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Review Article

An Overview on *Leishmania* Diagnosis

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Leishmaniasis is now accounted as a health problem and categorized as a class I disease (emerging and uncontrolled) by World Health Organization (WHO), causing highly significant morbidity and mortality with different clinical presentations. The incidence of human leishmaniasis is increasing and its geographic distribution in humans and animals is shown to be wider than estimated before. Indeed, more than 350 million people are at risk of *Leishmania* infection, and about 1.6 million new cases occur causing more than 50 thousand death annually. Control of leishmaniasis is highly dependent to the early diagnosis and treatment of the disease. In recent years, there have been advances in diagnosis of *Leishmania* infection. However, the main challenge in *Leishmania* diagnosis is the lack of a gold standard test in order to establish an effective strategic program to control and eradicate the disease. This review provides the latest information regarding the diagnosis of the disease, which is based on a combination of clinical features (supported by epidemiologic data) and laboratory tests including direct parasitological (microscopy, histopathology, and parasite culture), serological and molecular tests. *J Med Microbiol Infec Dis, 2017, 5 (1-2): 1-11.*

Keywords: Leishmaniasis, *Leishmania*, Diagnosis.

INTRODUCTION

Leishmaniasis is a protozoan disease, which is the most prevalent infectious disease after HIV/AIDS, tuberculosis, and malaria, and postulated to be amongst the six endemic diseases with high priorities worldwide [1]. According to WHO, over 20 *Leishmania* species are causing leishmaniasis and approximately 0.7-1 million new cases and 20000 to 30000 deaths occur amongst a susceptible population of 350 million in 88 countries on five continents each year [2-5]. Environmental changes such as building of dams, deforestation, urbanization and irrigation schemes and crises in the society such as immigration and war are postulated to be linked to Leishmaniasis and poor people who usually suffer from displacement, malnutrition, poor housing, weakness of the immune system and lack of financial resources are the main targets for the disease [6].

The parasite is transmitted by the bite of over 90 species of female sandflies from two *Phlebotmine* genera (*Phlebotomus* and *Lutzomyia*) in zoonotic or anthroponotic models [7-13].

Vaccination remains the most appropriate opportunity for the prevention and safe treatment of all forms of the disease, however, no safe and effective vaccine has yet been developed against *Leishmania*. Diagnosis is based on clinical criteria, detection of the parasite and immunological and molecular tests. This article provides the latest findings regarding diagnosis of leishmaniasis.

Clinical symptoms

Leishmaniasis has three forms of clinical manifestations and may appear similar to a wide variety of other conditions (Table 1).

The most serious form of leishmaniasis is VL (visceral leishmaniasis), which is also known as kala-azar, black fever and Dumdum fever. This form of leishmaniasis is caused by *Leishmania donovani* complex that mainly consists of *Leishmania infantum*, *L. donovani*, and *Leishmania chagasi*. More than 90% of *L. infantum* and *L. donovani* cases do not show clinical symptoms [14]. In endemic regions such as northwestern Iran, asymptomatic human carriers of *L. infantum* act as the reservoirs of the infection [15]. These *Leishmania* species can circulate in asymptomatic blood donors for more than a year after exposure to the parasite [16].

The other two forms of leishmaniasis are CL (cutaneous leishmaniasis), which is caused by *Leishmania amazonensis*, *Leishmania mexicana*, *Leishmania braziliensis*, *Leishmania panamesis*, *Leishmania peruviana* and *Leishmania guayanensis* (New World CL), *L. infantum*, *L. chagasi* (Mediterranean and Caspian Sea regions) and *Leishmania major*, *Leishmania tropica*, *Leishmania aethiopica* (Old World CL), and Mucocutaneous leishmaniasis (MCL) or espundia, which is usually caused by *L. brazilensis*, *L. panamensis*, *L. guyanensis* in the New World. However, the MCL is occasionally caused by *L. infantum* and *L. donovani* [17].

