

Laboratory Diagnosis of Visceral Leishmaniasis

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The group of diseases known as the leishmaniasis are caused by obligate intracellular protozoa of the genus *Leishmania* (39). Natural transmission of leishmania is carried out by a certain species of sandfly of the genus *Phlebotomus* (Old World) or *Lutzomyia* (New World). These are present in three different forms: (i) visceral leishmaniasis (VL), (ii) cutaneous leishmaniasis, and (iii) mucocutaneous leishmaniasis. The visceral form, also known as black sickness or kala-azar in Asia, is characterized by prolonged fever, splenomegaly, hepatomegaly, substantial weight loss, progressive anemia, pancytopenia, and hypergammaglobulinemia and is complicated by serious infections. It is the most severe form of the disease and, left untreated, is usually fatal. Although confirmed cases of VL have been reported from 66 countries, 90% of the world's VL burden occurs on the Indian subcontinent and in Sudan (12, 21, 65, 80). After recovery, some patients (50% in Sudan and 1 to 3% in India) develop post-kala-azar dermal leishmaniasis (PKDL), which requires prolonged and expensive treatment (57, 83). PKDL patients also play an important role in VL transmission (77). VL is typically caused by the *Leishmania donovani* complex, which includes three species: *L. donovani*, *Leishmania infantum*, and *Leishmania chagasi*. The clinical features of VL caused by different species are different, and each parasite has a unique epidemiological pattern. On the Indian subcontinent, the disease is almost exclusively caused by *L. donovani*. The initial report of *Leishmania tropica* causing VL in India (61) was refuted by us and others (74, 78). *L. infantum* is responsible for VL in children in the Mediterranean basin. However, due to increasing prevalence of human immunodeficiency virus (HIV) infection in this region, HIV-VL coinfection in the adult population is being reported frequently. *L. chagasi* causes VL in children in Latin America, where lymphadenopathy is a dominant clinical feature. *L. tropica*, the causative organism of Old World cutaneous leishmaniasis, is reported to produce visceral disease in nonimmune persons (41). Similarly, visceralization by *Leishmania amazonensis*, has also been reported (28). Clinical manifestations of all forms of VL change from time to time, and this is the case more so in AIDS patients (8, 21, 42, 43, 48).

EPIDEMIOLOGY

Leishmania infections are worldwide in distribution: they are found in five continents. The disease is endemic in the tropical and subtropical regions of 88 countries. There are an estimated 12 million cases worldwide; 1.5 to 2 million new

cases occur every year. Cutaneous forms are most common (1 to 1.5 million cases per year), representing 50 to 75% of all new cases, and 500,000 cases of VL occur every year (81). The geographical distribution of leishmaniasis is limited to the areas of natural distribution of the sandfly, the vector for the disease. Economic development, including widespread urbanization, deforestation, and development of newer settlements, besides migration from rural to urban areas, is responsible for the spread of the sandfly as well the reservoir system of leishmania (76). Moreover, the number of new host populations, i.e., populations of immunodeficient HIV-infected patients, is increasing, especially in southern Europe and Africa (21, 22).

Leishmania-HIV coinfection is regarded as an emerging disease especially in southern Europe, where 25 to 70% of adults with VL have AIDS as well; leishmaniasis behaves as an opportunistic infection, and it has been proposed that it be included as an AIDS-defining illness. Moreover, the presence of the leishmania parasite outside the reticuloendothelial system, e.g., in the peripheral blood, in HIV-infected patients makes these patients a reservoir and source of infection for the vectors. The parasite load in peripheral blood is generally so high that transmission among intravenous drug users by use of shared syringes has also been demonstrated (4). The resurgence of leishmaniasis, its emergence in newer geographical areas and in newer hosts, besides changing the clinical profile of infected patients, has put forward newer challenges in the areas of diagnosis, treatment, and disease control.

PRINCIPLES FOR DIAGNOSIS OF LEISHMANIASIS

The diagnosis of VL is complex because its clinical features are shared by a host of other commonly occurring diseases, such as malaria, typhoid, and tuberculosis; many of these diseases can be present along with VL (in cases of coinfection); sequestration of the parasite in the spleen, bone marrow, or lymph nodes further complicates this issue.

