Laboratory diagnosis of human visceral leishmaniasis

Hercules Sakkas, Constantina Gartzonika & Stamatina Levidiotou

Microbiology Department, Faculty of Medicine, School of Health Sciences, University of Ioannina, Greece

ABSTRACT

Visceral leishmaniasis (VL), caused by the *Leishmania donovani* complex, is a vector-borne systemic disease, with a worldwide distribution causing high morbidity and mortality in the developing world. VL patients may be asymptomatic or they may present symptoms and findings of a systemic infection. The positive predictive value of clinical diagnosis in patients with typical symptoms is usually high, but more often, the signs and symptoms are inconclusive and mistaken with other co-endemic diseases. The fact that HIV co-infections often produce atypical presentations and the heterogeneity of *Leishmania* species, which is common in many endemic regions, also complicate the diagnosis. Despite that, some of the parasitological methods are still considered to be the reference standard for VL diagnosis due to their specificity. The development of serological and molecular tests has further enhanced the diagnostic approach of VL. Recombinant antigens have improved the performance of serodiagnostic tests, with DAT and the rK39 antigen based immunochromatographic test being the most appropriate methods for the serological diagnosis of VL. Molecular techniques, despite the fact that their implementation is often difficult and infeasible, have become increasingly relevant due to remarkable sensitivity and specificity, and to the variability of tested samples. Quantitative polymerase chain reaction (qPCR) has been shown to be superior than conventional PCR for the differentiation between active VL and asymptomatic infections, such as for the detection of VL-HIV coinfection. This review summarizes the available methods with their applications in the diagnosis of VL, and focuses on the recent developments in VL diagnostics.

Key words  Diagnosis; HIV coinfection; Phlebotomus; visceral leishmaniasis

INTRODUCTION

Leishmaniasis is a complex of vector-borne diseases caused by > 20 trypanosomatid protozoan species of the genus *Leishmania*, belonging to the class *Kinetoplastida*, family *Trypanosomatidae*. The main mode of transmission occurs primarily after the bite of infected female sandflies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World. Ninety-three out of about 800 different species of phlebotomines and flies that can spread leishmaniasis have been described. More rarely, it can also be transmitted to humans by blood transfusion and congenitally from mother to infant. Dogs are the most important reservoirs and, to a lesser extent, other mammals, such as rodents, carnivores, or lagomorphs are implicated for zoonotic transmission, while infected and asymptomatic individuals are the sole reservoirs in anthroponotic cycles. Leishmaniasis consists of cutaneous clinical form (CL), which is the most prevalent disease that manifests as skin lesions; visceral leishmaniasis (VL), also known as kala-azar (black fever), representing the most severe form of the disease; muco-cutaneous leishmaniasis (MCL) and post-kala-azar dermal leishmaniasis (PKDL). *Leishmania* infections have been reported in 101 countries and about 350 million people live in areas characterized by active transmission of the parasite. There are an estimated 1.3 million cases occurring worldwide annually, especially of CL and MCL. Among all parasitic diseases, leishmaniasis is the third most common cause of mortality after malaria and schistosomiasis. The cases of VL range from 0.2 to 0.4 million people per year, with > 90% of these occurring in India (mainly in the states of Bihar, West Bengal and Uttar Pradesh), Bangladesh, Sudan, Ethiopia, Brazil and Nepal, while mortality is high, estimated up to 40,000 deaths per year. Despite the increasing number of the infected people being registered worldwide, many cases are believed to be under-reported.