Cutaneous leishmaniasis is usually found in two forms: Antrhroponotic Cutaneous Leishmaniasis (ACL), mostly

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caused by *L. tropica*, and Zoonotic Cutaneous Leishmaniasis (ZCL), mainly caused by *L. major* [18]. The severity of symptoms depends on the species of the parasite and the host immune system. Diffuse cutaneous leishmaniasis (DCL), which is caused by *L. amazonensis* and *L. aethiopica*, is a form of the disease, which is usually categorized as cutaneous leishmaniasis [17]. Post-kala-azar dermal leishmaniasis (PKDL) is another form of CL. This form of the disease is a dermal manifestation of VL, which is characterised by a macular, maculopapular, and nodular rash in patients recovered from VL [19]. Viscerotropic leishmaniasis is another form of the disease, mainly reported in soldiers served in *Leishmania* endemic regions. This form of leishmaniasis is caused by cutaneous causing species such as *L. tropica*, and sometimes affects internal organs [20]. Clinical manifestations of leishmaniasis in patients with VL, PKDL, and DCL may become more severe in immunocompromised patients [21]. Clinical presentation of leishmaniasis in immunocompromised patients particularly HIV-infected individuals can be atypical, so that the infection in gastrointestinal tract and other involved organ systems may easily be misdiagnosed as a flare-up of the underlying disease [22].

Table 1. Major species of *Leishmania* and their geographic distribution

Species	Disease form	Reservoirs	Vector	Distribution
		***Old World Leishmaniasis**		
L. major	LCL	Desert rodent (Psammomys, Meriones, Gerbillus)	*Phlebotomus papatsi*	North Africa, the Middle East, central Asia and the Indian subcontinent
L. tropica	LCL	Human Rock hyraxes Unknown animals	*P. sergenti*	North Africa, the Middle East, central Asia and the Indian subcontinent
L. ethiopica	LCL, DCL	Hyraxes	*P. pedifer* *P. longipes*	Ethiopian highlands, Kenya
L. infantum	VL, LCL	Domestic dog, wild canines	*P. perniciosus* *P. ariasi* *P. tobbi* *P. jangeroni*	Mediterranean basin, Middle East, and central Asia
L. donovani	VL	Humans	*P. argentipes* *P. orientalis* *P. martini*	Kenya, Sudan, India, Pakistan and China
		***New World Leishmaniasis**		
L. Mexicana	LCL, DCL	Forest rodents	*Lutzomyiaolmecaolmeca* *Lu Cruciata*	Southern Texas through Mexico and northern Central America
L. amazonensis	LCL, DCL	Forest spiny rats	*Lu. faviscutellata*	South America in the Amazon basin and northward
L. pifanoi	LCL, DCL	Probably rodents	*Unknown*	Venezuela
L. grnhami	LCL	Unknown	*Lu. youngi*	Venezuela
L. venezuelensis	LCL	Unknown	*Lu. Olmeca bicolor*	Venezuela
L. braziliensis	LCL, ML	Forest rodents Opossums Sloths Domestic doges Donkeys	*Ps. wellcomei and others*	South America from the northern highlands of Argentina and northward to Central America
L. panamensis	LCL, ML	Sloths	*Lu. trapidoi* *Lu. ylephiletor*	Panama, Costa Rica Colombia
L. guyanensis	LCL	Sloths Lesser anteater	*Lu. umbratilis*	Guyana, Surinam, Northern Amazon Basin
L. peruviana	LCL	Unknown	*Lu. peruensis*	Peru, Argentinian highlands
L. chagasi	VL, LCL	Domestic dogs Foxes	*Lu. logipalpis*	Mexico (rare) through Central and South America

*Geographic distribution, reserviors, vectors and forms of the disease caused by *Leishmania* species [23]

The most prominent symptom of CL is changing the skin appearance manifested as destructive mucosal inflammation (mucosal leishmaniasis, ML), ulcerative skin lesions at the site of sand fly bite (localized cutaneous leishmaniasis, LCL) and multiple nonulcerative nodules (diffuse cutaneous leishmaniasis, DCL) [24].

CL usually begins with a papule at the site of the vector sandfly bite on the epidermal layer of the skin. The papule then grows in size and turns to crust form, which may also ulcerates. After 2-10 months, majority of cutaneous cases heal on themselves unless the lesion is complicated by secondary infections. In mucocutaneous leishmaniasis, the incubation period is 1-4 months and the lesions extend from the skin to the nose, oral cavity and pharynx. This form of the disease is normally associated with difficulties in respiration and eating with considerable risks of mortalities [25]. In VL, following a period of 2-6 months, patients may develop symptoms of a persistent systemic infection. Symptoms varies in severity from fever, skin pigmentation (kala-azar; black disease), loss of appetite, weakness, fatigue and weight loss to hepatosplenomegaly, lymphadenopathy, pancytopenia and death [26-27].