Laboratory diagnosis of leishmaniasis can be made by the following: (i) demonstration of parasite in tissues of relevance by light microscopic examination of the stained specimen, in vitro culture, or animal inoculation; (ii) detection of parasite DNA in tissue samples; or (iii) immunodiagnosis by detection of parasite antigen in tissue, blood, or urine samples, by detection of nonspecific or specific antileishmanial antibodies (immunoglobulin), or by assay for leishmania-specific cell-mediated immunity.

Demonstration and isolation of parasite. The commonly used method for diagnosing VL has been the demonstration of parasites in splenic or bone marrow aspirate. The presence of the parasite in lymph nodes, liver biopsy, or aspirate specimens or the buffy coat of peripheral blood can also be demonstrated. Amastigotes appear as round or oval bodies measuring 2 to 3

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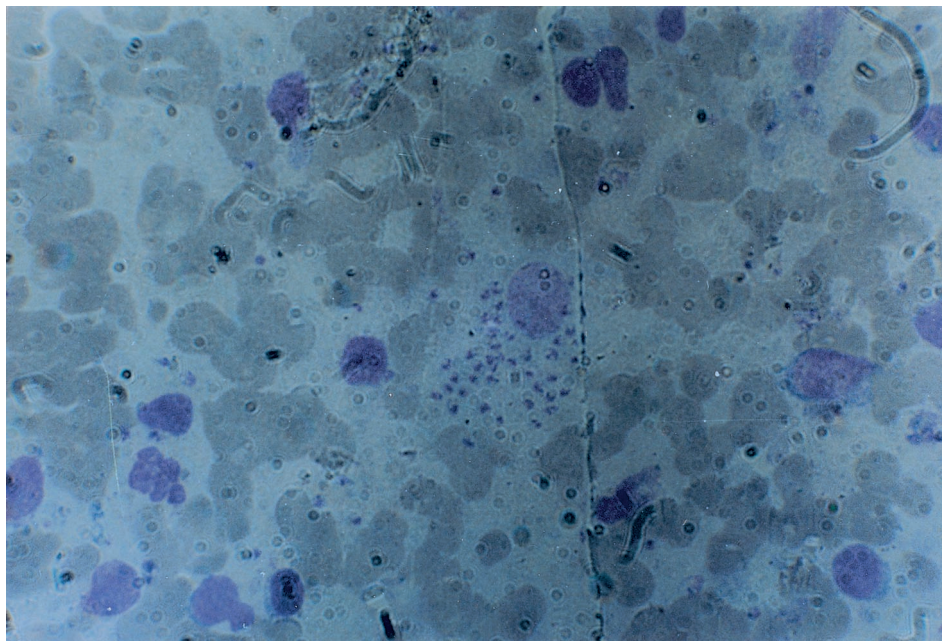


FIG. 1. Microphotograph showing intracellular and extracellular *L. donovani* bodies in splenic aspirate from a patient with visceral leishmaniasis.

μm in length and are found intracellularly in monocytes and macrophages. In preparations stained with Giemsa or Leishman stain, the cytoplasm appears pale blue, with a relatively large nucleus that stains red. In the same plane as the nucleus, but at a right angle to it, is a deep red or violet rod-like body called a kinetoplast (Fig. 1). After identification, parasite density can be scored microscopically by means of a logarithmic scale ranging from 0 (no parasite per 1,000 oil immersion fields) to +6 (>100 parasites per field) (19). The sensitivity of the bone marrow smear is about 60 to 85%. Splenic aspirate, though associated with risk of fatal hemorrhage in inexperienced hands, is one of the most valuable methods for diagnosis of kala-azar, with a sensitivity exceeding 95%. It requires no special equipment, from the patient's standpoint is generally preferable to the more painful bone marrow aspirate, and has proven to be safe and relatively easy to perform in experienced hands. For patients suspected to have VL, splenic aspirate can be performed even when spleen is not palpable, after demarcating the area of splenic dullness by percussion. The only risk of splenic puncture is bleeding from a soft and enlarged spleen. At our treatment center, fatal bleeding has occurred only twice in 9,612 splenic aspirate procedures performed over the last 10 years. To avoid the risk of excessive blood loss, splenic puncture should be avoided in patients with a platelet count of less than 40,000 platelets/ μl and a prothrombin time of more than 5 s over the control.

