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Molecular Diagnosis of Visceral Leishmaniasis

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Abstract

Visceral leishmaniasis (VL), a deadly parasitic disease, is a major public health concern globally. Countries affected with VL have signed the London Declaration on Neglected Tropical Diseases and committed to eliminate visceral leishmaniasis as a public health problem by 2020. To achieve and sustain VL elimination, it will become progressively important not to miss any remaining cases in the community who can maintain transmission. This requires accurate identification of symptomatic and asymptomatic carriers using highly sensitive diagnostic tools at primary health service setting. rK39 rapid test (RDT) is the most widely used and is the first choice for decentralized diagnosis of VL in endemic areas, it has good sensitivity and specificity. This test however cannot discriminate between current, subclinical or past infections and is useless for diagnosis of relapses and as prognostic (cure) tests. Importantly, as the goal of VL elimination as a public health problem is approaching, the number of susceptible people to infection will increase. Therefore, correct diagnosis using highly sensitive diagnostic test is crucial to apply appropriate treatment and management of cases. Recent advances in molecular techniques have improved the *Leishmania* detection and quantification and become increasingly relevant due to possible application to a variety of clinical samples. Most importantly, given current problems in identifying asymptomatic individuals because of poor correlation between the main methods of detection, molecular tests are valuable for VL elimination programs, especially to monitor changes in burden of infection in specific communities. This review provides a comprehensive overview of the available VL diagnostics and discusses the usefulness of molecular methods in the diagnosis, quantification and species differentiation, and its clinical applications.

1 Introduction

Leishmaniasis has been identified as high-priority disease by the World Health Organization [1]. It is caused by protozoa belonging to the genus *Leishmania*, transmitted by the bite of a 2 to 3 millimeter long insect vector, the phlebotomine sand fly found throughout the world's inter-tropical and temperate regions [2]. Around 21 species of *Leishmania* are known to be pathogenic to humans [3]. The disease occurs in three forms: self-healing or chronic cutaneous leishmaniasis (CL), mutilating mucosal or muco-cutaneous leishmaniasis (ML or MCL) and life threatening visceral leishmaniasis (VL). Each form varies in degree of

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severity, with visceral leishmaniasis (VL) being by far the most devastating with highest mortality.

VL, also known as Kala-azar is the most severe form of leishmaniasis, caused by the obligate intracellular protozoan parasites *Leishmania donovani* / *L. infantum*. It is estimated by WHO that worldwide 200-400 thousands new cases of VL occur annually. 90% of VL cases occur in three geographical regions: i) South East Asia: India (especially Bihar), Bangladesh, Nepal; ii) Latin America: mainly North Eastern Brazil; iii) East Africa: Sudan, Ethiopia, Kenya, Uganda and Somalia [4, 5][6, 7]. In the Indian Subcontinent, VL is now being reported in 54 districts in India, 16 upazila in Bangladesh, and 12 districts in Nepal [8]. In Europe, VL is endemic in nine countries and account for less than 2% of the global burden [9]. In Brazil, VL is endemic in 21 out of 26 states and a total of 14,859 cases were reported between 2001-2014 in 25% of Brazilian municipalities [10]. In Sudan, 17 localities in seven states are endemic for VL [11].