Differential diagnosis is critical and usually achieved by using several diagnostic tests due to similarities between clinical spectrum of different forms of leishmaniasis and other diseases (*e.g.* leprosy, skin cancers, and tuberculosis for CL and malaria and schistosomiasis for VL) which are also present in *Leishmania* endemic areas [3, 28].

Diagnosis of CL, MCL and VL

The infection is growingly reported in tourist-visiting endemic tropical and subtropical countries. The extensive clinical signs of the disease as well as inadequate knowledge of the illness among practitioners and patients may lead to an incorrect diagnosis [29]. Differentiation between conditions that mimic CL such as leprosy and fungal infections may require microbiological, cytological and histological evaluation. Diagnosis in the laboratory is made microscopically by observation of amastigotes in Giemsa-stained lesion smears of biopsies, scrapings or impression smears. Amastigotes are observed as 2-4 μm round or oval bodies, with adistinctive nucleus and kinetoplast. When microscopic and protozoal culture techniques are used, the diagnostic sensitivity increases up to more than 85 percent [30]. There is not a significant difference in the diagnostic outcomes when samples are taken from the center or the border of the ulcer [31]. Parasite cell culture and DNA detection by PCR method are sensitive, but not currently practical in some developing countries.

Laboratory diagnostic methods of VL include microscopic observation, culturing the parasite, DNA detection and serological tests. Laboratory tests should be able to make clear distinction between acute disease and asymptomatic infection and have high sensitivity (>95%) for the diagnosis of VL, as the clinical appearance of VL lacks specificity, and the current drugs used to treat VL are toxic. On the other hand, such tests should be straight forward and affordable [27]. Simple diagnostic tools are necessary for clinical use in developing countries with large number of patients in rural areas [32].

Parasitological diagnosis (microscopic examination and parasite culture)

In CL and MCL cases, the sensitivity of the microscopic examination is relatively low, with a range of approximately 15-70%. Detection of amastigotes by microscopic methods is mainly based on obtaining a smear from the skin lesion biopsy. In this method, after staining with Giemsa or Leishman stain, aspirated amastigotes are detectable as oval shaped cells with a pale bluecytoplasm, a relatively large nucleus that stains red and a deep red or violet rod-like kinetoplast [33].

Rasti *et al* (2016) compared the sensitivity of microscopic examination, parasite culture and molecular methods for diagnosis of CL, and concluded that despite the convenience and accessibility of the microscopic method, it did not show sufficient sensitivity for the diagnosis of CL [34]. The sensitivity of microscopic methods may increase up to 85% when accompanied by a parasite culture [35]. Even with visualization of amastigotes (Leishman-Donovan bodies), which has a sensitivity of 50-70%, a species-specific diagnosis cannot be ascertained. However, Giemsa-stained smears could be readily used as a sample for PCR [36]. In acute CL, there may be epidermal hyperplasia and ulceration. In the early stages of the disease, inflammation with a dense and diffuse dermal infiltration, often with a narrow area of uninvolved papillary dermis, ”Grenz Zone”, is present.

The infiltrate primarily contains macrophages (some with parasites in their cytoplasm), but lymphocytes and plasma cells may also be present. Dense dermal infiltrates often lead to destruction of adnexal structures [37]. An important step in the histopathologic analysis is finding amastigotes within macrophages which usually can be found beneath the epidermis [38]. The derm usually contains increased collagen deposition. In about 30% of acute CL cases, epithelioid cell granulomas with giant cells and a rim of lymphocytes may develop. This is associated with a good response to treatment and resolving ulceration [37]. Amastigotes (2 to 4 μm in diameter) are found in clusters in the cytoplasm of dermal macrophages [39] with a dull blue-gray color when stained with hematoxylin & eosin (Figures 1 and 2). After treatment and clinical cure of patients, a moderate inflammatory process with elevated levels of the anti-inflammatory cytokines especially interleukin-4 and interleukin-10 may be indicated [40].

In chronic relapsing CL caused by *Leishmania recidivans*, infection occurs within a prior scar. This may produce epidermal changes such as pseudoepitheliomatous hyperplasia, which may be seen if no Grenz zone is present. The epidermis may also undergo hydropic degeneration of the basal lamina and loss of pigments along with an extensive superficial and deep dermal lymphocytic infiltration, and loss of elastic fibers [41].