A tissue specimen, e.g., a spleen, liver, or lymph node tissue specimen, may be subjected to imprint cytology by the repeated pressing of its cut flat surface on microscopic slides. The smear is fixed with absolute alcohol and stained with Giemsa stain. In imprint cytology, a monolayer of cells is formed and amastigotes are easily identifiable. The results are expressed as the number of leishmania per 100 host cell nuclei.

Tissue specimens can also be subjected to histology, and the presence of parasites can be demonstrated by standard hematoxylin and eosin stain. Tissue specimens are usually uneven in thickness; consequently the amastigotes are unevenly distributed. Long searches may be required to demonstrate the parasite. The sensitivity of the test can be increased by staining the specimen with fluorescent dye-tagged antibodies to the surface receptors of the parasite. Fluorescein isothiocyanate isomer- or rhodamide B isothiocyanate-conjugated antiserum is usually used for this purpose. Fluorescent dye-conjugated monoclonal antibodies are also used for speciation of the parasite.

Culture of parasite can improve the sensitivity of detection of parasite, but leishmania culture is rarely needed in routine clinical practice. However, cultures are required for (i) obtaining a sufficient number of organisms to use an antigen for immunologic diagnosis and speciation, (ii) obtaining parasites to be used in inoculating susceptible experimental animals, (iii) in vitro screening of drugs, and (iv) accurate diagnosis of the infection with the organism (as a supplement to other methods or to provide a diagnosis when routine methods have failed). Leishmania strains can be maintained as promastigotes in artificial culture medium. The culture media used may be monophasic (Schneider's insect medium, M199, or Grace's medium) or diphasic (Novy-McNeal Nicolle medium and Tobias medium). We prefer diphasic medium containing modified diphasic rabbit blood agar overlaid with RPMI 1640 (Gibco BRL, Grand Island, N.Y.) (74) for primary isolation, and we prefer M199 medium containing 20% fetal calf serum to amplify parasite numbers (74). Hockmeyer's medium, which is Schneider's commercially prepared culture medium supplemented with 30% heat-inactivated fetal calf serum with 100 IU of penicillin and 100 μg of streptomycin, is simple to use and

satisfactory for diagnosis of VL, but it is expensive (29). Culture tubes are inoculated with 1 to 2 drops of bone marrow or splenic aspirate and incubated at a temperature between 22 and 28°C. The tubes are examined weekly for the presence of promastigotes by phase-contrast microscopy or by wet mount of culture fluid for 4 weeks before being discarded as negative. If promastigotes are present, they are maintained by weekly passage to fresh medium. Blood can also be used to isolate the parasite, but the method is slow and takes longer. Aseptically collected blood (1 to 2 ml) is diluted with 10 ml of citrated saline, and the cellular deposit obtained after centrifugation is inoculated in culture media. Contamination of the culture media by bacteria or yeast species or other fungi usually complicates the culture but can be avoided by use of good sterile techniques and by the addition of penicillin (200 IU/ml) and streptomycin (200 µg/ml) to the medium (for bacteria), as well as 5-flucytosine (500 µg/ml) (as an antimycotic agent) (64).

In vitro culture of the amastigotes is done for chemotherapeutic studies and to study the interrelationship of the amastigotes and macrophages. The amastigotes are grown in tissue or macrophage culture. These cell lines are produced from (i) human peripheral blood monocytes, after these are set apart by density sedimentation with lymphocyte separation medium (LSM; Organon-Teknika, Durham, N.C.), in which case a new batch of macrophages must be produced anew (24); (ii) macrophage cell lines, e.g., P388D and J774G8 lines from mice; and (iii) dog sarcoma and hamster peritoneal exudates of cell lines, in which case continuous culture can be achieved (64).

