A systematic review on control of leishmaniasis. Part I: Unveiling challenges and advanced diagnosis

Review Article

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ABSTRACT

Leishmaniasis is a neglected tropical disease ranked next to malaria as a deadly protozoan disease, induced by an intracellular protozoan "*Leishmania*", that belongs to the family Trypanosomatidae. It affects the poorest populations in over 90 countries throughout Africa, Asia, the Middle East, and Central and South America, representing a significant obstacle toward the attainment of the Sustainable Development Goals by 2030 in various countries. The presence of multiple clinical forms, socioeconomic factors, several *Leishmania* spp., various vector species, and complex life cycle, mark leishmaniasis as a complicated disease. These major obstacles impede the implementation of control measures and hinder the efficiency of preventive approaches. Misdiagnosis, timing of the diagnosis, cost and side effects of anti-leishmanial drugs, and drug resistance are the top challenges facing disease control. Therefore, advances in different diagnostic methods are a vital initial step towards effective control. They involve molecular techniques, proteomic-based approaches, immunological assays, and nano-based tools. The present review aims to highlight challenges facing leishmaniasis control, and advanced diagnostic methods as a crucial step towards such control.

Keywords: challenges; control strategies; immunological tests; leishmaniasis; molecular diagnosis; nanotechnology; proteomics.

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INTRODUCTION

Leishmaniasis is a neglected tropical disease ranked by the WHO next to malaria for mortality and morbidity. It represents a significant obstacle toward the attainment of the Sustainable Development Goals by 2030 in various countries[1]. It is a vector-borne, zoonotic disease induced by an obligate intracellular, protozoan "*Leishmania*", belonging to the family Trypanosomatidae. Additionally, it is a multifaceted disorder caused by multiple subspecies with diverse clinical manifestations, leading to confusion even among medical experts. It induces many clinical forms, emerging from two major types. The visceral type (VL or Kala-azar) has a mortality potential of about 95% in untreated cases. Currently, its prevalence is approximately 50,000–90,000, primarily in India, Brazil, and East African countries[2,3]. Estimates of annual VL are currently less than 100,000 which presents a significant decrease in comparison to the prior estimate (400,000)[4]. Yet, cutaneous leishmaniasis (CL) is the most prevalent type, inducing a main public health illness, responsible for 95% of global leishmaniasis cases. While likely under-reported, the current incidence of CL ranges from 700,000 to 1.2 million cases per year[4]. About 82% of CL cases are reported in the Eastern Mediterranean region and the remaining in the Americas, Africa, and Europe. Its prevalence is estimated at 0.6–1 million cases, however, this figure is a fraction of the actual number[3,5]. Owing to the large variety of species, leishmaniasis has been divided geographically into the Old World and the New World. The Old World involves the Eastern Hemisphere (Asia, the Middle East, Africa, and Southern Europe). Conversely, the New World refers to the Western Hemisphere (Mexico, Central America, South America, and the USA)[6,7].

Around 22 species of the *Leishmania* parasite have been distinguished and are transmitted by the bite of almost 70 different types of phlebotomine sand flies: *Phlebotomus* in the Old World and *Lutzomyia* in the New World, belonging to the Family Diptera[8]. According to Pan American Health Organization (PAHO), sand flies are recorded across the globe, and while tropical species can complete the life cycle throughout the year in the subtropical zone species can only complete their life cycles during warm months[9].

Over the African continent, the prevalence of leishmaniasis varies from 14% to 50% in different regions so that the frequency of different *Leishmania* species exhibit variations depending on the specific district. Leishmaniasis is endemic in many countries across the continent, particularly in African countries of the Mediterranean basin[10,11], and East Africa[12], and certain areas of South Africa[13]. Among the recognized species, *L. donovani* and *L. infantum* are the most frequently responsible for VL in Africa. Countries such as Ethiopia, Somalia, Sudan, and South Sudan report alarmingly high rates of VL, while Algeria experiences a notable percentage of CL cases[14,15].

