Diagnosis of Visceral Leishmaniais: Review approach on development of different techniques from invasive to noninvasive nature after more than three decades of research.

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Abstract:

For the efficient management of infectious disease, in the present scenario, rapid, sensitive, specific and confirmatory identification of the pathogen is very important. Accurate diagnostic test plays a key role in clinician trust and patient management. Visceral Leishmaniasis (VL) is a neglected tropical infectious disease. Poor access to diagnosis stymies control of VL. In light of the study published regarding the rapid development of Leishmania detection technology and their application in VL diagnosis, In this review, the author discusses critical issues in the development of diagnostic method as well as promises and challenges for validation & transition of new innovations.

Key words: VL, Pathogen, Sensitive, Specific, Diagnosis

Inroductcion

Diagnostic testing is traditionally considered as a tool to rule out a condition or infection when the clinical presentation in a patient is non-specific [1]. Diagnostic tools " of sufficient sensitivity and specificity to detect levels of infection that can lead to transmission " were identified as one of the essential requirements for disease elimination and eradication [2] . Visceral Leishmaniasis (VL) is a vector- borne disease caused by the Leishmania donovani complex, which includes: L. donovani and L. infantum. This infectious disease is also referred in the category of Neglected Tropical infectious disease. WHO defines the case of Visceral Leishmaniasis as "a person showing clinical signs (mainly prolonged irregular fever, splenomegaly and weight loss) with serological and/or parasitological confirmation". These clinical features can easily be mistaken for other common febrile illness such as malaria, enteric fever, tuberculosis, etc. If left untreated, it is almost always fatal (www.who.int/tdr).

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The mean period from the onset of the disease symptoms to diagnosis (mean diagnostic lag period) is 7.7 ± 5.96 months [3]. In this period because of multiplication of complications patient not only suffers but also continues to spread the disease. Since humans are the only reservoir for L.donovani in the Indian subcontinent, control of VL programme require early case detection and further treatment . Clinical diagnosis already rely heavily on different techniques for patient disease classification, management and informing treatment & care pathways. The estimated annual global incidence of VL is 200,000–400,000 and >90% of these cases occur in India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil [4]. The visceral leishmaniasis (commonly called ´kala azar`) in the Indian subcontinent has been endemic for many decades of the estimated mortality is 50,000-200,000 per year [5] . From 1987 through 2011, a total of 6,70,897 VL cases were reported officially from Bihar only. Patient management, screening of asymptomatic infections and epidemiological studies are some of the areas where diagnostic tests play a major role. Some time rare presentation of active VL also comes [6, 7]. Evaluating a diagnostic test is particularly challenging when there is no reference gold standard, which is easy to perform. Therefore, at present scenario VL diagnosis remains a challenge. It is essential that the diagnosis should be sensitive as well as specific as much as possible. To be useful, diagnostic methods must be accurate, simple and affordable for the population for which they are intended. Researches are going on in this aspect since decades. Recent developments in new diagnostic tools, however, have opened new avenues for a vast improvement in parasite detection. Recent technological developments have led to the proliferation of new, rapid diagnostic tests that hold promise

for the improved management and control of infectious disease. In light of this, developments in diagnosis from traditional invasive to nonivassive approaches addressed here.