The parasite can also be demonstrated after inoculation of laboratory animals (such as hamsters, mice or guinea pigs) with infected specimen (42). Animal inoculation is not usually employed as a diagnostic test, since several months may be required to obtain a positive result. Golden hamster is the animal of choice for maintaining *L. donovani* complex (15). It can be infected via many routes, including across mucous membranes, but intraperitoneal and intrasplenic routes are preferred. Both amastigotes and promastigotes can infect the animal. After inoculation, the animal is examined weekly for signs of infection, such as cutaneous lesions, hepatosplenomegaly, or metastatic lesions. Amastigotes can be harvested by biopsy from the spleen and the liver of an animal that is under anesthesia and that is allowed to survive following the procedure as a source of infective parasite. In the absence of signs of obvious infection, the animal is generally sacrificed after 4 months, at which point liver and spleen samples are examined for the presence of the parasite.

In areas of endemicity, recognition of species of leishmania is rarely required. However, identification of an organism to the species level is helpful epidemiologically and is also important for the treatment of and prognosis determination for global travelers who are not immune to the parasite and tend to develop unusual manifestations of the disease (41). Identification of species of the *L. donovani* complex is particularly difficult, because morphologically the species are almost indistinguishable from each other. For species-level identification, a large amount of promastigotes is obtained by culture of the organism and the species-specific isoenzyme pattern is analyzed by cellulose acetate electrophoresis (35). Typing of washed live promastigotes by direct agglutination test with species-specific monoclonal antibodies is another highly sensi-

tive taxonomic tool frequently utilized for this purpose (33). Species-level identification can also be done by analysis of amplified minicircle kinetoplast DNA (KDNA), by choosing primers from conserved regions of different leishmania species KDNA minicircles (61, 71). Yet another method used for identification of species of leishmania is the analysis of the in vitro promastigotes' released antigenic factors, which are different for different leishmanial species (32).

Although demonstration of even a single amastigote upon microscopic examination of tissue smears or multiple promastigotes in cultures is considered sufficient for positive diagnosis of the disease, the sensitivity of the tissue examination, except in the case of splenic aspirate, is low. Moreover, the procedure(s) for obtaining tissue specimen(s) is traumatic and associated with considerable risk. Identification of amastigotes requires considerable expertise and training and is subject to the ability of the observer. Besides, culturing parasites is expensive and time consuming and requires expertise and costly equipment, severely restricting its use in routine clinical practice.

DNA detection method. Due to the limitations inherent in techniques used for detection of parasites, new approaches to the detection of parasites, such as DNA hybridization, have been attempted since the early 1980s. Although these methods had considerable sensitivity (detecting as few as 50 to 100 parasites) (40), their potential use in routine diagnosis is hampered by the complex procedure of hybridization. The development of PCR has provided a powerful approach to the application of molecular biology techniques to the diagnosis of leishmaniasis. Primers designed to amplify conserved sequences found in minicircles of KDNA of leishmanias of different species were tested in various tissues of relevance. Such a target was eminently suitable because the kinetoplast is known to possess thousands of copies of minicircle DNA. In recent years, PCR-based diagnostic methods with a wide range of sensitivities and specificities have been described (1, 5, 51, 54). In a study reported from Sudan, PCR was found to be more sensitive than microscopy for the detection of *Leishmania* parasites in lymph node and bone marrow aspirations. However, its sensitivity for the detection of *Leishmania* DNA in the blood of parasitologically proven VL cases was only 70% (51). In another study reported from India, in which a species-specific primer for *L. donovani* (LDI primer) was used, the sensitivity of PCR with whole blood from VL patients was 96% and *Leishmania* DNA was detected in skin specimens from 45 of 48 patients with PKDL (sensitivity, 93.8%) (54).