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Despite the availability of data on leishmaniasis in Africa, unfortunately, more than half of the African nations lack precise and comprehensive information. This is mostly attributed to insufficient resources, and limited access to healthcare facilities in such nations. Sensitive diagnostics, effective therapeutics, and efficacious vaccines are fundamental to accelerate advancement toward elimination programs and reducing both morbidity and mortality. The current review is intended to shed light on the obstacles hindering the control of one of the important neglected tropical diseases "leishmaniasis". Advances in diagnostic methods that improve disease detection and species identification were also discussed.

Challenges

The presence of multiple clinical and epidemiological forms of leishmaniasis, socioeconomic factors, several *Leishmania* spp., and complex life-cycle make this parasite unique and sophisticated. These represent major obstacles impeding the implementation of control measures and so hinder the efficiency of preventive approaches. Herein, there are brief hints for such challenges categorized according to measures undertaken to control leishmaniasis.

Challenges facing accurate diagnosis

Misdiagnosis: Leishmaniasis mimics a large spectrum of health conditions and infections (fungal, viral, bacterial). For instance, lupus vulgaris, sporotrichosis, tuberculosis, mycobacterial ulcers, zoster and wart viruses, cutaneous myiasis, ecthyma, foreign-body granuloma, acute furunculosis, and skin carcinoma[16]. Moreover, CL lesions could be associated with cutaneous granulomatous disease and skin cancer[17]. Accordingly, misdiagnosis can occur, and serious consequences usually develop. In addition, the low sensitivity of some of the assigned diagnostic tests hinders the early detection of cases, so the infection progresses. Notably, most of the patients who received late treatment presented either poor or no response to the conventional standard therapy[18].

Timing of diagnosis: Late diagnosis was usually associated with death due to VL[19]. Consequently, along with vector control and reservoir management, early case identification was highly recommended, followed by appropriate treatment. Therefore, national public health care systems consider primary health care centers, basic care units, and emergency care units essential for timely VL suspicion and confirmation. These centers should then redirect patients to tertiary care centers (referral hospitals) according to their clinical picture[20]. Thus, early recognition is important for better prognosis, and reduction of death rate in VL cases.

Challenges facing proper treatment

Cost of anti-leishmanial drugs: Notably, the first and second-line therapeutics of leishmaniasis are often costly and unaffordable for individual patients and control programs, especially in developing nations[21].

Route of drug administration and side effects: Most of the conventional, commercially available formulations are parentally applied, painful, and associated with complex treatment regimens and serious adverse effects. These lead to poor treatment outcomes and may result in chronicity and exacerbation of the disease condition[18]. Although the control of leishmaniasis depends mainly on chemotherapy, poor treatment consequence is a widespread phenomenon in developing countries[22].

Drug resistance: It is a basic determinant in leishmaniasis treatment failure. The different species are unique for their remarkable genomic plasticity and their ability to undergo genetic mutations producing drug-resistant genotypes. This allows them to survive even in the presence of the standard drugs' concentrations in the body. The phenomenon of drug resistance has been evident in anthroponotic leishmaniasis whether VL[23] or CL[24] in the Old World where human-to-human transmission frequently occurs.

Weak health system: In developing countries, there are insufficient supplies, inappropriately qualified and poorly delivered healthcare services[25]. This causes uncertainty and discourages patients compliance to healthcare requests and impairs adherence to the treatment[22].

Challenges facing effective prevention

Lack of anti-leishmanial vaccine: There are key obstacles restricting the development of an effective vaccine against leishmaniasis. Difficulties include improper understanding of host-parasite interactions and development of a vaccine that is effective against different forms of leishmaniasis (CL, MCL, and VL). Additionally, the deficiency of reliable methods and laboratory animal prototypes evaluating the efficacy of vaccinations is a critical problem. The scarcity of highly effective delivery systems and supreme adjuvants to trigger such a protective immune response is an additional hindering factor[26].