The leishmania parasite is transmitted by female *Phlebotomine* sand flies as a flagellated, metacyclic promastigote, which is phagocytised by host macrophages and then differentiates into the non-flagellated, replicative amastigotes [12]. The commonly affected organs during VL are the bone marrow, liver and the spleen [12]. Thus, clinical symptoms include hepatosplenomegaly, which is characterized by an enlarged abdomen with palpable spleen and liver. Other symptoms include long-term, low-grade fever, muscle wasting, anemia, leukopenia, polyclonal hyper-gammaglobulinemia and weight loss [13, 14]. If left untreated, it has a mortality rate of almost 100%. During an epidemic in the early 1990s in Sudan, there was an estimated 100,000 deaths. Risk of an epidemic still exists in the horn of Africa, at the junction of Eritrea, Ethiopia and Sudan, a highly endemic region where tens of thousands of refugees, returnees and agricultural workers have been resettled. Especially in Sudan, Ethiopia, Kenya, Uganda and Somalia, VL is the cause of much morbidity and mortality, and only a small minority of patients have access to diagnosis and treatment [15]. VL is endemic in several tropical and subtropical regions and has been reported from 56 countries around the world (Figure-1). Importantly, the disease affects mostly poverty stricken people with over 80% of patients living below the poverty threshold (daily income < US\$) whose source of income is agriculture and /or animal husbandry [16]. More than 75% live in mud or grass covered houses. These patients are thus completely dependent for diagnosis or treatment upon charity or public health services, which remain grossly deficient in endemic areas [17, 18].

Importantly, on the Indian Subcontinent, the three countries affected by VL, India, Nepal and Bangladesh, aspired to eliminate visceral leishmaniasis (VL) by 2015 (a deadline later reset to 2020). The aim is to reduce the incidence to less than 1 per 10,000 of population at sub-districts level (i.e. block level in India and Nepal; and upazila level in Bangladesh) through early diagnosis & complete treatment of cases and integrated vector management [19]. Furthermore, as countries move towards elimination goals, number of VL and Post kala-azar dermal leishmaniasis (PKDL) (characterized by skin lesions in which parasites can be identified, in a patient who is otherwise fully recovered from VL) cases will decrease, and a low number of such cases will almost inevitably lead to a decreasing awareness in the communities and in health providers. If cases of VL and PKDL are ignored or missed in

such context, a new epidemic phase may start. To avoid such a scenario, there is need for development and validation of an innovative set of tools for VL and PKDL case finding, outbreak management strategy, and surveillance for infection measurement. Thus, there is a direct need for new tools to allow monitoring infections, treatment effectiveness and drug resistance. Molecular detection tools would constitute a more rapid and high throughput alternative to detect parasites. In the present review, we discuss the various molecular methods focusing on the recent developments and its clinical application in leishmania detection, absolute quantification, species differentiation and phylogenetic analysis.

2 Standard diagnostic tools and their limitations

A major challenge in the clinical management of VL is the weakness of health systems at primary care levels in many affected countries with multiple challenges and numerous constraints [8, 20]. Despite multiple techniques for confirming VL cases being available, they are all still far from being ideal. To date, observation of parasites in splenic aspirate is considered the gold standard for VL diagnosis [21]. Though, microscopic examination of spleen aspirates is rapid and cheap approach with high sensitivity and specificity, but is not practical at the PHC level. However, in Kenya, VL policy specifies that all serologically-proven leishmaniasis be confirmed by spleen aspirate, a procedure that can only be performed in referral hospitals [22]. Alternative parasitological diagnostic techniques are the lymph node or bone marrow aspirates (reviewed in [23]), the standard means of diagnosing VL in most countries. Sensitivity of such diagnostic method is highly variable and dependent on sampling procedure and technical skills of physician or personnel's performing the tests. Although sensitivity of bone marrow is lower than splenic aspiration, the diagnostic potential in combination with serology is adequate for clinical purposes [24, 25]. Again these methods cannot be performed at PHC setting in endemic areas because of requirement of skill persons, high cost and less simplicity. Most importantly, now a day's, bone marrow/splenic aspirates examination are recommended only when the rK39 RDT test is negative but the suspicion of VL disease is high or in VL patients diagnosed by rK39 who do not respond to first line treatment [23]. Importantly, very few practitioners currently have the skills to perform these dangerous aspirates and very limited numbers of these biopsies are taken in the ISC. Culturing leishmania promastigotes from tissue biopsies/ PBMC/ whole blood is another method of diagnosis but is expensive and requires a sophisticated laboratory [26].