A PCR-enzyme-linked immunosorbent assay (ELISA) technique using a primer that was able to identify 33 *L. infantum* strains from 19 different zymodemes has been developed. It has a sensitivity higher than that of other diagnostic techniques, e.g., indirect fluorescent-antibody (IFA) test, parasite culture, or microscopy, and was able to detect a minimum of 0.1 promastigote or 1 fg of genomic material. This PCR-ELISA technique can potentially be used for diagnosis of VL from peripheral blood samples (44). PCR done from blood spots on filter paper can also be used as a screening test to identify *Leishmania* infection in immunocompromised patients with high parasite loads in peripheral blood. The sensitivity of this technique for detecting leishmania (75%) was considerably higher than the respective sensitivities of microscopy (26.3%) and blood culture (42.3%) (17). However, PCR assay

with buffy coat preparations to detect *Leishmania* was 10 times more sensitive than that with whole-blood preparations, and particularly good results were obtained when proteinase K-based methods were used. Proteinase K-based PCR was able to detect 10 parasites/ml (37). A fluorescent DNA probe specific for a conserved region of the small subunit rRNA gene of *Leishmania* and a pair of flanking primers, when used for DNA amplification in one assay, proved to be a highly specific and rapid diagnostic modality to detect infection with *Leishmania* (82). Using this rapid fluorogenic PCR technique, DNA could be amplified from 27 strains of cultured *Leishmania*, and the turnaround time from fresh human tissue biopsy to test result was found to be less than 24 h (82). Besides being a highly sensitive and specific tool for diagnosis of both VL and PKDL and a useful method for species identification (46), PCR can also be used to distinguish between relapse and reinfection in treated VL patients. Restriction fragment length polymorphism analysis of the PCR-amplified minicircle of leishmanial DNA can be utilized for this purpose (47). PCR could also prove to be an important tool in assessing the success of VL treatment: of patients treated for VL who tested negative by PCR with lymph node tissue, none relapsed or developed PKDL, while more than half of patients who tested positive by PCR with lymph node tissue either relapsed or developed PKDL after apparent cure of disease following supervised treatment (50, 52). On the other hand, a substantial number of the patients who tested positive by PCR, after apparent cure, did not relapse or develop PKDL, a result that suggests the limitation of PCR in deciding the end point of treatment. The PCR positivity observed in these patients may be due to non-viable parasite. Similarly, PCR results for healthy endemic controls may be positive (38, 54), which may lead to the erroneous conclusion that they suffer from VL. In these healthy endemic controls, a combination of direct agglutination test (DAT) (which shows low titers in healthy endemic controls) and PCR may be helpful in defining the status of these patients.

Immunodiagnosis. (i) Antigen detection. Antigen detection is more specific than antibody-based immunodiagnostic tests (20, 79). This method is also useful in the diagnosis of disease in cases where there is deficient antibody production (as in AIDS patients). De Colmenares et al. (20) from Spain have reported two polypeptide fractions of 72-75 kDa and 123 kDa in the urine of kala-azar patients. The sensitivities of the 72-75-kDa fractions were 96%, and the specificities were 100%. Besides, these antigens were not detectable within 3 weeks of anti-kala-azar treatment, suggesting that the test has a very good prognostic value (20).

A new latex agglutination test (KATEX) for detecting leishmanial antigen in urine of patients with VL has showed sensitivities between 68 and 100% and a specificity of 100% in preliminary trials. The antigen is detected quite early during the infection and the results of animal experiments suggest that the amount of detectable antigen tends to decline rapidly following chemotherapy. The test performed better than any of the serological tests when compared to microscopy. Large field trials are under way to evaluate its utility for the diagnosis and prognosis of VL (6).

(ii) Antibody detection. For several decades, nonspecific methods, which depend upon raised globulin levels, have been

used in the diagnosis of VL. Some of the tests used for detecting these nonspecific immunoglobulins are Napier's formol gel or aldehyde test and the Chopra antimony test. Since these tests depend upon raised globulin levels, results can be positive in a host of conditions (13, 14). Lack of specificity, as well as varying sensitivities, renders them highly unreliable.

Several immunodiagnostic methods which are more sensitive and specific have been developed. They are useful in identifying specific cases and can be used for community surveillance. The human body makes an attempt to fight against VL by producing some of the highest levels of antibodies found in response to any disease, all to no avail. This is due to polyclonal activation of the B cells, resulting in marked elevation of levels (in serum) of immunoglobulin G (IgG) and IgM against various nonspecific proteins and haptens (23). The consistent presence of high levels of antibodies against parasite antigens can simplify diagnosis of VL. Several serological techniques are based on detection of these antibodies. The specificity of the antibody depends upon the antigen or epitope used in the test, as the parasite stimulates production of a wide array of antibodies, including group-, genus-, and species-specific antibodies. Therefore, the sensitivity may depend upon the test and its methodology, but the specificity will depend on the antigen rather than the serological procedure used. In most serological tests, the sensitivity and specificity data are compared against demonstration of parasites in various tissues.