Although, leishmaniasis is considered to be a tropical disease, two *Leishmania* species are endemic in Europe: *L. infantum*, responsible for zoonotic cutaneous and systemic disease within the Mediterranean region, with a reservoir in the domestic dog; and *L. tropica*, which occurs sporadically in Greece, causing anthroponotic cutaneous disease. Visceral leishmaniasis is typically caused by the *L. donovani* complex, comprising three species: *L. donovani*, *L. infantum* and *L. chagasi*. After transmission into host, the parasites differentiate from flagellated and metacyclic promastigotes to non-flagellated and replicative amastigotes which are phagocytised and prolif-
erated into host macrophages, and finally released by cell lysis, in order to infect other macrophages. The organs that are usually affected are bone marrow, liver and spleen. The manifestation of the newly acquired infection varies from subclinical, to oligosymptomatic and to fully established (kala-azar). The occurrence of asymptomatic and subclinical illness has been considered as an important aspect of VL epidemiology. In the symptomatic infection, the most common symptoms and clinical findings include prolonged fever, weakness, night sweats, anorexia, weight loss, pallor, lymph node enlargement, hepatomegaly and splenomegaly. Characteristic laboratory findings are normocytic normochromic anemia, leucopenia, neutropenia, thrombocytopenia, increased transaminases, hypoalbuminemia and polyclonal hypergammaglobulinemia. Despite appropriate treatment, relapse of VL may occur 6–12 months later. Untreated VL can produce multisystem disease, susceptibility to secondary infections and death.

Over the last years, VL cases in Europe have increased. This is related to several factors, such as immunodepression, increase of international travel, migration and trade, additionally the global warming affects the ecology and distribution of phlebotomine vectors. Moreover, high rates of Leishmania-HIV-coinfected patients have resulted in an increase of VL in Europe. In southern Europe, up to 70% of VL cases are associated with HIV infection, while, in HIV patients, VL is the third most frequent opportunistic infection in several parts of the world, and the coinfection is now reported from 35 countries. Coinfection of Leishmania donovani and L. infantum with HIV has been recognized as a significant clinical problem, and when compared to other coinfections, it demonstrates higher mortality rates.

Although, VL in patients especially from known endemic areas, is usually diagnosed clinically, more often the signs and symptoms are inconclusive and mistaken with other coendemic diseases. The differential diagnosis of VL includes haematological malignancies and various infections. The available methods for the diagnosis of VL involve several techniques, such as demonstrating the parasite in tissues, in vitro culture, animal inoculation, serological and molecular techniques. The review summarizes the main laboratory methods that have been used over the last years and highlights on the recent developments in VL diagnostics.

Laboratory diagnostic methods

Parasitological methods

Parasitological diagnosis can be confirmed by demonstrating the amastigote form of the parasite in tissues, isolating the promastigote form in cultures, or with molecular assays. This remains the gold standard in VL diagnosis due to its high specificity. Splenic, lymph node and bone marrow aspirates, buffy coat of peripheral blood as well as liver biopsy have been used for the direct visualization of amastigotes by microscopic examination with variable sensitivity. Amastigotes (also called ‘Leishman-Donovan bodies’), which are round or oval bodies, 2–4 µm in diam, with characteristic nucleus and kinetoplast, can be seen within mononuclear or macrophages cells on microscopic examination of Giemsa-stained blood or aspirate specimens. The specificity of the method is high, while the sensitivity varies according to the examined aspirates. The sensitivity of splenic aspiration ranges from 93 to 99%, however, it can be associated with life-threatening hemorrhages in about 0.1% of the patients. In a study carried out in India, it was reported that fatal bleeding occurred in two out of 9612 splenic aspirate procedures during a 10-yr period. Additionally, one death occurred out of 671 patients subjected to splenic aspiration in Kenya and three out of 3000 in India. Liver biopsy is safer, although it is less sensitive than the splenic aspiration. Bone marrow aspiration is safer method than both the splenic aspiration and liver biopsy, but it is less likely to demonstrate parasites in stained film. The sensitivity ranges from 53 to 86%, but it is higher in HIV-coinfected patients. Lymph node aspiration can be diagnostic, especially when enlarged lymph nodes are observed, as in case of VL patients from Sudan and the reported sensitivity of direct microscopic examination ranges from 52 to 58%. The lowest sensitivity of this method is observed in peripheral blood smears, as parasitaemia in immunocompetent individuals with VL is low.