Human VL is associated with high level of plasma antibodies. Although it is useful in diagnosis, but the role of antibodies in VL pathogenesis is not clear [27][28]. A number of non invasive serological tests to detect Leishmania antibodies are now available: Direct Agglutination Test (DAT) extensively validated in endemic areas and recommended by the World Health Organization (WHO) for VL control programs [21, 29]. The requirements of relatively specific material and expertise make it difficult for their use in peripheral health centers. Similarly, the Indirect Immunofluorescent Antibody Test (IFAT) requires an immune-fluorescence microscope which restricts its use to referral hospitals. Much hope is now laid on a rapid immunochromatographic test based on a recombinant 39-amino acid repeat antigen (rK39 dipstick), which despite the variability initially observed among different producers and countries, seems to be the first choice for decentralized diagnosis of

VL with a good sensitivity and specificity [30, 31]. However, it shows decreased sensitivity in East Africa when compared to the Indian subcontinent [31, 32]. rK39 dipstick is stable, easy to use and it is a “rapid test” (results available in 10 minutes). The performance of this test in various studies has been comprehensively reviewed recently [23, 24, 31]. In India, Bangladesh and Nepal, the VL elimination initiative has adopted the rK39 RDT as the main tool, but its limitations are sorely felt as it cannot be used to diagnose relapse or to assess response to treatment (test of cure)[23, 33]. Approximately 10-20% of healthy people living in endemic areas are positive with this test [34, 35]. Importantly, despite its limitations, the rK39 RDT has been and still a great asset in the struggle against VL as it allows decentralizing diagnosis and treatment as close as possible to the villages where the patients live. Although rK39 RDT test represents a sound approach in highly endemic areas, it fails in situations of low infection intensity. Furthermore, these antibody detection tests are of limited used in immunocompromised patients (i.e. HIV co-infection)[23]. Antigen detection is required as a means of identifying symptomatic infections in immunocompetent and immunocompromised patients (e.g. diagnosis of primary VL in Sudan where rK39 RDTs lack sensitivity and complex diagnosis of VL relapse) and as an indicator of cure. Latex agglutination test (KATEX) detects of leishmanial antigen in urine and record the results in scoring system that correlated well with the parasite load. However, KATEX is currently not an ideal test as it had a poor sensitivity when it was tested in different centers [32, 36-38].

2.1 Molecular diagnosis and detection of infection

Most of the *Leishmania* species have been sequenced revealing an overall conservation of gene order, chromosome structure and discrete differences in gene content. These recent research advancements helped in development of more appropriate rapid molecular diagnostic devices and platforms [39]. However, despite the technological development, there is huge difference in using a commercially available and standardized molecular diagnostic as opposed to in-house kits. So far, several molecular methods have been developed for detection, identification, quantification and phylogenetic analysis, and these are summarized in Figure-2.

One of the most sensitive and specific methods for diagnosis of clinical VL is the development polymerase chain reaction (PCR) kits that amplifies parasite DNA, and can be visually read without sophisticated equipment [44, 78-80]. Sensitivity of PCR assay mostly depends upon the biological sample used (e.g blood, bone marrow or splenic fluids etc.) and primers to amplify the target sequence (variable or conserved target region) [81, 82]. Most commonly used amplification targets are nuclear DNA such as small subunit ribosomal rRNA (*SSU rRNA*) gene [45, 83, 84], extra-chomosomal DNA such as repetitive kinetoplastid DNA (*kDNA*) [45, 46, 85], mini exon genes [86], and ribosomal internal transcribed spacer (*ITS*) region [52]. A comparative overview of frequently used PCR targets and its sensitivity and specificities in different tissue samples are summarized in Table-1. One of the major limitation of DNA based PCR is the counting dead parasite DNA (as half life of DNA is 24hrs within the body, which is still controversial and not proven); thus, RNA based amplification target is preferred [82, 87]. However, a reliable RNA extraction is difficult at PHC settings. Srivastava *et al* [44] validated *18S r-RNA* based PCR on the blood of largest number of patients and controls, and found sensitivity of 87.8%

