Diagnosis of visceral leishmaniasis

Pankaj Srivastava, Anand Dayama, Sanjana Mehrotra, Shyam Sundar*

Department of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India

ABSTRACT

Leishmaniasis is a vector-borne disease with up to 350 million people at risk of infection worldwide. Among its different clinical manifestations, visceral is the most severe form. Since clinical features of visceral leishmaniasis (VL) mimic several other common diseases, accurate diagnosis is crucial as the treatment is associated with significant toxicity. Invasive and risky techniques involving demonstration of the parasites in stained preparations from splenic and bone marrow aspirate is still the gold standard for VL diagnosis. Serological tests using rK39 in ELISA or rapid immunochromatographic format, Direct Agglutination Test (DAT), immunoblotting have issues related to a significant proportion of asymptomatic individuals being positive with these tests and their inability to diagnose relapses as these remain positive for several months to years after cure. PCR is the most common molecular technique successfully used for diagnosis and differentiation of species. Through this review we focus extensively on the comparative utilities of the various diagnostic tools currently available for VL, describing in depth their advantages and disadvantages, addressing the recent advances attained in the field. A simple, rapid, non invasive, accurate and cost effective marker of active VL, which can be used in field conditions, is necessary to improve diagnosis of VL.

© 2010 Royal Society of Tropical Medicine and Hygiene. Published by Elsevier Ltd.

1. Introduction

Visceral leishmaniasis (VL), is a vector-borne disease caused by replication of parasites in macrophages, mononuclear phagocytic system. It is caused by the Leishmania donovani complex, which includes L. donovani and L. infantum. It is endemic in large areas of the tropics, subtropics and the Mediterranean Basin. VL is a systemic disease and is fatal if left untreated. The transmission characteristics of VL differs in different geographical regions; in the Mediterranean Basin, Brazil and parts of Africa, the dog is the main reservoir and VL is zoonotic; while in the Indian subcontinent and parts of Africa, it is anthropogenic.

Post kala-azar dermal leishmaniasis (PKDL) is a sequel of VL, characterized by amacular, maculo-papular or nodular rash and is frequently observed in Sudan and the Indian subcontinent.2

Clinical features of VL can be easily mistaken for other febrile illnesses such as malaria and enteric fever. Reliable laboratory methods become mandatory for accurate diagnosis. Early case detection followed by adequate treatment is central to the control of VL.3,4 Patient management, screening of asymptomatic infections, surveillance including verification of elimination, and epidemiological studies are some of the areas where diagnostic tests play a major role. Ideally, a test should make the distinction between acute disease and asymptomatic infection, as most of the antileishmanial drugs are toxic. Moreover, such tests should be highly sensitive and specific, simple and affordable, but unfortunately some commonly used tests like rK39 immunochromatographic strip test (ICT) and Direct Agglutination Test (DAT) carry some significant disadvantages: the inability to differentiate between clinically active and asymptomatic infections and showing positive long...
after cure. Molecular diagnostic tools like PCR and real-time PCR are quite sensitive and specific but are cumbersome to perform and have a high cost. DNA-based tests are available in strip formats but these cannot be used in the field. The DAT assay has shown similar diagnostic performance but is not as user-friendly as the rK39. A study from Sudan compared the diagnostic performance of rK39 and DAT for VL both qualitatively and semi-quantitatively and recommended the combined application of these two tests for optimizing diagnosis and simultaneously assessing the magnitude of immune response to L. donovani infection. Qualitatively both rK39 and DAT demonstrated comparable reliability for VL detection (sensitivity = 96% and specificity = 98.7% or 99.3%).

We searched Medline through PubMed for articles on the diagnosis of VL (Box 1).