Clinical samples may also be cultured into monophasic (Schneider’s insect medium, M199, or Grace’s medium) or diphasic culture media (Novy-McNeal-Nicolle medium and Evans modified Tobie’s medium). After incubation at 22–28°C, cultures are examined weekly for four weeks until amastigotes transform into promastigotes. Culture from splenic or bone marrow aspirates has high specificity, although it is tedious, time-consuming, requires expertise, expensive equipment and is, therefore, restricted to referral research centers and hospitals. The diagnostic sensitivity can be improved, especially with the development of a more sensitive micro-culture method (MCM) for the parasite’s isolation, while a recent modification involves the use of buffy coat and peripheral blood mononuclear cells isolated from the VL patients’ blood. Although, the culture method is...
rarely used, when it is performed, the parasites obtained are generally used as source of antigens for immunologic tests, in animal inoculation, for in vitro screening of drugs and for an accurate diagnosis of the infection supplemented to other routine methods.

Specimens can also be inoculated into laboratory animals, such as hamsters, mice, guinea pigs, or rodents, but this is not considered to be a diagnostic tool, since several months may be required to demonstrate the parasite in such animals.

Immunodiagnostic methods

Several assays using different antigens have been used for the detection of specific antileishmanial antibodies. Over the last years, numerous recombinant antigens have been used in VL serologic diagnosis, since the use of crude antigens was often limited. Direct agglutination test (DAT), indirect fluorescent antibody test (IFAT), indirect hemagglutination assay (IHA), enzyme-linked immunosorbent assay (ELISA) and immunochromatographic tests (ICTs) are currently used for VL diagnosis. Serological tests are most commonly used for the diagnosis of VL, and, generally, they are highly sensitive, however the specificity of these methods may vary. Antileishmanial antibodies remain detectable for months or years after the patient’s treatment, therefore, relapse of the infection is difficult to diagnose. Furthermore, comparisons of several serological tests usually demonstrate low levels of agreement with the results obtained among asymptomatic individuals in endemic regions. Additionally, in endemic areas healthy individuals may be positive in these antileishmanial antibody detection tests, owing to asymptomatic infection or to incomplete elimination of the parasite in past infection. Moreover, in Leishmania-HIV coinfection, serological methods show variable low sensitivity as the determination of antibodies is difficult due to the immunosuppressive action of the virus. Methods for the detection of the leishmanial antigen in urine samples have also been evaluated with varying results.

Antigen detection

Agglutination tests were employed for determining antigens in VL patients. Antigen detection tests offer an alternative to antibody detection methods, especially in immunocompromised patients, such as in Leishmania-HIV-coinfected patients, who present deficient antibody production. Theoretically, these methods should be more specific than antibody detection tests, since they avoid cross-reactivity and can distinguish current from past infections. However, serum is not the most suitable specimen, since several immunological factors may cover important antigenic determinants or inhibit competitively the binding of antibodies to free antigen. Therefore, several methods of antigen detection in urine have been used, mostly the latex agglutination test (KAtex), which is a simple, noninvasive, rapid, reliable and easily executable test for the diagnosis of VL. KAtex detects a low molecular weight (5–20 kD) glycoconjugate, heat-stable, carbohydrate antigen in the urine of VL patients, that is present in both promastigote and amastigote forms of the parasite. The antigen may be detectable from one to six months after treatment. The method is appropriate for the diagnosis of primary VL, for monitoring the efficacy of treatment and for the detection of subclinical infection. KAtex displays positive predictive value, high specificity from 82 to 100% and variable sensitivity from 47 to 95%. For immunocompromised patients, the sensitivity and specificity have been reported at 85–100% and at 96–100%, respectively. Both DAT and KAtex provide better, non-invasive immunodiagnostic tools in HIV-coinfected patients, due to their high sensitivity and specificity.

Direct agglutination test

DAT is a semi-quantitative, simple, reliable and cost-effective test that has been extensively validated in most endemic areas, particularly in developing countries. The antigen usually consists of promastigotes and the test can be carried out on serum, plasma and whole blood. Whole, trypsinized, coomassie-stained promastigotes can be used either as a suspension or in freeze dried form. The test uses microtiter plates, in which increasing dilutions of a patient’s specimen are mixed with stained killed promastigotes. If specific antibodies are present, agglutination is visible after 18 h. The DAT titer is indicated as the highest dilution at which agglutination is still visible and it is considered to be positive at 1 : 3200, although different definitions of a positive test have been reported, with threshold titers ranging from 1: 800 to 1 : 6400. The method has the advantage of detecting low levels of antibodies due to the mosaic of the used antigens, some of which may be cross reactive; therefore, attention is needed on determining the cut-off values for a positive test. DAT is considered to be a highly sensitive and specific method; however, the cut-off value should not be based only on sensitivity and specificity, because the predictive values of the test also depend on the epidemiological context and the prevalence of the infection.