(95%CI 84.1-89.8) and specificity of 94.6% (95%CI 92.8-96.1). Leishmanial DNA has been detected by PCR in peripheral blood of persons with asymptomatic infection in Brazil and recently this was also documented in India and Nepal [88-90]. To date, several cohort studies conducted in India, Nepal, Bangladesh, Italy, Ethiopia, Sudan and Brazil for detection of asymptomatic *L.donovani* infection in endemic villages has confirmed the increased capacity of PCR tests to detected infection in healthy individuals [89-94]. PCR assays has been also performed in non invasive samples like buccal swab and urine with sensitivity of 83% and 88% respectively [95, 96]. Molecular diagnosis using PCR is very useful in HIV-VL patients where clinical picture is confusing and, serological as well as immunological tests are not reliable due to low sensitivity [97]. Furthermore, sensitivity and specificity of PCR for detection of low level parasitemia has been shown to be significantly improved by performing nested and semi nested PCR which involves two sets of primers (targeting single gene locus) used in two successive runs. Second set of primers amplify the secondary target within the product of first PCR product [49]. Furthermore, nested PCRs are prone to contamination and not recommend except in accredited laboratories. Sensitivity and specificity of nested PCR using *SSU-rRNA* in diagnosis of VL is reported as 97% and 100% respectively [98]. Similarly, multiplex PCR involve amplification of different DNA targets at the same time [99]. Although such assays are more sensitive than conventional PCR, their high costs make this test not appropriate in a field setting. Another form of PCR such as OligoC-test [100], PCR-ELISA [101] and Nucleic acid sequence based amplification (NASBA) have been developed and found to be more sensitive than conventional PCR [43]. More recently, rapid and highly specific loop mediated isothermal amplification (LAMP) has emerged as powerful tool for point of care diagnosis and has been validated on VL & PKDL in several countries [102, 103]. One of the advantages of this assay is that the test can be performed without requiring sophisticated equipments, making it a more attractive tool for field based diagnosis. This assay is rapid and cost effective than conventional PCR, but limited in utility due to false positivity. Importantly, PCR-Oligo and LAMP are the only available commercially and this offers huge benefits over in-house kits in terms of reliability, of course this comes at a price. Most recently, recombinase polymerase amplification (RPA) assay (simple and molecular assay as mobile suitcase laboratory) was developed for canine VL [104]. This assay has been tested and proved promising diagnostic method for VL which tremendously decreases the cost associated with testing [105].

Importantly, in view of mounting drug pressure, PCR diagnostic assays play a key role in monitoring drug efficacy and early reporting of drug resistance which are essential to bring corrective actions in drug policy; this is even more important when the drug arsenal is limited, as in the case of VL [122-124]. The molecular assays are the only standard, rapid and high throughput and easy method to track parasite resistance that can completely replace tedious *in vitro* susceptibility assays. Furthermore, such tools should be simple as much as possible to be applicable and affordable in the endemic countries. Recently, Srivastava *et al* identified single nucleotide polymorphism in *cysteine proteinase B* gene associated with amphotericin-B drug resistance [125].