Direct Parasitological confirmation

Diagnostic in parasitology has traditionally centered on morphology using light microscopy and various histochemical stains. In visceral infection Leishmania mainly resides in visceral organ , i.e spleen. Therefore, gold standard techniques for VL diagnosis is manual observation of amastigote in splenic smear of VL patients. Amastigotes look like round or oval bodies measuring 2 to 3 m in length and are identified intracellularly in monocytes and macrophages. In preparations colored with Giemsa or Leishman stain, the cytoplasm looks pale blue, with a quite large nucleus that stains red. At the same plane as the nucleus, but at a right position, it is a deep red or violet rod-like body known as a kinetoplast [8]. Parasite density in splenic aspirate smears was graded on a logarithmic scale from 0 (no parasites in 1,000 microscopic fields) to 6+ (greater than 100 parasites per microscopic field) as no disease to highly severe form [9]. Sensitivity of amastigote detection by microscopy has been reported 96-98%, 70% and 58% for splenic, bone marrow and lymph node tissue smears, respectively[10-14]. Amastigotes (called LD bodies) of Leishmania parasite are demonstrated using light microscope in splenic/bone marrow aspirate or lymphnode aspirate by others also[15, 16], or the buffy coat of peripheral blood (in HIV coinfection) [17, 18]. 2% positivity of buffy coat smear suggested use of blood for parasitological confirmation based dignostic test in VL [19]. Sensitivity of patient spleen biopsies for Leishmania parasites culture in NNN-tube is around 97-100%, whereas the sensitivities of bone-marrow and lymph-node aspirate are estimated at 53-86% and 53-65% respectively [14]. Patient's peripheral blood contains living amastigotes [20], which could be a very good source for diagnosis of VL and as it has been shown the sensitivity of buffy coat culture is around 84% [21]. Culture showed positivity of 98.52-100 % from splenic aspirates and 85.29% and 91.48% respectively from buffy coat and PBMC in the Indian VL endemic region[22],[24]. Skin slit smear microscopy is the only confirmatory test for PKDL.

Antigen-Antibody based detection

Development of an immune-epidemiological tool to detect L.donovani (LD) infection greatly benefit the VL control and elimination programme. RDTs have been introduced to increase VL screening, enabling same day testing and treatment of patients. Most RDTs use recombinant K39 antigen, a 39-amino-acid antigen conserved in the kinesin region of Leishmania chagasi syn. Infantum [23-25]. rK39 RDT have almost 100% sensitivity, but in areas with high transmission, reported positivity for the rK39 rapid test is 15 to 32% in healthy individuals[26-28]. In VL endemic region rK39 ELISA showed 93-100% sensititvity and 97-98% specificity [29, 30]. Meta analysis report for rK39 RDT estimated sensitivity of 93.9% (95%CI , 87.7-97.1) with specificity 95.3% (95%CI, 88.8-98.1). However, less accuracy of rK39 RDT has been found in East Africa and Sudan [31]. One of the Brazilian studies showed rK39 RDT more sensitive than IFAT & elisa [32]. This rK39 RDT has been developed for use with serum but now are routinely performed on whole blood in endemic region of India, Nepal & Bangladesh [33]. rKk39 RDT performed on blood vs serum have good agreement with kappa value of 0.88, but it was found to be chances of getting negative result with blood and positive with serum when titres of anti rK39 antibodies low [34]. Non invasive approaches on urine & Saliva by rK39 RDT were also performed on Indian VL samples [35, 36]. rK28 antigen had similar sensitivity and specificity in ELISA format as rK39 [37]. In search of other antigen based detection methods, rKE16 antigen-based rapid test also found to be of good sensitivity and specifity in Indian population40 [38]. ELISA based on recombinant 70kDa HSP showed both good sensitivity and specificity but recombinant 12.6 kDa had good specificity and low sensitivity when performed on sera in Indian VL endemic population [39-41]. Filter papers impregnated with patient blood have been also used without affecting the performance [42]. Though serum antibody levels decreases after successful treatment [43, 44] they remain detectable up to several years after cure [45, 46]. Leishmania recombinant proteins (rgp63, rK39, gene B protein, rH2A and rH2B histones proteins, rLACK, rPSA-2, rP20) have also been tested for potential use in diagnosis with sensitivities varying between 85% and 100% [47]. As with any antibody based test, Direct Agglutination test (DAT) remains positive for a long time after the cure of the disease, 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[48]. This semiquantitative test DAT was the first real field application of a diagnostic test for VL[49-51]. The antigen detection is considered more specific than antibody based immunodiagnostic tests[52, 53]. There are existence of circulating antigens and immune complexes in VL[54-57]. Urine based antigen study was also performed to diagnose active VL[32, 58, 59]. But the question always arises for their sensitivity and specificity.

The LST result is usually negative in active kala azar (symptomatic VL) patients and becomes positive several months or longer after successful treatment in a proportion of patients[31, 60].