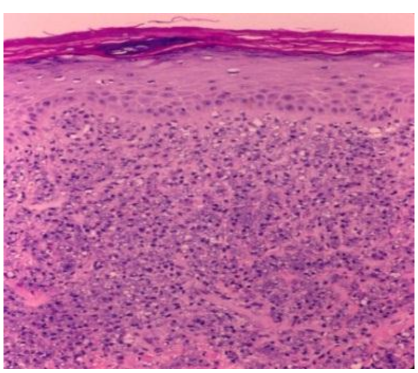

Fig. 1. A nodule on the forearm of a *leishmania* infected person showing chronic inflammation with dense infiltration of mononuclear cells in the connective tissue of the dermis. Hematoxylin & eosin x40 [42]

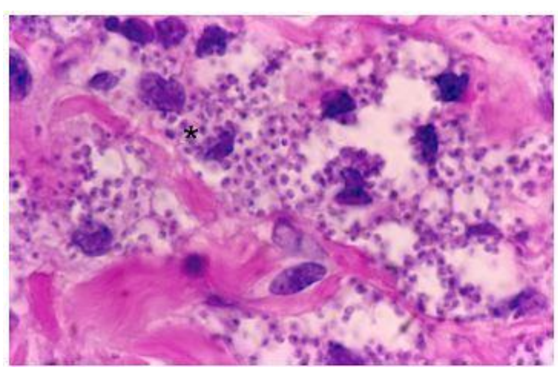

Fig. 2. Higher magnification of Fig. 1 demonstrating numerous, basophilic intracellular amastigotes of the parasite (asterisk) in macrophages. Hematoxylin & eosin, x1250 [42]

In VL, the amastigotes can be easily detected in monocytes or macrophages in Giemsa stained smears of aspirates derived from lymph nodes, bone marrow, liver or spleen (Figure 3). Depending on the type of sampled tissue, the specificity of this technique is high, and the sensitivity is higher for liver and spleen (93-99%) than for aspirates of bone marrow (53-86%) or lymph node (53-65%) [43]. For recovering the parasite, the aspirate can be cultured [44]. The culture method is usually time-consuming, which makes it not an ideal method for field use, however using culture media such as Novy-Mcneal-Nicolle medium (NNN) is relatively simple, low-cost and sensitive. The sensitivity of culture-based methods and direct microscopic examination in CL depends on the parasite species, the clinical figure of disease and the technical expertise applied for the tests. The range of sensitivity is estimated to be 42-74% for direct stained smear and 33-76% for histological sections [45]. In mucocutaneous leishmaniasis in particular, the sensitivity of microscopic and culture-based methods is quite low, as the organisms are often scarce [46]. However, by employing both microscopic study and parasite culture, the sensitivity may increase even up to 83%, and the specificity of the methods is reported to be as high as 100% [47]. Also, it has been reported that in post-kala-azar dermal leishmaniasis (PKDL), the sensitivity of tests for skin lesions was low (17%), but was higher (30%) for lymph node aspirates [48]. The sensitivity of these methods for detection of VL is as high as 98% with splenic aspiration, but is lower for other organs, indicating a very high level of infection in splenic macrophages. Since parasitemia in VL patients is rare, the sensitivity of direct blood smear testis low (Table 2) [49]. Parasitological diagnosis methods have higher sensitivity in immunocompromised patients and VL caused by *L. donovani*. In sub-clinical disease, both direct microscopy and culture have low sensitivity and are not able to distinguish between the amastigotes of different species [50-51].

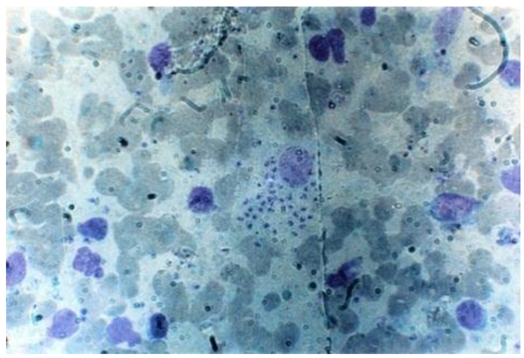

Fig. 3. Intra and extracellular Leishman-Donovanbodies (center of the image) in splenic aspirate from a patient with visceral leishmaniasis [52]

Table 2. Sensitivity and specificity of various laboratory tests used for diagnosing VL