Conventional methods for antibody detection included gel diffusion, complement fixation test, indirect hemagglutination test, IFA test, and countercurrent immunoelectrophoresis (2, 13, 14, 25, 30, 69, 80). However, aside from practical difficulties at peripheral laboratories, the sensitivities and specificities of most of the above tests have been the limiting factors. Except for the IFA test, which is used on a limited scale, these tests are rarely used for routine diagnosis of VL. In 1988, a modified DAT was reported to be useful in kala-azar and is being used in several countries of endemicity (27). In this test, the trypsinized whole promastigotes are formalin fixed and stained with Coomassie brilliant blue; serum from the patient is then incubated with the antigen, and agglutination is observed the next day. Use of an 0.8% concentration of 0.1 M 2-mercaptoethanol in the sample diluent further improves its performance (66). DAT in various studies has shown to be 91 to 100% sensitive and 72 to 100% specific (45, 67, 72, 84, 85). In Sudan, in specially set up field laboratories, Medecins Sans Frontieres uses DAT for diagnosis of VL; patients with high titers receive treatment, and a confirmatory parasitic diagnosis is done in those with low titers (10). From India, several laboratories reported satisfactory sensitivity and specificity levels for this test (72, 79). Although DAT showed a high degree of repeatability within the centers, its reproducibility across the centers was quite weak (11). Moreover, difficult field conditions, the fragility of aqueous antigen, the lack of cold chain, and batch-to-batch variations in the antigen, along with the nonstandardization of test readings, have severely limited its widespread applicability in regions of endemicity. Freeze-dried antigens developed in Belgian and Dutch laboratories are likely to overcome some of these handicaps (10, 49). Unless this improved antigen is produced indigenously to make it affordable and DAT is made user friendly with one-step dilution and reduced incubation time, its field use is unlikely in countries of ende-

micity like India. Like most antibody-based tests, DAT may yield positive results for a long time after complete cure and thus has not proved to be of much prognostic value (27).

ELISA has been used as a potential serodiagnostic tool for almost all infectious diseases, including leishmaniasis. The technique is highly sensitive, but its specificity depends upon the antigen used. Several antigens have been tried. The commonly used antigen is a crude soluble antigen (CSA). It is prepared by repeated freezing and thawing (four to six cycles) of a suspension of promastigotes in phosphate-buffered saline, followed by cold centrifugation at 10,000 to 20,000 $\times g$. The supernatant is used as soluble antigen and is used to coat ELISA plates after estimation of protein content (100 to 5,000 ng/ml). The sensitivity of ELISA using these concentrations of CSA is reported to range from 80 to 100%, but cross-reactions with sera from patients with trypanosomiasis, tuberculosis, and toxoplasmosis have been recorded (13, 14, 18, 36, 67, 70). On the other hand, when various selective antigenic masses (116 kDa, 72 kDa, and 66 kDa) were used, a specificity of 100% could be achieved, but only at the cost of sensitivity, which went down to as low as 37.5% (20, 79). Palatnik-de-Souza et al. (53) described the use of fucose-mannose ligand as the antigenic molecule. It is a 36-kDa glycoprotein present throughout the life cycle of leishmania (amastigote and promastigote stages). Its use in ELISA has been found to result in 100% sensitivity and 96% specificity (53). In a recent study, it was found that the sensitivity and specificity of ELISA in diagnosing VL could also be increased by the use of soluble antigens derived from promastigotes cultivated in a protein-free medium. One study, done with 129 VL and 143 cutaneous leishmaniasis patients, showed a sensitivity of 95% (56, 60).