Molecular diagnosis

Several nucleic acid based detection methods are evolved as Molecular techniques for parasitological confirmation based diagnosis of VL as well as other Leishmania infection. Molecular assay offers superior diagnostics performance compared to the limit of detection of

immunoassay. Appropriate design and workflow are major considerations for any molecular diagnostic laboratory.

Parasitological confirmation is important for reinfection/relapse and PKDL. When the discussion is about immunosuppressed patients molecular diagnosis is choice of priority. Based on different targeting region in conventional PCR sensitivity ranges from 70% to 100%[61-72]. These methodologies comprised of different targeting regions of Leishmania parasite and also samples as PBMC, whole blood, buffy coat with primers targeting several multicopy genes and internal spacers like ITS region, gp63 locus, telomeric sequences, sequence targets in rRNA genes such as SSU-rRNA and both conserved and variable regions in kinetoplast DNA (kDNA) minicircles, tryparedoxinperoxidase[67, 73-77]. PCR from blood samples obviate the cumbersome and risky process of bone marrow and splenic aspiration. Polymerase chain reaction (PCR) based assays form the mainstay of molecular diagnosis, especially for HIV-VL co-infections[78, 79]. The sensitivity of PCR using conjunctival swab for VL diagnosis in dogs was found 90% by kDNA primer and 83.3% by ITS1 primer. On the other hand, for blood samples, the positivity of ITS1 PCR was significantly higher than the one obtained by the kDNA PCR-hybridization, indicating that sensitivity of PCR methods can vary according to the biological sample examined[80]. In Indian VL non invasive PCR based assay has been used for the first time using buccal samples [81]. PCR assay show 86.3 % sensitivity &98.3 specificity in buccal swab of HIV-VL coinfection[82]. Comparative study of conventional microbiologic techniques, and a PCR assay, using peripheral blood and bone marrow aspirate samples for VL had shown the sensitivities of 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 Italy[83].

PCR based diagnostic assay targeting SSU rRNA has been developed and validated on a large sample size using minimal amount of blood with 87.8% sensitivity [84]. PCR also used as prognostic marker for the development of relapse or PKDL after apparently successful treatment [85]. Although the cnPCR tests are parasitological confirmation with varying sensitivity one limitation of these techniques is their varying specificity and unable to discriminate between patients and positivity of endemic healthy persons. Taking advantage of the accessibility PCR was also performed on urine samples for VL diagnosis having sensitivity of 88% and 100% specificity [86]. The Comparative study of human & dog in northwestern in iran show parasitemia of human was significantly less compared to Dog [87]. Conjuntival swab, nasal, ear & oral swab for CVL were also reported [88]. kDNA PCR also performed on serum to detect Leishmania specific DNA [89]. Single step duplex kDNA PCR assay also preferred which showed high sensitivity for L. donovani complex DNA detection from blood of VL patients [90]. kDNA cnPCR finding showed 91-95.5-% from Bone marrow while 68% & 29.4% of blood and sera respectively [91].

Quantitative PCR (qPCR) is an emerging diagnostic technique in detection of different pathogen infection. Quantitative PCR based on REAL TIME PCR theory is not only able to detect but also quantify Leishmania in infected subjects[92-95]. qPCR have advantages over PCR is that it also differentiate sps. of Leishmania [96]. Leishmania load is found to be lower in blood than spleen in CVL[97]. The sensitivity of Real Time PCR in ear hair was similar to that obtained in blood. Moreover, the presence of L. infantum kDNA was also detected in the hair of all the analyzed body zones[98, 99]. Although parasite load by qPCR in blood and oral fluid have no significant relation, but sensitivity to detect Leishmania in these 2 different samples reported same[100]. They suggested, Parasite load equal to or greater than 100-1000 parasites/ml in bone marrow are detectable in blood whereas lower parasite load 1-100 parasite/ml in bone marrow are usually negative in the blood in CVL [101]. qPCR also have a role in diagnostic and prognostic purpose to see effective treatment[102]. There is a positive correlation between microscopy based splenic score grade (invasive type) with that of qPCR finding from blood (non-invassive type) [103, 104]. In 2014 , qPCR study also showed its usefulness in early detection of VL and differentiate symptomatic from asymptomatic[104, 105]] . Different cytokines IL-10, IL-4, IFN-γ, TNF-α were correlated with parasite load by qPCR in different Leishmania infection presentation[106]. Parasite burden also studies in urine in canine VL using qPCR [107]. But, the main draw back of targeted pathogen specific PCR is that it is only able to identify predefined targets.