Investigation	Sensitivity	Specificity
Splenic aspirate smear	80-98%	100%
Splenic aspirate culture	70-98%	100%
Bone marrow smear	60-85%	100%
Bone marrow culture	40-50%	100%
Liver aspirate smear	50-75%	98%
Lymph node smear	40-50%	95%
Buffy coat culture	0-30%	100%
Complement fixation test	70-80%	60-73%
Immunodiffusion test	60-75%	90-95%
counter current immunoelectrophresis test	80-90%	50-70%
Indirect haemagglutinationtest	73-75%	80-95%
mmunofluorescence assay	55-96%	70-98%
Direct agglutination test	90-100%	80-95%
ELISA	36-100%	85-100%

Sensitivity and specificity of various VL laboratory tests [53]

Culture is the best method for isolating the parasite, although is not easy. Parasites may be obtained from scraping, aspiration or a punch biopsy specimen. Dermal scraping is a quick and simple method, which may be employed for slide evaluation or culture [54].

Inoculation of animals, most commonly hamsters and mice, may be used for *in vivo* culture of the parasite; however, it is not the standard practice for diagnosis of the infection. The detection level is a little higher by *in vitro* culturing samples (44-58%) than by inoculation into hamsters (38-52%) [55]. Several different culture media have been used to isolate *Leishmania*, including NNN medium containing sodium chloride in blood agar, Evans' modified Tobie's medium containing fetal calf blood serum, L-prolineand antibiotics, and Schneider insect medium containing salts, sugars, amino and organic acids [56].

Roswell Park Memorial Institute (RPMI) medium contains several nutrients necessary for the growth of the fastidious *Leishmania* organisms.

Leishmanin Skin Test

Leishmania Skin Test (LST), also known as the Montenegro reaction, is a delayed hypersensitivity reaction in cutaneous forms of leishmaniasis. An intradermal injection of *Leishmania* antigen, phenol-killed amastigotes, is used to detect cell mediated immunity [57]. After subclinical infection and within weeks to months after successful therapy against VL, results of the test become positive indicating a healing or protective response [58]. A reaction must be measured after 48 to 72 hours, much like the tuberculin skin test [39]. This test does not differentiate between the past and present infection. Moreover, active VL, PKDL, and DCL are characterized by a negative skin test [59]. This test is shown in different disease-endemic areas to detect asymptomatic infection [60]. In VL-endemic areas, the sensitivity of LST in asymptomatic *Leishmania* infections is similar or even greater than that of serologic tests [61]. This makes the LST a valuable tool in detecting exposure to *Leishmania* parasites and distinguishes asymptomatic cases in epidemiologic surveys [62].

No cross-reaction with Chagas disease occurs, but there may be cross-reactivity with cases of glandular tuberculosis and lepromatous leprosy [53]. The LST is commonly used as an indicator of the prevalence of CL and MCL in human and animal populations and successful cure of VL, as it remains negative during active VL and will be converted to positive after treatment. This test is not useful in PKDL patients because the results are not associated with the presence of the infection [63]. In these patients, within weeks to months after successful therapy against VL, the LST results still become positive [58].

Antigen detection

Antigen detection in the serum or urine can be used for the diagnosis of *Leishmania* infection, particularly in the immunocompromised patients, where the immune response is poor. However, due to the presence of circulating immune complexes, serum amyloid, autoantibodies, rheumatoid factor and high level of antibodies, detection of antigens in the serum may be complicated [64].

Several studies have demonstrated leishmanial antigens in the urine of VL patients. In a study, two polypeptide fractions of 72-75 kDa and 123 kDa in the patients' urine were reported [65]. Also, a urinary 5-20 kDa carbohydrate-based antigen from VL patients has been described [66]. In another study, in the urine sample of a VL patients, a heat-stable carbohydrate with low molecular weight has been detected by an agglutination test [67]. Recently, the A2 antigen derived from *Leishmania* amastigotes and crude antigens derived from *L. infantum* promastigotes have been used in a latex agglutination test for rapid detection of anti-leishmanial antibodies.

Immunological tests

Immunological tests are based on detection of anti-leishmanial antibodies and are used in both individual diagnosis and epidemiological surveys. However, due to cross-reactivity with other pathogens such as Plasmodium, Trypanosoma, Schistosomaor Mycobacterium leprae, the prevalence of the antibody in endemic areas particularly in post-infected cases, or absence of antibody during the incubation period can produce short comings in serodiagnosis of leishmaniasis [68].