According to a meta-analysis that included 30 studies, the sensitivity and specificity of DAT were estimated at 94.8 and 97.1%, respectively, while the performance
of the method was not influenced by the region or the parasite’s species. Nevertheless, when DAT was performed in patients from East Africa, it showed inconsistent and lower performance in comparison to the Indian subcontinent in immunocompetent patients. Moreover, DAT has some limitations, which include the long incubation time from 12 to 18 h and the serial dilutions of samples. Additionally, specific antibodies may be positive for up to five yr after recovery in more than 50% of VL patients and that may limit the use of the method in endemic areas. In Bangladesh, the reported ratio of seropositive individuals without VL to those with VL was 6:1. A positive test in the absence of symptoms may be attributed to past or asymptomatic infection, pre-clinical VL, or cross-reactivity, as it has been reported among patients with leprosy, Chagas disease, malaria and schistosomiasis. To circumvent these disadvantages, a fast agglutination screening test (FAST) for rapid and early detection of specific antibodies has been developed over the last years. As it requires only one serum dilution and three h of incubation, it makes the test suitable for the screening of large populations. The sensitivity and specificity of FAST have been reported at 95.4% and 88.5%, respectively.

Indirect fluorescent antibody test (IFAT)

IFAT is based on detecting antibodies which are demonstrable in the very early stages of infection and undetectable six to nine months after treatment, while persistence of low titers is usually an indication of a probable relapse. It has shown acceptable estimates for sensitivity (87–100%) and specificity (77–100%) in many VL-endemic countries. The sensitivity and specificity of IFAT have been reported at 95.4% and 88.5%, respectively.

Indirect hemagglutination assay

In IHA the patient’s serum is mixed with human erythrocytes, which are sensitized with the soluble, purified L. donovani antigen. IHA is considered to be positive at a titer of >1:64. The sensitivity and specificity have been reported at 90–100% and 86%, respectively, although titers continue to remain high after recovery.

Enzyme-linked immunosorbent assay

ELISA has been used as a diagnostic method for almost all infectious diseases, including leishmaniasis. The technique requires a highly specific antigen as the starter reagent in order to capture a specific antibody; therefore, it is based on several antigenic molecules, such as surface antigens, ribosomal or nuclear proteins, histones and kinesin-related proteins. The sensitivity and specificity of ELISA depends on the antigen used. One of the most commonly used antigens is a crude soluble antigen (CSA) in VL. The sensitivity and specificity of the method using CSA range from 80 to 100% and from 84 to 95%, respectively, while cross-reactivity among patients with tuberculosis, trypanosomiasis and toxoplasmosis has been reported. Recently, an L. donovani specific 12.6 kDa soluble (in phosphate-buffered saline) promastigote antigen (BHUP3) has been used in diagnosis of VL with high specificity (95%), but lower sensitivity (88%). Additionally, 3% of cross-reactivity with patients suffering from malaria and tuberculosis was reported. A member of the heat shock protein family of 70 kDa from L. donovani, designated as BHUP1, has been found to having a good potential in the diagnosis of VL. The sensitivity of BHUP1 was found to be 95%, the specificity was 96%, and, furthermore, it turned negative in 54% of cured VL patients after one year, while DAT remained positive in 93% of treated individuals after one year’s follow-up. Over the last two decades, recombinant antigens such as rK39 (a 39-aminoacid-repetitive immunodominant B-cell epitope of kinesin-related antigen from L. chagasi), have been used for the diagnosis of VL, demonstrating high sensitivity and specificity. The rK39 based ELISA has shown high sensitivity ranging from 93 to 100% and specificity from 97 to 98% in many VL-endemic countries, such as India and Nepal, but lesser in East Africa, while patients from Sudan demonstrated lower titers of antibodies against rK39 than that in Indian patients. Therefore, a new recombinant antigen (rKLO8), from an autochthonous strain of L. donovani in Sudan, was evaluated in VL serodiagnosis. The rKLO8 ELISA showed significant sensitivity (98.1%) and specificity (96.1%) as com-
pared to rK39 ELISA in a study that included Sudanese patients. The strong antigenicity of rKLO8 and rK39 also demonstrated high detection of specific antibodies in HIV-coinfected patients, displaying remarkable sensitivity (81.8%)18.