Fluorescence Resonance Energy Transfer-based real-time PCR assay (FRET) also performed for Leishmania detection [108]. Quantitative nucleic acid sequence-based amplification (QT-NASBA) detects RNA in a background of DNA and measure viable parasites which might significantly increase assay sensitivity and decrease required sample volume[109]. Leishmania OligoC-TesT in Kenya 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 [110]. A few studies employing LAMP assay to diagnose Leishmania infection are known .However, these assays are limited in their utility because of the false positivity due to cross contamination or prolonged reaction time or the use specialized equipment[111-117]. Though strip format of DNA based test is available but it cannot be used in field[118].

A growing number of promising diagnostic tools are based on nanotechnology. The application of nanomaterials to detect host or pathogen biomarkers has the potential to yield ultrasensitive assays. Considering this Nano structured nickel oxide based DNA biosensors for detection of Kala azar targeting region 18s rRNA of Leishmania was also developed[119].

Challenges in Visceral Leishmaniaisis diagnosis

The different algorithms proposed to identify infections presents specific advantages & limitations. In the current review, we are trying to highlight the crucial knowledge gap after 25 years as well as the obstacles in research on leishmania infection diagnosis. Challenges in different aspects for VL are as follows :

VL and HIV co-infection: Although a clinical practical guidelines for VL-HIV diagnosis has been published by WHO. But it is mainly based on Buffycoat , PBMC, Bone Marrow PCR/culture as serology have limited sensitivity. Therefore, future research to improve the existing format would be necessary to obtain a noninvasive tool for diagnosis & treatment monitoring.

Relapse : Antibodies remain detectable up to several years after cure therefore VL relapses cannot be diagnosed by serology . Combining clinical signs and parasitological/molecular confirmation are necessary for prediction of clinical relapse. Therefore new markers to access cure and to help to predict relapses are still needed.

PKDL: Confirmatory test for PKDL, A sequale of VL is only parasitological confirmation by microscopy or molecular tests. If there is a previous history of VL serology test is not appropriate and clinical sign may mislead (skin lesions have sometimes similarity with other skin diseases).

Asymptomatic: Asymptomatic infections could present a major challenge for the VL control program if its infectiousness is confirmed. The greatest challenge of this investigation was to understand the disagreement among the diagnostic methods used to identify asymptomatic infections.

Operational characteristics of diagnostic test include the time taken to perform the tests, its functional simplicity or case of use, user acceptability and stability of test under user condition. The standards for Reporting of Diagnostic Accuracy (STARD) initiative has developed a sequenced checklist to help to ensure that all relevant information is included when the result of studies on diagnostic accuracy are reported. Different approaches are now a days in this area of research to define accuracy in comparison. Therefore, there is need of meta-analysis study to theconfirm actual diagnostic application.

As a summary, There is continued need for the development and further application of rapid methods for the detection as well as quantification of pathogen. Morphological interpretation is subjective and require significant expertise, Although till present standard diagnosis is morphology , but advances in other techniques, especially molecular approaches like quantitative PCR suggest another paths to travel for proper and correct diagnosis and thereby early treatment. A new generation of immunoassays, molecular and nanotechnology plateform has been developed in the recent year that can improve patient management and disease surveillance. Such technologies provide real time results to inform patient management decisions. Increased sensitivity of detection using these advances has made it possible to have tests that make detection from noninvasive samples also for VL. With rapid technological innovation in the last 25 years and donar investments in the development of improved diagnostics for infectious disease of public health importance , it is time to re-examine our point of weakness and explore the promises and challenges of VL diagnostics in this elimination era and age of new technology.

Conflict of Interest

We have no conflicts of interest to report.

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