Antibody-detection tests

Several tests are available to detect anti-leishmanial antibodies, though with two limitations: (1) although serum antibody levels decrease after successful treatment [69], they remain detectable up to several years after cure [70], therefore, VL relapse cannot be diagnosed by serological methods. (2) A significant proportion of apparently healthy individuals living in endemic areas with no history of VL and due to asymptomatic infections are positive for anti-leishmanial antibodies. The seroprevalence in healthy populations varies from <10% in low to moderate endemic areas [71], to >30% in high-transmission foci or cases of household contacts [72]. Antibody-based tests should therefore always be used in combination with a standardized clinical case definition for VL diagnosis.

Serological tests based on indirect fluorescent antibody (IFA), enzyme-linked immunosorbent assay (ELISA) or western blotting have shown high diagnostic accuracy in most studies, but are poorly adapted to field settings [73]. Two serological tests have been specifically validated, the direct agglutination test (DAT) and rK39-based immuno chromatographic test (ICT).

IFA

The IFA is a sensitive test available for diagnosis of leishmaniasis in humans and animals with 96% sensitivity and 98% specificity [67]. Promastigote forms should be the antigens of choice for diagnosis of VL by the IFA because of minimizing cross-reactivity with sera from *Trypanosoma* infected patients [74]. This can be overtaken by using amastigotes instead of promastigotes [53]. The antibody response can be recognized in early stages of the infection. The antibody level declines six to nine months after treatment, but low titers of the antibody usually indicate a relapse of the disease [53]. A Titer of 1:120 or above is significant and1:128 is diagnostic.

ELISA

ELISA is a useful tool for serological diagnosis of VL. This method has high sensitivity, and its specificity depends on the type of used antigen. This assay can detect many antigenic molecules. In VL, recombinant protein K39 (rK39) has been shown to be a useful antigen to be utilized in ELISA. However, crude SLA still seems to be a potent alternative [75]. In contrast, rK39 does not show detectable antibodies in CL or MCL [69].

At a time when the disease is active, the titer of antibody to rK39 has a good correlation with the effectiveness of chemotherapy in the treatment of VL [69]. Also, rK39 ELISA has a high predictive value for detecting VL in immunocompromised patients, like those with HIV/AIDS [76]. Some other antigens such as gene B protein (GBP) and recombinant major surface glycoprotein (gp63) from *L. major*, have been tested for detection of cutaneous leishmaniasis [53]. ELISA of crude SLA or the patient's serum is a valuable test with a sensitivity as high as 94.7-100%, in detection of MCL. On the other hand, due to the cross-reactivity with Chagas disease and malaria, the specificity of the test is lower [77]. In addition, ELISA of rK39 detects asymptomatic infection earlier than the DAT [78]. However, due to the requirement of skilled personnel, laboratory equipment, and electricity, using ELISA for diagnosing VL is not typical in many endemic areas [79]. Antibody titers have been shown to decline steeply at the end of treatment and during follow-up, with successful therapy; in contrast, patients who relapsed showed increased titers of antibodies to rK39. This can be used as a marker application for rK39 ELISA in monitoring drug therapy and detecting relapse of VL [75]. rKE16 is another recombinant protein used in ELISA. This antigen has been very sensitive and specific as rK39, for VL diagnosis, when tested in patients from China, Pakistan, and Turkey [80]. A new experiment has been developed based on the detection of the K28 fusion protein in studies performed in Sudan (with 96% sensitivity) and Bangladesh (with 98% sensitivity) [81].

DAT

This test is one of the best methods to diagnose *Leishmania* infection and is more specific than antibody-based immunodiagnostic tests [82]. To detect the antigen, DAT has extensively been evaluated in clinical trials and well-defined cases and controls from endemic and non-endemic regions (Table 3). This test is based on direct agglutination of *Leishmania* promastigotes that react specifically with anti-*Leishmania* antibodies in the serum specimen. Whole, trypsinized, Coomassie-stained promastigotes can be used either as a suspension or in a freeze-dried form that can be stored at room temperature for at least two years, facilitating its use in field [83].

