and two died. Seaman et al,\textsuperscript{52} in a large study (200 patients), compared Sb\textsuperscript{v} 20 MKD alone for 30 days with a combination therapy of Sb\textsuperscript{v} and aminosidine (15 MKD) for 17 days. In Sb\textsuperscript{v} group 81% and in the combination group 95% were cured at the end of 17 days, however at the end of 30 days, cure rate rose to 93.4% in Sb\textsuperscript{v} group. Mortality and drug
toxicities were similar in the two groups. No serious side effect was observed even though a transient rise in urea and creatinine was seen. Other side effects include nephro and otoxicity as observed in any other aminoglycoside, but these were very uncommon. Main advantage was the short duration of therapy, but no significant benefit was achieved as far as cure rate was concerned. Another advantage projected in some studies is that the patients receiving this drug may have lesser degree of infective diarrhoea and pneumonia because of its antibacterial action and its action against intestinal protozoa - giardia and amoeba. A recent study in India by Jha et al shows aminosidone alone at 16 mg/kg/day for 21 days cured 93% of patients. However, this drug is not available commercially. A new manufacturer IDA Pharmamed of Malta is now producing this drug, but a pivotal phase III trial is needed in India before this drug can be approved.

CYTOKINE THERAPY

It is now well established that leishmania infection progresses to kala-azar in individuals who are unable to elicit a Th1 type of immune response, which is initiated by IL-12 and mediated through IL-2 and IFN-γ. IFN-γ is one of the principle activators of macrophages and has shown its ability to control leishmanial infection in animals. Results of studies in India have been disappointing with IFN-γ with cure rate less than 50%. Decline in the response rate of antimony rendered the addition of IFN-γ ineffective. In a developing country like India field applicability of these products remains a distant possibility.

ORAL AGENTS

Oral agents have got obvious advantages and for decades oral drugs had been tried in leishmaniasis without success. Several agents like allopurinol, atovaquone, fluconazole, ketoconazole etc. have been tried but found to be not sufficiently leishmanicidal to induce permanent cure when used alone or in combination with Sb⁺. Two new oral antileishmanial compounds, miltefosine and WR-6026 (sitamaquine) are likely to succeed in the treatment of visceral leishmaniasis.

MILTEFOSINE (HEXADECYLPHOSPHOCHOLINE)

It is an alkyl phosphocholine analogue which was initially developed as an anticancer drug. But later it was found to be having little clinical efficacy against tumour cells when administered orally. However, in vitro and animal studies indicated it to be an effective antileishmanial agent. Exact mechanism of its leishmanicidal activity is not known. Probably it acts by modifying the cell signaling pathways and interfering with membrane synthesis. Miltefosine was first tried in 30 patients phase I dose escalating trial which included 14 patients with Sb⁺ failure. When administered orally for 28 days in doses of 100-150 mg/day were found to be very safe and resulted in a cure rate of 93%. Doses < 100 mg/day were only partially effective and those ≥ 200 mg/day were poorly tolerated. Three following phase II studies in adults (including a multicentre trial), established that doses of 100-150 mg for 3-4 weeks were well tolerated and were associated with high cure rates (95%). Gastrointestinal side effects such as vomiting and diarrhoea were frequent but mild to moderate in severity and none of the patients discontinued therapy because of this. Asymptomatic transient elevation of hepatic enzymes was common, however in high doses it was nephrotoxic. This drug is teratogenic and cannot be used in pregnant females or those who refuse contraception for the duration of therapy plus three months as a safeguard considering its long half life.

A WHO sponsored phase III trial with 100 mg for four weeks is now nearing completion and results are encouraging. Recently it has been approved for marketing in India for kala-azar in doses of 50 mg/day for those weighing ≤ 25 kg and 100 mg daily for those weighing > 25 kg for four weeks (50 and 100 mg capsules will be available). Studies in children are underway and preliminary data are similar to adults. The likely dose in children will be 2.5 mg/kg body weight for 28 days.

WR 6026 (SITAMAQUINE)

It is a primaquine analogue with high antileishmanial activity. It was first developed against malaria in Walter Reed Army Institute of Research (USA) several decades ago. In a phase II clinical trial done in Kenya, it was found that the drug cured 50% in a dose of 1 mg/kg/day. Methemoglobinemia up to a maximum of 55% and mild elevation of hepatic enzymes are notable adverse reaction. In another phase two dose study in Brazil, the cure rate was 67% (four out of six) at a dose of 2 mg/kg/day. The drug demonstrated some unusual clinical features like lack of increased efficacy against Brazilian kala-azar with increased dosing above 2 mg/kg/day and nephrotoxicity that was not present in previous investigations. Phase II trials are in progress in India.

HIV-VISCERAL LEISHMANIASIS COINFECTION

Treatment is essentially the same as in an immunocompetent patient. Conventional amphotericin B may be more effective in achieving initial cure than antimony compounds. By using high doses of AmBisome, a high cure rate is possible. The approved regimen is 4 mg/kg/day 1-5, 10, 17, 24, 31 and 38 for a total of 40 mg/kg. These co-infected patients have a tendency to experience relapse within a year. For the prevention of relapse the role of maintenance chemotherapy needs further study.

REFERENCES