Vector and reservoir control unfeasibility: Outstandingly, *Leishmania* spp. are maintained by various hosts (70 animal species) belonging to seven mammal orders which are Primata, Carnivora, Rodentia, Marsupialia, Cingulata, Pilosa, and Chiroptera. They are responsible for the maintenance of the parasite in the domestic and wildlife habitat. The only confirmed vectors of human disease are species and subspecies of *Phlebotomus* and *Lutzomyia* in the Old World and the New World, respectively. Of the phlebotomine sandflies, 70 species are recognized as disease vectors[27]. The control of leishmaniasis is multifactorial depending on the clinical form of the disease, parasite species, geographic location, vector and reservoir dispersion, and density. Chemical and environmental management have so far been neither practical nor eco-friendly[25,28].

Canine leishmaniasis: Stray dogs become a public health problem in developing countries. Due to

the zoonotic potential of canine leishmaniasis, diagnosis, treatment, and vaccination of infected dogs are challenges facing control measures against leishmaniasis. In Egypt, a study[29] was conducted to determine seroprevalence of canine leishmaniasis, and recorded 21.3% with the highest rates in Cairo and Giza Governorates. Eventually, an efficient monitoring program and effective control measures are essential to reduce the risk of human infections.

Miscellaneous challenges

Public health awareness: Lack of knowledge and appropriate health information among residents in leishmaniasis endemic areas hinders effective management and diminishes the efficiency of both vector and reservoir host control programs[30]. Accordingly, health education and improved awareness among such communities represent an initial step toward disease control and elimination.

Socioeconomic status and human behavior: Leishmaniasis is a poverty-related disease. Poor housing circumstances and improper sewage disposal enhance the breeding places and encourage sandfly resting, facilitating human contact and disease transmission[25]. Sand flies are readily attracted to unsanitary and overcrowded residences where they bite and feed on human blood and transmit the disease[31]. Human behavior is directly correlated with leishmaniasis burden. For instance, sleeping on the ground outdoors in endemic communities enhances the exposure of individuals to the vectors[2,32].

Nutritional status: Protein-energy malnutrition, vitamin A deficiency, and deficiency of minerals such as iron and zinc can bring about a full-scale disease condition. Notably, the host nourishment can affect the preference of sand flies to bite a particular host[33].

Host immunity: Human immunosuppression induced by chronic diseases, malignancies, immunosuppressive drugs, and HIV infection seriously affects the disease progression. In HIV-infected patients with super-imposed VL, a definite diagnosis of leishmaniasis using serological tests is a problem[27]. Furthermore, atypical manifestations and severe consequences were reported in such cases[34]. Unfortunately, treatment failure is a significant phenomenon in immunocompromised leishmaniasis cases. On the other hand, VL negatively affects the response to the anti-retroviral therapy where co-infected patients hardly get cured, especially when their CD^{+4} cell count is <200 cells/mm^3[35]. Additionally, VL is a remarkable immunosuppressive illness that predisposes to opportunistic microbial and parasitic infections such as tuberculosis and leprosy[36], malaria[37], and schistosomiasis[38]. In most co-infections, disease severity frequently proceeds to a fulminating variety and leads to a high mortality rate.

Climate changes: Leishmaniasis is significantly affected by climatic conditions. Global warming remarkably impacts vector abundance and the dispersion of leishmaniasis. Additionally, climate variations force people to migrate to new destinations, often in the outskirts where economic and sanitary situations are poor[39]. These can accelerate the transmission of such a vector-borne disease and result in emergence of new hotspots of the infection[40].

Natural events and population displacement: The incidence of leishmaniasis is extremely affected by natural disasters. Earthquakes, tsunamis, and floods provide suitable breeding conditions for the propagation of vectors and spread of parasitic infections among susceptible hosts[41]. Furthermore, population movements are significantly accompanied by the spread of neglected tropical diseases (NTDs), including leishmaniasis. Such diseases frequently deteriorate by poor health facilities and insufficient medical infrastructure[42].