### Box 1. Search criteria, data extraction and quality assessment.

<table>
<thead>
<tr>
<th>Search Criteria</th>
</tr>
</thead>
</table>
| We searched Medline through PubMed ([http://www.ncbi.nlm.nih.gov/pubmed/](http://www.ncbi.nlm.nih.gov/pubmed/)) for articles on the diagnosis of VL. Methods compared were microscopy, direct agglutination test (DAT) rK39 immunochromatographic (ICT) assay, ELISA used on serum or blood samples and PCR assay for VL diagnosis. The search terms were ‘visceral leishmaniasis’, ‘kala-azar’, with ‘diagnostic accuracy’, ‘sensitivity’, ‘specificity’. This search generated 384 papers, which we subsequently combined with the search terms ‘DAT’, ‘ELISA’, ‘ICT dipstick’ and ‘PCR’. Additional studies were identified through back tracing of reference lists and subsequent reports from the same longitudinal studies. Data extraction and quality assessment We abstracted information from published journals, compared and summarized their sensitivity and specificity along with their merits and demerits of different assays. For quality assessment we took into account different diagnostic markers, their field evaluations and the role of diagnostic tools in treatment failure.

## 2. Existing diagnostic methods for diagnosis of VL

### 2.1. Parasitological Diagnosis

Parasitological diagnosis remains the gold standard in the diagnosis of leishmaniasis because of its high specificity. The amastigote forms (called LD bodies) can be seen in tissue smears from lymph nodes, bone marrow or spleen. 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. Splenic smears have the sensitivity of 93.1–98.7%. Bone marrow and the lymph node smears have lower sensitivity ranging from 52–85% and 52–58% respectively. Bone marrow aspiration (BMA) or splenic aspiration are painful and risky techniques. Serious or fatal bleeding after splenic aspirate is not uncommon, however, in skilled and experienced hands, serious bleeding is rare. At our treatment centre (Kala-azar Medical Research Centre, Muzaffarpur, Bihar, India), fatal bleeding has occurred only twice in 9612 splenic aspiration procedures performed over the last 10 years. The use of microscopy in the diagnosis of VL offers the benefits of high specificity and the possibility of grading the parasite on a logarithmic scale (0–6+) in splenic smears. But like all microscopic procedures it suffers from variability of detection sensitivity and the inevitable need for an expert microscopist. Culture, too, suffers from the same deficiencies and the tedious, time consuming nature of the technique and the high cost are prohibitive and thus, except in dedicated research laboratories, it is seldom used for clinical diagnosis. In a modification in the form of microtitre culture, sensitive and reproducible detection of parasites was possible using buffy coat (WBC rich layer) and peripheral blood mononuclear cells (PBMC) isolated from patient blood. *Leishmania* strains can be maintained as promastigotes in artificial culture medium. The culture media used may be biphasic (Novy-McNeal Nicolle medium and Tobies medium) for conversion of amastigotes to promastigotes while monophasic medium (Schneider’s insect medium, M199, or Grace’s medium) is preferred for amplifying parasite number.

### 2.2. Serological Diagnosis

The serological diagnosis is based on the presence of specific humoral response. A wide range of serological methods varying in sensitivity and specificity are available for the diagnosis of VL. These serological methods can be grouped into non-specific and specific tests.

**Non specific tests:**
- Tests like formal gel test have been in use in the past but should be abandoned because of their poor specificity and sensitivity.

**Specific tests:**
- Indirect Fluorescent Antibody Test (IFAT): 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. If the antibodies persist in low titres, it is an indication of a probable relapse. It is sensitive (96%) and specific (98%) but the requirement of sophisticated laboratory conditions prohibit its application in the field.
- Enzyme Linked Immunosorbent Assay (ELISA): ELISA has been used in the serodiagnosis of VL. Sensitivity and specificity of ELISA depends upon the antigen used. Most promising results are shown by antigen rK39 with sensitivity and specificity of 100% and 96% respectively. The antibody titres to this antigen directly correlate with active disease and have potential in monitoring the chemotherapy and in predicting the clinical relapse. In addition, rK39 ELISA has a high diagnostic and prognostic utility in HIV-infected patients. Due to the requirement of skilled personnel, sophisticated equipment and electricity, ELISA is not used in the endemic regions for the diagnosis of VL.