In addition, together with classical clinical features, a cut-off point of 1:12, 800 for DAT can be used for diagnosis of VL in endemic areas. Although DAT is simpler than many other tests, the reproducibility of results is problematic and depends on antigen elaboration [84]. A similarity between the results of DAT and rK39-ICT has also been indicated in recent studies for diagnosis of VL. However, higher positivity rates have been reported for DAT compared with rK39-ICT in asymptomatic populations. In addition, combination of DAT and LST or rK39-based ELISA tests has showed better results for detection of asymptomatic infections when applied in VL endemic areas [61, 78]. However, the DAT test for serological diagnosis of VL with high sensitivity and specificity, still has some limitations, among those are the relatively long incubation time (18 hours) and the serial dilutions of the samples that must be made. A faster method, fast agglutination screening test (FAST) utilizes only one serum dilution and requires three hours incubation, which make the test suitable for screening of large populations. A sensitivity and specificity of 91.1%-95.4% and 70.5%-88.5% , respectively, have been reported for the FAST [85]. Another method of DAT has also been investigated using patients' urine in endemic and non-endemic areas, with a comparable sensitivity and specificity to that performed with serum (Table 3) [86, 87].

Table 3. DAT results for anti-*Leishmania* antibodies in suspected and confirmed VL patient

	Serial dilution series (reciprocal)								
Patient group	800	1600	3200	6400	12800	25600	51200	102400	>102400	Total
Confirmed VL	-	-	-	-	-	5	4	2	13	24
Suspected VL	1	-	1	1	-	1	1	-	-	4

Immunoblotting (Western blotting)

This method can detect the infection but is only used in research laboratories. This test is based on the detection of *Leishmania* antigens. For this test, promastigotes are cultured to log phase, lysed, and the proteins are separated on SDS-PAGE. Separated proteins are electro transferred to nitrocellulose membrane and probed with serum from the patient. This technique provides an antibody response to various antigens of *Leishmania* [88]. However, due to the low-level of antibody in CL patients, this method is mostly being used in the diagnosis of VL [89]. The western blotting technique is more sensitive than the IFA and ELISA, especially in co-infected HIV patients with VL [89].

ICT

The technique is usually based on unpurified or recombinant antigens and can achieve sensitivities of >90% [90]. A recent study showed that the detection of circulating antigens could be introduced as a new method. This technique is a simple, rapid, and reliable method which can be easily carried out by inexperienced personnel under field condition [91]. However, in a previous study using Dipstick test, two proteins, A-colloidal gold conjugate, and rk39 *Leishmania* antigen were used. The combination of these two proteins can detect anti-leishmanial antibody in serum or plasma. The rK39 IC revealed 90% sensitivity and 100% specificity in Brazil [92], 100% sensitivity and specificity in the Mediterranean area [93], and 100% sensitivity and 93%-98% specificity in India [94]. In other reports from southern Europe, the rK39 IC test was positive in only 71. 4% of the VL cases [95]. In Sudan, rK39 IC showed a sensitivity of 67% [96]. Therefore, the various racial groups may lead to differences in antibody responses and ultimately result in differences in sensitivity. In a significant proportion of healthy individuals in endemic regions and for long periods after treatment, IC is positive like the DAT assay, as this test cannot differ between a case of VL relapse and other pathologies; this limits its usefulness in individuals with a previous history of VL which present with recurrence of fever and splenomegaly [97].

Molecular techniques

Due to the specificity of molecular detection techniques for *Leishmania* compared to parasite cell culture and histopathological methods, these techniques are advantageous [98]. Detection of *Leishmania* DNA can be done by PCR, which allows sensitive, accurate and fast detection of minute amounts of the pathogen DNA [99]. In PCR-based techniques, the primers target *Leishmania*-specific regions or genes in the DNA [100] such as gp63 gene, mini-exon-derived RNA genes, β-tubulin gene region [101], genomic repeats, internal transcribed spacer (ITS) regions [102] and kinetoplast DNA (KDNA) [24]. In these techniques the primers are designed to amplify conserved sequences of DNA found in genomsor mini-circles of KDNA of *Leishmania* species. Mini-circles of KDNA is eminently suitable because the kinetoplastis known to possess thousands of copies of mini-circle DNA. The sample type normally affects the test sensitivity, for example, the sensitivity is highest (near 100%) in spleen or bone marrow. Peripheral blood is also an ideal sample due to its non-invasive characteristic and 70-100% sensitivity [50]. The sensitivity of this test in CL (up to 100%) and MCL (86.4%) is shown to be higher than other techniques [46]. In PKDL, PCR with samples from lymph node or skin aspirates is more sensitive than microscopic examination [48]. The specificity of the test is 100%, which is even higher than ELISA. The sensitivity of PCR in PKDL patients is also 93.8-96%[103]. In a study, a combination of PCR-ELISA, was used to diagnose VL in HIV-negative patients. This method using peripheral blood samples was more sensitive than conventional PCR with aspecificity of 100% and 87.2% for healthy controls who had never traveled to a VL endemic area and controls from a VL endemic area, respectively [104]. After apparent cure, a substantial number of the patients who tested positive by PCR did not relapse or develop PKDL, a result that suggests the limitation of PCR in deciding the end point of treatment. PCR becomes positive in these patients perhaps due to existence of the nonviable parasite; similarly, PCR results for healthy endemic controls may be positive [105] leading to incorrect conclusions. The combination of DAT (which shows low titers in healthy endemic controls) and PCR, could help to identify patient's status [52].