Although, numerous recombinant antigens, such as rK9, rK26 and rKRP42 have been evaluated on symptomatic VL cases in endemic areas, the rK39 antigen displays the best diagnostic accuracy46. Nevertheless, specific antibodies remained detectable after recovery for up to 2, 4, and 12 yr in Sudanese, Indian and Brazilian individuals, respectively26. In a comparative study that included patients from the endemic population of Bihar (India), rK39 ELISA was the most suitable method for the VL serodiagnosis. The sensitivity and specificity for CSA, rK9, rK26 and rK39 were: 80 and 72%, 78 and 84%, 38 and 80%; and 100 and 96%, respectively47. However, the rK28 antigen that has recently been introduced as a candidate for VL diagnosis, showed similar sensitivity and specificity, when compared to the rK39 antigen, in individuals from Bihar. In non-endemic healthy controls, the sensitivity and specificity for both rK28 and rK39 ELISA were 99.6 and 100%, respectively, while in endemic healthy population, the rK39 ELISA demonstrated a slightly lower specificity (94.2%) than that with rK28 (92.2%)46.

**Immunochromatographic strip test (ICT)**

A simple, rapid and accurate ICT based on the rK39 antigen has also been developed for VL serodiagnosis5. The rK39 rapid test is a noninvasive and cost effective method that has shown high sensitivity and specificity, both up to 100%, even in areas of low VL endemicity48. It has also been used in the serodiagnosis of HIV-coinfected patients48; however, its lower sensitivity has limited its use for VL diagnosis in these patients22. Although there are several available recombinant antigens for VL serodiagnosis, most rapid tests are based mainly on the rK39, since they are quite effective in VL diagnosis in Brazil and the Indian subcontinent, while their use in East Africa is not satisfactory45. Anti-rK39 antibodies have also been detected from urine and sputum, showing to be useful in the VL diagnosis. Recently, an rK39 rapid test using saliva demonstrated low sensitivity; therefore, saliva is not a suitable sample for the VL diagnosis49. An rKE16 recombinant antigen from an Indian strain of *L. donovani* has been used as the base of another rapid test, showing similar sensitivity (92.8–100%) with an rK39 strip test in Indian individuals, but their sensitivity was remarkably lower (36.8–92%) in Brazil and East Africa45. Additionally, in the endemic regions of Bangladesh, rK39 and rK16 antigen-based rapid tests have been effectively performed on serum and whole blood specimens, demonstrating high sensitivity and specificity50. Recently, an important rapid detection test (TRALd) using rK39 and rK26 antigens has been introduced in VL serodiagnosis with high sensitivity (100%) and specificity (98%)10.

**Diagnostic performance of serological methods**

Several studies have been carried out to demonstrate the sensitivity and specificity of serological methods. A systematic review with meta-analysis of the literature showed that rK39 ELISA, rK39 strip test and DAT were the most accurate diagnostic methods, when compared with IFAT and CSA based ELISA23. Data from a prospective study conducted in the endemic states of Brazil showed that DAT and the rK39 rapid test demonstrated better performance than rK39 ELISA and IFAT38. In the endemic area of Nepal, DAT and the rK39 dipstick test were more sensitive than IFAT32 and both tests were highly sensitive for detecting newly-diagnosed cases of VL33, while IHA was more effective than IFAT and the rK39 strip test in Kuwait, where the disease is not endemic37. In India, the sensitivity and specificity of DAT and the rK39 strip test were comparable, whereas the lower sensitivity of KAtex did not promote its use as a first-line diagnostic test in the field-setting51. In another study in Iran, where VL is endemic in some areas, authors suggested that KAtex could be also used as a reliable diagnostic test, when compared with other two diagnostic tests, CSA ELISA and DAT52. As several authors propose, DAT and the rK39 rapid test are the most appropriate methods for the serological diagnosis of VL in the field, a meta-analysis showed good to excellent diagnostic performance of both tests in populations not known to be infected with HIV, however, their sensitivity was lower in the studies from East Africa than in those from South Asia35.