**Immunoblotting:** Provides detailed antibody responses to various leishmanial antigens. This test is more sensitive than IFAT and ELISA, but expensive and time consuming. It also requires considerable skill and is therefore only sparingly used in the diagnosis of VL.
Direct Agglutination Test (DAT): In this test, Coomassie brilliant blue stained whole promastigotes are incubated with sera of the patients and agglutination observed after an overnight incubation. It is a highly specific, sensitive, inexpensive and simple test. Initially, aqueous antigen was used but it had the drawback of cold chain requirement, and short life. Now, freeze dried antigen has been developed which can be transported at ambient temperature. In a meta-analysis of studies using DAT, it had sensitivity and specificity estimates of 94.8% (95% confidence intervals (CI), 92.7–96.4%) and 85.9% (95% CI, 72.3–93.4%), respectively. However, the major disadvantage of DAT is the need of multiple pipetting, relatively long incubation time, high cost of antigen and limited production facility of quality controlled antigen in two European laboratories. As with any antibody based test, DAT remains positive for a long time after the disease is cured, and thus cannot be used as a test of cure or for diagnosis of relapses. Furthermore, about 20–30% of healthy individuals living in the endemic areas test positive with DAT, and an illness mimicking VL might be mistaken for VL if DAT is positive in this particular individual.

Immunochromatographic (ICT) strip test: Immunochromatographic strips using K39 antigen have become popular in recent years. K39 antigen contains 39 amino acids encoded by a 117 base pairs gene encoded in the highly conserved kinesin region of L. chagasi. In micro-ELISA format this antigen showed remarkably high sensitivity. Using its recombinant product, an immunochromatographic based strip test has been developed in which rK39 is fixed on a nitrocellulose paper, and colloidal gold-protein A is used for detection. A drop of serum or blood obtained by finger prick is smeared over the tip of the strips and dipped in a small amount of buffer, with the results read within 15 minutes. In the initial clinical evaluation 100% sensitivity and 98% specificity was observed. The rK39 strip test has been found highly sensitive and a reliable indicator of kala-azar. In a meta-analysis of rK39 strip test studies, the results were quite uniform with very high sensitivity 98.4–100% and specificity 81.2–96.4% in Sudan, however, the sensitivity (69.2–85.6%) of strip tests has been low compared with other regions. However, a new version produced by DiaMed AG, (Cressier sur Morat, Switzerland) has been reported to have satisfactory results from Sudan. Unfortunately the market of strips in disease endemic countries remains unregulated, and several formats are available which have not been evaluated for their performance. ICT suffers from the same disadvantage as DAT: being positive in a significant proportion of healthy individuals in endemic regions and for long periods after cure of VL. TDR/WHO are carrying out a worldwide multicenter evaluation of commercially available rapid tests for VL.

Antigen Detection: Antigen detection is an excellent method of diagnosing an infection. It is more specific than antibody-based immunodiagnostic tests. Antigen levels are expected to broadly correlate with the parasite load. This method of diagnosis should be a better alternative to the antibody detection, particularly in HIV-VL coinfection, where antibody response is very poor. Two polypeptide fractions of 72-75 kDa and 123 kDa were detected in the urine of kala-azar patients. The sensitivity of the 72-75 kDa fractions were 96% and the specificity was 100%. These antigens were not detectable within three weeks of successful antileishmanial treatment, suggesting that the test has a very good prognostic value. Another urinary leishmanial antigen, a low-molecular-weight, heat-stable carbohydrate was detected in the urine of VL patients. An agglutination test to detect this antigen has been evaluated extensively in clinical trials, using urine collected from well-defined cases and controls from endemic and non-endemic regions. This test showed 79.1–94.1% specificity and sensitivity of 60.4–71.6% in India. However, the sensitivity of this test was low in clinically suspected patients. Efforts are being made to improve the performance of this technique, as it holds promise as a test of cure, for which none of the current serological tests can be employed.