Fluorogenic PCR technique, using a fluorescent DNA probe for a conserved rRNA gene that is amplified using flanking primers can be used with great sensitivity and specificity [106]. Also, the Real-Time PCR for the follow-up of treatment and allowing for the assessment of the parasite burden is helpful [107].

Diagnosis of VL-HIV co-infection

With regards to WHO, an estimated 35 million people worldwide are living with HIV. Leishmaniasis has been emerged as an opportunistic disease in HIV patients in endemic areas. The similarity of clinical symptoms of VL in HIV-infected patients poses a considerable diagnostic challenge. Symptoms including, fever, splenomegaly, and hepatomegaly are found in less than half of such patients. Latent *Leishmania* infection may reactivate due to immunosuppression in asymptomatic patients and among HIV/AIDS patients [108]. The diagnostic principles remain essentially the same as those for non-HIV-infected patients. Amastigotes may exist in buffy coat and sometimes may be found in unusual locations, such as pleural fluid, biopsy specimens from the gastrointestinal tract and specimens from bronchoalveolar lavage [109]. Due to the low sensitivity of serologic tests for VL in HIV-infected patients, several serologic tests must be done to increase the sensitivity of antibody detection for each patient [110]. The detection of polypeptide fractions of 72-75 kDa and 123 kDa of *Leishmania* antigen in the urine of patients could be ideal. In an evaluation study on VL, the test was 96% sensitive and 100% specific, nevertheless, these antigens were not detectable after three weeks of treatment [65]. For accurate evaluation of infection in these patients, it is recommended to use both molecular and serological methods.

Species identification

Identifying the involved species of *Leishmania* is necessary to predict patients' status and provide appropriate treatment approach. *Leishmania* species have many similarities under a microscope. In the past years, isoenzyme analysis is used to identify the species of *Leishmania*. This has allowed the construction of phylogenetic classification, and differentiation between anthroponomical and zoonotic variants within a single species [111]. This procedure is based on variation in the electrophoretic mobility of enzymes isolated from *Leishmania* parasites. As it is costly, time-consuming, and requires large quantities of cultured promastigotes, this method is only performed in a few reference laboratories [112]. Another approach is the use of molecular techniques. The kinetoplast DNA is unique to each species of *Leishmania* [113]. When the sample contains only few amastigotes, PCR results in detection rates of up to 97% [113]. In addition, several target genes such as mini-exon gene, HSP70, hexokinase, and *phosphoglucomutase* genes have been used for PCR. HSP70-based species identification method (as a globally applicable approach) could become the reference method for identification of *Leishmania* species in clinical specimens [114]. Detection of ITS1 followed by HaeIII restriction enzyme digestion is also used for identification of *Leishmania* species in *Leishmania* endemic areas of Iran [115-116]. Kinetoplast DNA is another gene, which has recently been used for detection of *L. major* and *L. tropica* in some provinces of Iran [117].

Different methods including parasitological, immunological and molecular methods are used for diagnosis of leishmaniasis. Parasitological methods are simpler and easy to perform. However, their sensitivity and specificity are often low. The combination of microscopic detection and the parasite culture increases the specificity of the techniques. Immunological methods show higher specificity in comparison with parasitological techniques. Molecular methods have the highest sensitivity and specificity among all techniques for detection of *Leishmania* species, though these techniques are more expensive and complicated and need higher expertise and special equipment.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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