2.2 Quantification of parasites (Severity of Disease)

As an analytical technique the conventional PCR method has some limitations. By first amplifying the DNA sequence and then analyzing the product, quantification was exceedingly difficult since the PCR give rise to essentially the same amount of product independently of the initial amount of DNA template molecules that were present. Therefore conventional PCR (qualitative analysis) test shows only *Leishmania* presence or absence without quantification of parasite load. With the highly efficient detection chemistries, sensitive instrumentation, and optimized assays that are available today by Real Time PCR (also known as quantitative Real Time PCR if DNA is the starting genetic material for quantification of parasites), the number of DNA molecules of a particular sequence in a complex sample can be determined with unprecedented accuracy and sensitivity sufficient to detect a single molecule. However, quantitative PCR (qPCR) does not directly measure the number of viable parasites circulating in the blood, but rather the amount of circulating parasite DNA. Therefore, sensitivity of qPCR depends on assay design (primer and target region), chemistry used (SYBER Green or TaqMan), nature of clinical samples (blood, skin, bone marrow or splenic fluids) and the DNA extraction methods (manual vs commercial kits) [126] (Table-2). Using this technique, it was earlier demonstrated that the simultaneous quantitative evaluation of *Leishmania* DNA and cytokine by Real Time PCR assay allows prediction of the development of disease in asymptomatic infected dogs [127]. Using qPCR, we have shown that the parasite load decreases during treatment in treated VL cases. Amplification of *18S rRNA gene* sequence from a small volume of heparinised whole blood by Real Time-PCR revealed a wide range of blood parasitemia in VL patients prior to treatment that in each case began to decline within a few days of the start of their antileishmanial drug therapy [128], and thus can be used as a marker of treatment response as well as measurement of parasitic burden over time. Recently, Hossain *et al*, evaluated the use of real time PCR and revealed the difference in parasite loads between primary VL and relapse VL [129]. Subsequently on a larger cohort of asymptomatic subjects, we established the threshold parasitemia (>5 *L. donovani* parasite genomes detected/ml) in blood for clinical symptoms of VL to occur [61]. Later on, in the enlarged cohort of 1,606 healthy individuals of whom 442 were recent sero-converters with DAT and/or rK39, the risk for progression of disease was found much higher in qPCR positives (odds ratio: 14.8, 95% CI 5.1-42.5) (*Chakravarty et al* unpublished data).

Elevated levels of IL-10 during active disease is a hallmark of VL, and this overproduction of IL-10 promote parasite replication and disease progression. Verma *et al* evaluated the parasitic burden measured by qPCR and its association with IL-10 production in VL and found that high qPCR load is strongly correlate with plasma IL-10 levels, making it suitable for biomarker of disease severity[145]. Later on, Wilson group developed several qPCR methods and strategies for leishmania species differentiation and quantification in clinical specimens[63]. Leon *et al* evaluated the analytical performance of qPCR methods (designed on primers directed to *kDNA*, *HSP70*, *18S* and *ITS-1* targets) and found that *18S* marker presented the high sensitivity and specificity [146].

Since, qPCR assay usually provides a measure of parasite load of blood at a given time point, but it remains unclear how this load can be correlated to the load at infection because

the parasite load may vary with time, and likely reflect both host parasite interactions as well as initial load. A number of researchers are using PCR-ELISA for early detection and quantification which allows multiple sample testing using whole blood with sensitivity of 87% [101, 147]. However this method is tedious, expensive, and less sensitive than qPCR and tested on limited number of clinical samples [101, 148].

2.3 Species identification

In the Indian subcontinent, visceral leishmaniasis is the disease that mainly occur, however, recent identification of cutaneous leishmaniasis (CL) patients in Rajasthan (caused by *L. tropica*) and Himachal Pradesh (caused by *L. donovani* and *L. tropica*) [149-151], suggest that clinical profile of CL are different in these states. Therefore, species identification assays are useful in such areas for proper management of control program. Importantly, in the Indian subcontinent (mainly India and Bangladesh) as well as in Africa (mainly Sudan), where *L. donovani* is the causative parasite for VL, a common complication of VL is post kala-azar dermal leishmaniasis (PKDL) [152] which occurs in the months following treatment, in up to 50% of people who have recovered from VL. It is much less common in India, with an incidence of less than 5-10 %, and when it occurs, does so many years after the acute infection [153]. In Africa, PKDL is even more common but there are important intraregional differences; in Sudan it is most common: up to 50-60% of VL cases develop PKDL, usually within 6 months and virtually all cases develop within 12 months (mean 4.5 months). In Ethiopia, Kenya and Uganda PKDL is less common for reasons that are not well understood.

Through whole genome sequencing, Downing et al reported that there are a large amount of chromosome copy number variations between *L. donovani* strains and between leishmania species on the Indian subcontinent [71]. Therefore, better characterization of parasite strain (i.e species differentiation) is needed to resolve the mystery as to whether the disease is due to reactivation of persistent parasites following clinical cure of VL, or due to re-infection; and also to establish the cause of different forms of PKDL.