2.3. Molecular Diagnosis

PCR-based assays form the mainstay of molecular diagnosis especially for HIV-VL coinfections, with primers targeting several multicopy genes, e.g., rRNA genes, kinetoplast DNA (kDNA) minicircles. A comparative overview of sensitivities and specificities of various PCR based diagnostic assays targeting different regions of leishmania genome has been presented in Table 1. A recent comparative clinical study in Italy between conventional microbiologic techniques and a leishmania species-specific PCR assay, using peripheral blood and BMA samples had shown the sensitivities of the leishmania species-specific PCR to be 95.7% for BMA and 98.5% for peripheral blood samples versus sensitivities of 76.2%, 85.5%, and 90.2% for BMA isolation, serologic testing, and microscopic examination of bone marrow biopsy specimens, respectively. In PCR screening of blood samples of suspected cases of VL, sensitivity ranging from around 70% to around 100% has been reported. PCR from blood samples obviates the cumbersome and risky process of bone marrow and splenic aspiration. Recently epitope specific PCR and oligochromatographic dipstick assay has been reported for VL and PKDL detection with sensitivity to detect less than one parasite. Real-time PCR has made quantification of parasite burden possible, with a high degree of complex-specific diagnostic accuracy for clinical samples. Quantitative nucleic acid sequence-based amplification (QT-NASBA) detects RNA in a background of DNA and may thus serve to measure viable parasites which might significantly increase assay sensitivity and decrease required sample volume. In a recent report from Kenya the Leishmania OligoC-Test showed a sensitivity of 96.4% and a specificity of 88.8%, while the sensitivity and specificity of the NASBA-OC were 79.8% and 100%, respectively. These findings indicate high sensitivity of the Leishmania OligoC-Test on blood while the NASBA-OC is a better marker for active disease. The sensitivity of PCR using conjunctival swab (CS) as a sampling method for VL diagnosis by PCR of asymptomatic dogs was found 90% by kDNA primer and 83.3% by internal transcribed spacer 1 (ITS1) primer. On the other hand, for blood samples, the positivity of ITS1 PCR was significantly higher than the one obtained.
Table 1
Reported sensitivities and specificities of different targets using PCR protocols in the diagnosis of visceral leishmaniasis

<table>
<thead>
<tr>
<th>Target (bp) for PCR</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Reported by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood</td>
<td>BMA</td>
<td></td>
</tr>
<tr>
<td>kDNA (nr): L. donovani</td>
<td>90</td>
<td>NR</td>
<td>100</td>
</tr>
<tr>
<td>kDNA (204 bp): L. donovani</td>
<td>82.3</td>
<td>NR</td>
<td>100</td>
</tr>
<tr>
<td>kDNA (600 bp): L. donovani</td>
<td>96</td>
<td>100</td>
<td>96</td>
</tr>
<tr>
<td>kDNA (790 bp): Leishmania species</td>
<td>100</td>
<td>83</td>
<td>100</td>
</tr>
<tr>
<td>kDNA (600 bp): L. donovani</td>
<td>99</td>
<td>NR</td>
<td>100</td>
</tr>
<tr>
<td>SSU-rRNA n-PCR (358 bp): L. donovani</td>
<td>95.4</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SSU-rRNA</td>
<td>73.2 by conventional PCR; 83.9 by ELISA PCR</td>
<td>NR</td>
<td>87.2</td>
</tr>
<tr>
<td>SSU-rRNA n-PCR (358 bp): L. infantum</td>
<td>79</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SSU-rRNA real-time: L. infantum</td>
<td>100</td>
<td>NR</td>
<td>100</td>
</tr>
<tr>
<td>SSU-rRNA oligoC Test</td>
<td>93.2</td>
<td>NR</td>
<td>98.3</td>
</tr>
<tr>
<td>MedRNA (180 bp): L. donovani</td>
<td>96.8</td>
<td>NR</td>
<td>100</td>
</tr>
<tr>
<td>PCR mini-exon gene (450 bp): L. donovani</td>
<td>NR</td>
<td>83.3</td>
<td>100</td>
</tr>
<tr>
<td>n-PCR (100 bp): L. infantum</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

BMA: bone marrow aspirate; bp: base pair; kDNA: kinetoplast DNA; medRNA: multicopy mini-exon RNA; n-PCR: nested PCR; NR: not reported; SSU-rRNA: small subunit ribosomal RNA

by the kDNA PCR-hybridization, indicating that sensitivity of PCR methods can vary according to the biological sample examined.48

Reverse transcriptase-loop-mediated isothermal amplification (RT-LAMP) assay, based on nucleic acids, was recently developed as a point-of-care diagnostic tool. Amplification was visualized by the pre-amplification addition of fluorescent detection reagent (FDR) and a simple UV lamp. By using a reverse-transcriptase step, the system detected infections between 10 and 100 parasites per mL. The assay was tested on a range of nucleic acid extracts from Leishmania species VL patients from Sudan, and cutaneous leishmaniasis (CL) patients from Suriname. The sensitivity of RT-LAMP from the blood of VL patients was 83%.49