A recombinant antigen, rK39, has been shown to be specific for antibodies in patients with VL caused by members of the *L. donovani* complex (7, 9, 16). This antigen, which is conserved in the kinesin region, is highly sensitive and predictive of the onset of acute disease. The antigen is derived from *L. chagasi*, which in the United States is used for veterinary purposes, though it is not approved for human use. High antibody titers in immunocompetent patients with VL have been demonstrated. This antigen has been reported to be 100% sensitive and 100% specific in the diagnosis of VL and PKDL by ELISA (36, 55, 67). Another important facet of anti-rK39 antibody is that the titer correlates directly with the disease activity, indicating its potential for use in predicting response to chemotherapy. It was previously shown that anti-rK39 antibody titers were 59-fold higher than those of antibody against CSA at the time of diagnosis, and with successful therapy, it fell sharply at the end of treatment and fell further during follow-up monitoring. In patients who experience disease relapse, the titer rose steeply again (36). The diagnostic and prognostic utility of rK39 for HIV-infected patients has also been demonstrated (31).

Because of the conditions prevailing in areas of endemicity, any sophisticated method cannot be employed on a wider scale. There is a need for a simple rapid and accurate test with good sensitivity and specificity, which can be used without any specific expertise. A promising ready-to-use immunochromatographic strip test based on rK39 antigen has been developed as a rapid test for use in difficult field conditions. The recombinant 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 the finger is pricked, half a drop of blood is smeared at the tip of the strip, and the lower end of the strip is allowed to soak in 4 to 5 drops of phosphate-buffered saline, placed on a clean glass slide or tube. If the antibody is present, it will react with the conjugate (protein A colloidal gold) that is predried on the assay strip. The mixture moves along the strip by capillary action and reacts with rK39 antigen on the strip, yielding a pink band. In the strip of patients who are infected, two pinkish lines appear in the middle of the nitrocellulose membrane (the upper pinkish band serves as a procedural control). In the first extensive field trial in 323 patients, we found the strip test to be 100% sensitive (confidence interval, 98 to 100%) and 98% specific (confidence interval, 95 to 100%) (75). Several studies from the Indian subcontinent reported the test to be 100% sensitive (9, 73, 75). However, when evaluated in Sudan, the sensitivity of the test was only 67%. In the Sudan study, all the parasitologically confirmed VL patients who tested negative by the rK39 strip test showed IgG against rK39 by micro-ELISA (though at lower titers) (86). In a study done in southern Europe, the rK39 strip test results were positive in only 71.4% of the cases of VL (34). These differences in sensitivity may be due to differences in the antibody responses observed in different ethnic groups (67). When tested for PKDL, the test had a 91% sensitivity (63). High levels of specificity (97 to 100%) have been reported uniformly for this test; however, with a later version of the rK39-treated strips, some (12.5%) healthy endemic control subjects also tested positive (73). While such reactions might be considered to be false positive, these probably represent subclinical infections: PCR assay for *L. donovani* was positive in a few of these cases (62, 73). Anti-rK39 IgG may be present in serum for an extended period after successful treatment for VL; thus, patients with suspected relapse of VL with a past history of infection would not be candidates for diagnosis by strip testing. Another drawback of this format is that an individual with a positive rK39 strip test result may suffer from an illness(es) (malaria, typhoid fever, or tuberculosis) with clinical features similar to those of VL yet be misdiagnosed as suffering from VL. Notwithstanding these limitations, the rK39 immunochromatographic strip test has proved to be versatile in predicting acute infection, and it is the only available format for diagnosis of VL with acceptable sensitivity and specificity levels which is also inexpensive (~1 to 1.5 U.S. dollars) and simple and can be performed even by paramedics in prevailing difficult field conditions.

Specific antibodies can also be detected by Western blotting. For this type of testing, promastigotes of *L. donovani* are grown to log phase and lysed and the soluble protein is run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. The separated proteins are electroblotted onto a nitrocellulose membrane and probed with serum from the patient. The sensitivity of this technique can be enhanced using the chemiluminescent antibody probes. Using Western blotting, one can find even minor antigenic differences among various organisms and thus detect cross-reactive antigens. However, the process is time consuming, technically cumbersome, and expensive (68).