Commonly used target genes in leishmania for species identification includes ITS (non coding spacer DNA located between the *18S rRNA* and *5.8S rRNA*) [52, 154-156], repetitive nuclear DNA sequences [157], *cytochrome-b genes* [158, 159], mini exon genes [160], *G6PD* genes [161], *cpb* genes [162, 163], *gp63* genes [164] and *hsp70* genes [165, 166]. For example, digestion of *ITS-1* PCR product with *Hae-III* restriction enzyme differentiates most of leishmania species. Since, RFLP pattern dependent on restriction enzyme used, and thus it is suggested to go for sequencing for confirmation. RAPD is another molecular assay where amplification of DNA is performed using arbitrarily short primes without knowing the target sequences. Several studies have been done using RAPD for investigation of genomic diversity [167-170], but its use in leishmaniasis is restricted due to need of specific PCR standardization condition and poor reproducibility [171]. AFLP is more advanced assay for investigation of variations in strains or closely related species [172]. It uses restriction enzyme for genomic DNA digestion followed by selective PCR amplification of restriction fragments. Recently developed more sensitive PCR-fingerprinting techniques include multilocus sequence typing (MLST) which is based on the PCR amplification of multiple unlinked housekeeping genes followed by sequencing [68]. Moreover, a multilocus

microsatellite typing (MLMT) approach has been recently developed by which East African strains of *L. donovani* and Mediterranean strains of *L. infantum* could be resolved and assigned to genetically isolated populations [57, 74]. Srivastava *et al* explored the discriminatory power of different molecular assay and markers to detect genetic heterogeneity in clinical isolates of *L. donovani* from India [70]. Multilocus enzyme electrophoresis (MLEE) is protein based methods which differentiate leishmania parasites to species and subspecies levels based on electrophoretic mobility of enzymes [173]. This method has been known as gold standard for characterization and identification of parasite strains. However, requirement of mass cultivation of parasites, low differentiation power in homology population and developments of more sensitive molecular markers as an alternative methods are the major drawbacks of MLEE [174]. Hernandez *et al* identified six new world leishmania species through implementation of High Resolution Melting (HRM) genotyping assay which is another robust, highly sensitive and reproducible genotyping technique [175].

2.4 Phylogenetic analysis

The evolutionary pattern among species and taxonomic status of leishmania parasites is essential to understand the divergence among closely related species, designing of reliable diagnostic tools and development of novel control methods. The malaria field is driving much of the relevant technology for this type of work. A major limiting factor to leishmaniasis is a critical lack of expertise throughout endemic areas. So far, many leishmania strains have been typed by MLEE. On the other hand, introduction of numerous molecular typing methodologies with multicopy targets or multigene families have improved the analysis of phylogenetic, taxonomic and genetic studies. These includes DNA targets such as *ITS* [176], single copy gene for the catalytic polypeptide from *DNA polymerase α* (*polA*) [177], *cytochrome oxidase II* gene [178, 179], *cpB* genes [180], *7SL RNA* [181], and most recently *hsp70* subfamily sequence [165]. For example, Zhang *et al* investigated the phylogenetic relationship using *ITS1* and kinetoplast *cytochrome oxidase II* (*COII*) gene sequencing and hypothesized that phylogeny of Chinese *Leishmania* strains is associated with the geographical origin rather than clinical form of disease [73]. Fraga *et al* analysed the phylogenetic study of 43 leishmania strain from different geographic origins using *hsp70* sequence and found that monophyletic genus *Leishmania* consisted of three distinct subgenera, the *L. (Leishmania)*, *L. (Viannia)*, and *L. (Sauroleishmania)* [77].