**Molecular methods**

**Polymerase chain reaction (PCR)-based assays**

Molecular techniques have become increasingly relevant due to the remarkable sensitivity, specificity, the various choices of used samples, and also due to the limitations that are usually demonstrated by the conventional parasitological and serological methods10, 17. They have been successfully used to detect asymptomatic *Leishmania* infection and for the diagnosis and follow-up of VL patients33, since they are capable of identifying relapses and reinfections in treated VL patients22. The conventional PCR and the variations such as nested-PCR,
semisedented-PCR and quantitative real-time PCR have been demonstrated as important diagnostic tools\(^1\). These can be performed on bone marrow, splenic, lymph node smears, peripheral blood and serum samples\(^5\). PCR on bone and bone marrow samples is a highly sensitive method; while in immunocompetent individuals the sensitivities for both samples are almost equal\(^5\). In the last decade, a newly introduced PCR on urine samples from immunocompetent patients demonstrated high sensitivity (96.6\%) and specificity (100\%)\(^5\). More recently, an oral fluid-based real-time quantitative PCR showed sensitivity of 94.6\%, with a specificity of 90\%\(^2\). Several target sequences have been used, including kinetoplastid DNA, rRNA, mini-exon-derived RNA genes, the \(\alpha\)-tubulin gene region, glycoprotein 63 (gp63) gene locus and internal transcribed spacer (ITS) regions\(^1\). The conventional PCR shows a wide range of positivity in healthy subjects from endemic regions, but an important drawback of the conventional PCR as well as that of serology is their inability to differentiate between clinically active VL and asymptomatic infections. These drawbacks can be overcome by quantitative PCR (qPCR)\(^6\). Quantitative PCR, using primers from kinetoplastid DNA, has been used on blood samples with 100% sensitivity\(^2\). PCR targeting conserved sequences within the Leishmania kinetoplast or rRNA gene might be diagnostic, especially in HIV-coinfected, where the parasite load may occasionally be low\(^1\). PCR targeting on kinetoplast is probably the most sensitive, since this target is present in about 10,000 copies/parasite, however, the heterogeneity of its minicircles could be a limitation for accurate quantification\(^2\). Levels of circulating kinetoplastid DNA, detected in asymptomatic individuals, were reported to be nearly 500 times lower than those found in active VL, thus qPCR assay can be used as a tool to follow up asymptomatics\(^5\).

**Molecular assays other than PCR**

Innovative molecular assays, such as the nucleic acid sequence-based assay (NASBA) and the loop-mediated isothermal amplification (LAMP) have also been introduced for Leishmania DNA or RNA detection\(^1\). NASBA is a powerful tool, based on leishmanial RNA detection that combines high sensitivity and specificity and can be applied to blood and other specimens\(^5\). Quantitative NASBA (QT-NASBA) is a variation of the method that targets at the 18S rRNA, which is almost homologous for all Leishmania species, increasing its diagnostic value\(^9\). NASBA has also been coupled to oligochromatography (OC) to develop a simplified detection method for the VL diagnosis. The sensitivity and specificity of the method on blood samples from Sudanese patients have been reported at 93.3 and 100\%, respectively\(^5\). Similar specificity (100\%), but significantly lower sensitivity (79.8\%) was reported from another study carried out in Kenya\(^5\). Moreover, NASBA-OC method has been more effective than conventional PCR in Sudan, where parasitaemia is reported to be low\(^5\). The LAMP method has the advantage of amplifying DNA with rapidity and high specificity under isothermal conditions, using basic equipment\(^6\). LAMP has been developed as an alternate to PCR, since it is a faster, sensitive and less expensive technique\(^1\), having great potential for field application\(^7\). According to the results of a study that was carried out in India, \(L.\) donovani DNA was detectable in the blood of VL patients, giving sensitivity and specificity of 96.4\% and 98.5\%, respectively\(^6\). High diagnostic sensitivity (90.7\%) and excellent specificity (100\%) were also observed in a study of LAMP assay using buffy coat DNA from VL patients in Bangladesh\(^6\).