(iii) **Skin testing.** Delayed type hypersensitivity (DTH) or T-cell-mediated immunity is a group-specific immune re-

sponse. The Montenegro skin test (leishmanin skin test) is a test for DTH specific to leishmaniasis, but its role is limited (26, 43, 80). In this method, 0.5 ml of phenol-killed whole parasites (5×10^7 promastigotes) is injected on the volar aspect of the forearm of the patient. After 48 to 72 h, the size of induration is measured and compared with the size of induration produced by injection of a phenol-saline control in the other forearm. Presently, there is no available standardized leishmanin reagent. All leishmanins are said to be alike and nonspecific. The test is negative in acute cases of VL due to the absence of DTH and is positive only in cases where kala-azar has been cured (26, 42).

HIV-LEISHMANIA COINFECTION

Atypical clinical presentations of VL in HIV-infected patients pose a considerable diagnostic challenge. In fact, the clinical triad of fever, splenomegaly, and hepatomegaly is found in less than half of such patients, though more so in patients with low CD4 counts (<50 CD4 cells/mm³) (3, 4, 59). In these patients, leishmaniasis can present with gastrointestinal involvement (stomach, duodenum, or colon); ascites; pleural or pericardial effusion; involvement of lungs, tonsils, and skin; and even as widely disseminated disease (4, 58). The diagnostic principles remain essentially the same as those for non-HIV-infected patients. The presence of amastigotes may be demonstrated in buffy coat preparation. Sometimes the presence of amastigotes in unusual sites may be demonstrated (e.g., amastigotes may be present in specimens from bronchoalveolar lavage, pleural fluid, or biopsy specimens from the gastrointestinal tract). For HIV patients, the sensitivity of antibody-based immunologic tests like the IFA test and ELISA is low (3, 4). Since the parasite load is quite heavy in these patients, the presence of leishmania amastigotes in the bone marrow can often be demonstrated, but there are well-described instances in the literature where amastigotes were not demonstrable on bone marrow, though they were found at unexpected locations like the stomach, the colon, or the lungs. PCR analysis of the whole blood or its buffy coat preparation may prove a useful screening test for these patients, obviating the need for traumatic procedures.

CONCLUSIONS

Various noninvasive tests, with various specificities and sensitivities, are available for the diagnosis of leishmaniasis (Table 1); however, none have become popular in areas of endemicity. Very few are commercially available; generally speaking, they also are expensive, require skilled personnel, expensive equipment, and electricity, and are technically demanding. Parasite diagnosis by splenic, marrow, or skin lesion remains the "gold standard," with its usual limitations. DAT can be performed only in a few centralized laboratories that are equipped for the purpose (and have trained personnel); cost, multiple steps, incubation, and antigenic variations are limiting factors. The rK39 strip test has the potential to be used for diagnosis of VL under field conditions. Other tests, which are likely candidates

TABLE 1. Sensitivities and specificities of various methods used for diagnosis of visceral leishmaniasis

Method and test or tissue used ^a	Sensitivity (%)	Specificity (%)
Immunodiagnosis by:		
Antibody detection (13, 14, 25, 54, 80)		
Complement fixation test	70–80	60–73
Immunodiffusion test	70–75	90–95
CCIEP	80–90	50–70
Indirect hemagglutination	73–75	80–90
IFA test	55–70	70–89
DAT (67, 72, 84, 85)	91–100	72–95
ELISA with CSA (18)	80–100	84–95
ELISA with fucose-mannose ligand (53)	63–100	90–95
ELISA with rK39 antigen (36, 67)	100	100
Rapid strip test with rK39	100 ^b	88–98 ^b
Rapid strip test with rK39	67–71 ^c	97–100 ^c
Antigen detection		
KATEX (6)	68–100	Under evaluation
DNA detection by:		
PCR with LDI primer (62)		
Blood	96	Under evaluation
Bone marrow	100	
Skin	93.8	

^a References are cited in parentheses. CCIEP, countercurrent immunoelectrophoresis.

^b Values reported from the Indian subcontinent (73, 75).

^c Values reported from outside the Indian subcontinent (34, 84).

for diagnosis and prognosis of leishmaniasis in the future, are KATEX and a field-adaptable version of PCR, which would be simple, inexpensive, and easily available.

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