Civil unrest: Both CL and VL are closely associated with rising levels of conflict among nations. Owing to the ongoing wars in Syria, Libya, Afghanistan, Yamen, and neighboring countries, the burden of CL has substantially increased among the war-affected population[43]. Particularly, warfare induces empirical social deterioration, instability, and population displacement and consequently the emergence of leishmaniasis[44,45].

Diagnostic approaches

The present review is based on a systematic search of PubMed, Scopus, Science Direct, Web of Science and Google Scholar using the keywords: *Leishmania*, leishmaniasis, control, diagnosis, diagnostic biomarkers, immunological assays, serodiagnosis, strip test, species identification, molecular diagnosis, proteomics, nanoparticles, nanotechnology. The search covered the articles published during the period from January 2018 to June 2025. Research and review articles discussing control measures against leishmaniasis or evaluating advanced diagnostic approaches were carefully included.

The following are remarks that should be targeted to reach early and accurate diagnosis as an initial step towards successful control measures against leishmaniasis. Efficient diagnostic methods must be fast, reliable, easy to perform, highly sensitive, and specifically differentiate between similar parasitic diseases. Parasitological diagnosis, *i.e.*, detection of amastigotes in host samples, or promastigotes in culture, remains the gold standard for leishmaniasis, but sampling from either liver or spleen in VL cases may be accompanied by hemorrhage risk. On the other hand, serological techniques, which are simple, rapid, and non-invasive, have major drawbacks that include cross-reactivity between different parasitic infections and lower sensitivity detection of asymptomatic cases[46]. Recent approaches such as proteomics-based methods, flow cytometry, nano-diagnosis, and advanced molecular techniques proved to be revolutionary in diagnosing leishmaniasis due to their high sensitivity and specificity outcomes[47].

Leishmania antigens and diagnostic biomarkers

Although current serologic tests for CL diagnosis are restricted because of the poor humoral response, a study demonstrated that 78.6% of tested CL patients had elevated anti-*L. tropica* antibodies in their sera with a high positive predictive value. This study was designed to assess the serodiagnosis of CL using the vector salivary proteins as a biomarker of exposure to infection. The investigators tested the serum samples of patients with CL for anti-sand fly saliva IgG antibodies (*P. arabicus, P. sergenti,* and *P. papatasi*) using ELISA[48].

In another report, Kühne *et al*.,[49] claimed that accurate diagnosis of patients with VL utilizing current commercial kits is an obstacle in developing countries because their performance is suboptimal, *i.e.*, they are not suitable as point of care test. It is worth noting that analysis of results obtained from evaluating 86 assays using 80 antigens revealed only 20 native proteins, and 7 composed of antigen mixtures. The reviewers observed that most *Leishmania* antigens were not sufficiently evaluated, whereas non-protein antigens, and antigen mixtures were neglected. Only two tests, rK28-based ICT, and intact promastigote based indirect fluorescent antibody technique (IFAT), showed higher sensitivity and specificity (>94%, and >97%, respectively). In addition to Kinesin protein, several *Leishmania* antigens were suggested including 1) metalloprotease (gp63), 2) *Leishmania*-activated C-kinase antigen, a highly conserved protein among *Leishmania* spp., 3) Q proteins that activate phospholipase C to participate in a variety of cellular signaling pathways, 4) A2 protein that plays essential roles for intracellular survival of amastigotes in the visceral organs, and 5) Nucleoside hydrolase that is responsible for hydrolysis of host RNA nucleotides to nucleobases necessary for DNA synthesis utilizing salvage pathway[49].