3 Technical challenges and future prospects of molecular based assays

VL affected patients living in endemic areas will not have access to quality care unless efforts are made to integrate existing innovative diagnostic technology into clinical management. For example, in Sudan 3520 VL cases were reported in 2014 and only 62% were diagnosed as confirmed VL. Molecular diagnostics are not only beneficial for the patient, but if done through active case detection (as like sero surveys with k39 RDT) in the villages will also reduce the parasite reservoir in highly endemic areas since humans are the only host reservoir for *L. donovani* in the Indian subcontinent. Though sensitivity and diagnostic accuracy of molecular assays are reasonable to excellent in laboratory-based evaluations (in reference laboratories), these methods are not currently adapted to a primary

health care setting due to the expensive infrastructure and technical expertise required. Overall cost associated with PCR assay is less than US \$ 5 (INR 230.0) per sample [44]. Though, this cost is two times greater than rK39 RDT test, it is currently not very clear how such innovative techniques will replace k39 RDT testing and how it can be meaningfully applied within the health system context of VL endemic areas. However, assuming that such rapid and highly sensitive molecular tools to assist clinicians working in the frontline of the primary health care setting will help them to better manage their patients presenting with fever related clinical syndromes. Strengthening of early diagnosis and treatment capacities at primary health care settings may provide long-term sustainability of elimination effort through integrated case management as close as possible to the patient's village. Importantly, emergence and spreading of drug resistance is challenging the VL control program. Therefore, monitoring drug efficacy and early reporting are most essential as drug arsenal is limited. Molecular detection tools would constitute a more rapid and high throughput alternative to detect drug resistant parasites, but requires a standardized way to use them and a structure to implement them in the sentinel sites.

4 Conclusion

There is no preventive or therapeutic vaccine for VL and arsenal of antileishmanial drugs are limited, therefore, it is important to identify VL patients likely to relapse after drug treatment, as well as new ways to recognize individuals who have had recent exposure to live parasites. Effective clinical management, chemotherapy and control of transmission depend largely on early and unequivocal diagnosis. Molecular based methods have recently become popular in the field of diagnosis that can detect infection at the low level, pertaining to the goal of VL elimination. So far, several molecular based assays have been developed and evaluated, but PCR based assays are found to be simple, rapid and highly sensitive. The availability of such rapid test to be used to diagnose VL and as a marker of cure at peripheral health centers could have a great impact on the way VL is managed in endemic communities. The test could be an alternative to the current rK39 dipstick test for accurate diagnosis and it could be used to identify treatment failures and relapses.

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Key Points:

- ◆ Due to the limited number of currently available anti-leishmanial drugs, effective clinical management, chemotherapy and control of transmission depend largely on early and unequivocal diagnosis. Any patient residing in a VL endemic area, presenting with a history of fever of more than two weeks duration and with no response to antibiotics/antimalarials is to be tested for VL with an rK-39 dipstick test.
- ◆ The rK39 rapid diagnostic test (RDT) in strict clinical criteria is currently used with good results for diagnosis of VL, but this test cannot differentiate between active disease and past VL. Furthermore, a diagnostic algorithm of rK39 RDT for detection of asymptomatic infection has only been validated for high incidence settings in strict combination with clinical criteria (fever for more than two weeks duration plus an enlarged spleen).
- ◆ With the advent of technology, highly specific and sensitive molecular based tools have been developed for detecting infection, diagnosis and species differentiations, these hold considerable further promise for delivering better point-of-care diagnostic tests in elimination and post elimination setting.
- ◆ Molecular based methods play key role in early diagnosis, monitoring of treatment effectiveness and assessment of drug resistance in *Leishmania* parasites.

Figure-1.
Map of global distribution of visceral leishmaniasis

Figure 2.
Molecular tools and markers for visceral leishmaniasis diagnosis.

Table-1

Comparison of molecular methods in detection, differentiation, identification and quantification leishmania species.