3. Diagnosis of HIV-VL coinfection

VL is an important opportunistic infection in AIDS patients and atypical clinical presentations of VL in HIV-infected patients pose a considerable diagnostic challenge. In fact, the clinical symptoms of fever, splenomegaly, and hepatomegaly is found in less than half of such patients, though more so in patients with low CD4 counts (less than 50 CD4 cells/mm³).50,51 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.51 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).14 For HIV patients, the sensitivity of antibody-based immunologic tests like the IFA test and ELISA is low. 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

Table 2
Various diagnostic assays for visceral leishmaniasis

<table>
<thead>
<tr>
<th>Assay</th>
<th>Test time</th>
<th>Required skill level</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parasitological diagnosis:</td>
<td>Hours</td>
<td>Expert</td>
<td>Splenic aspirate 93.1-98.7%9-11; Bone marrow aspirate 52-85%11; Lymph node aspirate 52-58%9</td>
<td>100%</td>
<td>Restricted to endemic areas where clinicians are familiar with signs and symptoms, and for culture sophisticated laboratories are required.</td>
</tr>
<tr>
<td>Microscopic examination</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agglutination test (using urine)</td>
<td>Minutes</td>
<td>Medium</td>
<td>64-100%62,63</td>
<td>100%</td>
<td>Ideal method of diagnosing an infectious agent; antigen level correlates with parasite load. Requires equipped laboratory setup.</td>
</tr>
<tr>
<td>Indirect Fluorescent Antibody Test</td>
<td>Hours</td>
<td>Expert</td>
<td>96%15</td>
<td>98%</td>
<td></td>
</tr>
<tr>
<td>Direct Agglutination Test</td>
<td>Hours</td>
<td>Medium</td>
<td>94.8% (95% CI, 92.7-96.4)19</td>
<td>97.1% (95% CI, 93.9-98.7)</td>
<td>Usef ul for epidemiology studies.</td>
</tr>
<tr>
<td>Immunochromatographic Strip Test</td>
<td>10 minutes</td>
<td>Low - Lab technician</td>
<td>93.9% (95% CI, 87.7-97.1)19</td>
<td>95.3% (95% CI, 88.8-98.1)</td>
<td>Used for screening by using rK39 strip, not useful in treated patient. Cannot be used in field setting. Standardization is hindrance, lab to lab variation and contamination.</td>
</tr>
<tr>
<td>ELISA</td>
<td>Hours</td>
<td>Medium</td>
<td>100%23</td>
<td>96%</td>
<td></td>
</tr>
<tr>
<td>PCR</td>
<td>4-5 hrs</td>
<td>High</td>
<td>70-100%36,41-43,61</td>
<td>85-99%</td>
<td></td>
</tr>
</tbody>
</table>
instances in the literature where amastigotes were not demonstrable in 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.

Table 2 presents a concise summary and features of all the described techniques commonly used for VL diagnosis.

4. Conclusions

Accurate diagnosis of VL still remains a problem for clinicians and coordinators of kala-azar control programs. Though the gold standard remains the demonstration of parasites, it has several disadvantages and thus diagnosis in the field is delayed. Antibody based diagnosis, like rK39 strip test is being used world wide in the endemic countries despite their inherent disadvantage of being positive in significant proportion of healthy individuals, and remaining positive for long periods after cure. DAT based on whole promastigotes of \textit{L. donovani} or \textit{L. infantum} and the rK39-ICT are the two serological tests used widely for the diagnosis of VL. 

52–54 There is an urgent need to develop a marker of the active diseases, urine based latex agglutination test had all the qualities to detect only active patients, quickly turning negative after a successful response, however its low sensitivity needs to be improved. Molecular biology-based assays for detecting parasite DNA have been developed, but none have become popular in field diagnosis.

Authors’ Contributions: PS and AD conceived the idea of writing the review. PS, AD, SS and SM collected and organised the data and drafted the manuscript. All authors critically read, edited and approved the final manuscript. SS is guarantor of the paper.

Acknowledgements: The authors would like to thank all the research scholars working in Infectious Disease Research Laboratory, Department of Medicine, IMS, BHU.

Funding: This work was supported by NIAID, NIH Grant Number: 1P50AI074321-01

Conflicts of Interest: None declared

Ethical approval: Not required

References