**Species identification**

Molecular methods based on PCR or other amplification techniques, are also increasingly used in both diagnostic and epidemiological studies, for the identification of Leishmania parasites at species and subspecific levels. Techniques such as PCR amplification followed by restriction fragment-length polymorphism (PCR-RFLP) analysis, random amplified polymorphic DNA (RAPD), single-stranded conformation polymorphism (SSCP) analysis or sequence analysis have been developed for Leishmania genotyping\(^6\). A fluorogenic probe-based PCR assay called fluorescence-resonance energy transfer/melting curve analysis (FRET/MCA) has been developed as a significant alternative for the molecular typing of parasite species\(^6\). However, the current classification of the parasite is still based on isoenzyme typing by using the multilocus enzyme electrophoresis (MLEE). MLEE has been the most widely used technique during the last years and is still considered the gold standard method for the strain identification, separating it into groups so-called zymodemes, according to the identification of their enzymatic patterns\(^6\). However, the method is isolation and cultivation dependent, limited to specialized centers and lacks discriminatory power. It is also time consuming, therefore it is not rapid enough to guide therapeutic decisions although it is essential for epidemiological studies\(^6\). MLEE has now been surpassed by molecular techniques such as high-resolution multilocus sequence typing (MLST), which is based upon sequence analysis of several household genes\(^6\), and is currently considered the most powerful phylogenetic approach, with high discriminatory properties, reproducibil-

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ity and transportability of the results between laboratories. Multilocus microsatellite typing (MLMT) is also a highly discriminatory and reproducible method that has been used for *Leishmania* population genetic studies worldwide, which provides information on relapse or reinfection of the disease.

**CONCLUSION**

The diagnosis of VL remains a great challenge, especially in endemic and poor regions, where the available methods are often limited. Various invasive and noninvasive methods are currently available for the diagnosis of VL. The demonstration of the amastigote forms of leishmanial parasites from splenic aspirations is considered to be the reference standard for VL diagnosis, due to its high sensitivity and specificity; nevertheless, it is an invasive method that can be associated with life-threatening hemorrhages. Other parasitological methods, such as bone marrow and lymph node aspirations are safer than both splenic aspiration and liver biopsy, but they are remarkably less sensitive. Culture from splenic or bone marrow aspirates has high specificity; however, it is rarely used in routine. The sensitivity and specificity of serological methods, such as DAT, IHA, IFAT, ELISA and ICTs may be variable in different endemic areas worldwide. The main drawback of these methods is that antileishmanial antibodies may be detectable for a long period after recovery.

Additionally, in *Leishmania*-HIV coinfected patients, serological methods demonstrate low sensitivity, as the determination of antibodies is difficult. The most commonly used tests are DAT and the rK39 antigen based immunochromatographic test, which is a simple, rapid, reliable and cost-effective. Molecular methods may be performed in various samples and have been successfully used in VL diagnosis, demonstrating high sensitivity and specificity, also in asymptomatic and HIV coinfected patients, while they are capable of identifying relapses and reinfections in VL patients after recovery. However, the implementation of molecular methods is often difficult, especially in developing countries, because they are not cost-effective and they need well-established laboratories, trained and skilled staff.

The existence of several methods for the diagnosis of VL enables the distinction between active and asymptomatic infection, the diagnosis of relapse and also the diagnosis of VL-HIV coinfection. As the sensitivity and specificity of a method may vary in different endemic regions, the selection of the diagnostic tests should be based on several parameters, including the sensitivity and specificity as well as the cost, the availability of equipment and qualified personnel, and field applicability.

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**Correspondence to:** Dr Hercules Sakkas, Microbiology Department, Faculty of Medicine, School of Health Sciences, University of Ioannina, P.O Box 1186, Ioannina– 45110, Greece.

E-mail: isakkas@uoi.gr

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