Molecular methods	Capacity to detect leishmania parasites in clinical samples	Levels of leishmania discrimination#			Sample used	Sensitivity (%)	Specificity (%)	Ref.
		G	SG	S				
PCR methods	Yes	yes	yes	yes	Whole blood	70-100	85-99	[45, 46, 106]
					Buccal swab	79-83.00	86-90.56	[107, 108]
					Urine	88.0 -96.8	100	[95]
					Bone Marrow	95.30 -97	92.60-100	[109]
					Buffy coat cells	80-100	63-100	[110] [111]
					Serum	85- 96	100	[112]
					Blood spotted filter	60-98	100	[113]
					Bone marrow spotted filter	99-100	9.0-87%	[114]
PCR-ELISA	Yes	yes	yes	yes	Whole Blood	83.90 - 100	100	[101]
Real time PCR / qPCR	Yes	No	No	No	Whole blood	90 - 100	83.3-100	[61, 115, 116]
					Buffy coat cells	100	90	[117]
					Oral fluids	95	90-100	[117]
Oligo-C test	Yes	yes	yes	yes	Whole blood	96.2	90.0	[41, 43]
					Lymph node	65-96.8	70-100	[41]
					Bone marrow	89-96.9	57 -99	[41]
Gene sequencing	Yes	yes	yes	yes	Buffy coat	69	100	[118]
NASBA	Yes	No	No	No	Whole blood	79.8- 93.3	100	[41, 43, 116]
					Bone Marrow	85-99	50-98.9	[41]
					Lymph node	64-95	70-100	[41]
LAMP	Yes	No	No	No	Whole blood	83.0 -96.4	98.0 -99	[102, 116, 119, 120]
					Buffy coat	90.7-95.0	86-100	[103, 121]
PCR-HRM	Yes	yes	yes	yes	Biopsy	NA	NA	[144, 182]

#Levels of discrimination amenable; G: Genus; SG: Subgenus; S: species.

Abbreviations

PCR: Polymerase Chain Reaction; ELISA: Enzyme linked immunosorbent assay; IFAT: immunofluorescence antibody test; NASBA: nucleic acid sequence-based amplification; LAMP: Loop-mediated isothermal amplification; qPCR: quantitative PCR; Oligo C test: Oligochromatography PCR test; HRM: High Resolution Melting.

Table-2

Summary of the comparative analytical sensitivity of real time PCR assay targeting leishmania DNA region

Target Sequence	Tissue tested	Assay Chemistry	Analytical Sensitivity (parasite/ml or parasite DNA equivalence/reaction)	Reference
<i>kDNA</i>	Blood	Intercalating dye	0.07-0.1	[130]
		Fluorescent dye	0.001-0.002	[131, 132]
	Bone Marrow/Lymph Nodes	Intercalating eye	0	[133]
		Fluorescent dye	0.001-0.0125	[66, 132]
	Skin Biopsy	Intercalating dye	0.0001-0.5	[134, 135]
		Fluorescent dye	0.005	[136]
<i>rDNA</i>	Blood	Intercalating dye	0.5-10.0	[137, 138]
		Fluorescent dye	1.0 – 6.25	[139, 140]
	Bone Marrow/Lymph Nodes	Intercalating eye	0.1 – 10.0	[138, 141]
		Fluorescent dye	1.0	[140]
	Skin Biopsy	Intercalating dye	0.5 – 10.0	[137, 138]
		Fluorescent dye	1.0 – 1000.0	[64, 140]
<i>GPI</i>	Skin biopsy	Fluorescent dye	165.4	[142]
<i>G6PD</i>	Biopsy	Intercalating dye	0.005	[136]
<i>HSP70</i>	Parasite culture	Intercalating dye	10.0	[143]
<i>cpb</i>	Biopsy	Fluorescent dye	0.01	[144]

Abbreviations

G6PD: Glucose-6-phosphate dehydrogenase; *HSP70*: Heat-shock protein 70; *kDNA*: Kinetoplast DNA; *rDNA*: ribosomal DNA; *cpb*: cystein protease B; *GPI*: Glucose phosphate isomerase; qPCR: Quantitative real-time PCR