Since post-Kala-Azar dermal leishmaniasis (PKDL) serves as a reservoir for VL transmission, its diagnosis is a challenge because symptoms resemble those of other endemic diseases, *e.g.*, leprosy and vitiligo. A study conducted in India investigated the glycosylation profile of circulating immune complexes (CICs) in PKDL patients in comparison to patients with leprosy and vitiligo. Utilizing glycan differentiation kit, the study identified several glycan-rich PKDL-specific proteins of varying molecular weights. Accordingly, the investigators developed a colorimetric glycoprotein CIC assay that showed improved sensitivity (95.6%) and specificity (99.3%) in comparison to ELISA. Due to its low cost, it was concluded that the assay was efficient in the accurate diagnosis of PKDL and could be applied for prognostic evaluation of PKDL patients[50].

Recently, British investigators[51] identified a novel diagnostic biomarker with potential competence to accurately diagnose VL in humans. The identified candidate, type I membrane protein (D36) exhibited immunogenic reactions irrespective of the host species for both *L. donovani* and *L. infantum*. Proteomics analysis suggested that D36 has essential roles in both promastigote and amastigote survival. The study screened D36 against sera collected from Bangladesh, and it recorded 97.44% sensitivity and 97.67% specificity. Similarly, D36 maintained its performance against Ethiopian patients' sera with 95% sensitivity and 86% specificity. Its specificity was 86% against Kenyan samples, but with lower sensitivity (50%). The investigators attributed this regional difference to genetic variations in *Leishmania* spp., and/or co-infection with other pathogens. Accordingly, they recommended further studies to validate D36 value in distinguishing symptomatic from asymptomatic infections[51].

Immunological methods

Immunodiagnosis of leishmaniasis is based on the detection of *Leishmania* antigens or antibodies in serum or urine samples. Several immunological tests are available, particularly for VL, since it has an obvious humoral response.

Leishmanin skin test (LST or Montenegro skin test): This test was used for almost a century. It is based on a delayed hypersensitivity response to antigens of *Leishmania* promastigotes. It exhibits high sensitivity and specificity values (86–100% and >90%, respectively), being useful for epidemiological studies. However, it is negative in patients with diffuse cutaneous leishmaniasis (DCL) and active VL due to impaired cell-mediated immune response[52]. Furthermore, the accuracy of LST was evaluated in PCR-negative patients to diagnose American tegumentary leishmaniasis. It showed a high sensitivity (90.0%) in PCR-negative patients, which was ~10% higher than the sensitivity recorded in PCR-positive cases (79.66%). This reinforces the value of tests that detect cellular response to *Leishmania* antigens, especially in questionable diagnostic conditions[53].

Direct agglutination test (DAT): The test is based on the agglutination reaction between *Leishmania* antigen and patient antibodies in serum or urine samples. It was reported that the non-carbohydrate moiety of lipophosphoglycan antigen, and other non-carbohydrate epitopes on the surface of the DAT antigen, are responsible for its agglutination with antibodies in samples of VL patients[54]. It was useful as a marker of asymptomatic infection and in HIV co-infected cases, showing high sensitivity and specificity values[55]. Recently, a systematic review and meta-analysis[56] reported high estimated pooled sensitivity and specificity of DAT for VL diagnosis. However, the sensitivity and specificity were lower for different patient groups and geographical locations, due to the lack of DAT standardization, and the lack of data from some important geographical locations[56].

Indirect hemagglutination assay (IHA): Human erythrocytes sensitized with *Leishmania* antigens are used for sensitive IHA detection of anti-leishmanial antibodies[57]. This approach showed sensitivity and specificity percentages of 90-100% and 86%,

respectively. However, this assay is not suggested as the only screening approach for VL because clinical samples show high titer even after recovery[58].

Latex agglutination test (KAtex): This non-invasive assay for detecting antigens in urine samples of patients with VL, is an alternative approach to antibody detection methods where antibody production is low, as in immunocompromised individuals (*Leishmania* and HIV co-infection). It was reported that KAtex detected low molecular weight (5–20 kDa) carbohydrate-based *Leishmania* antigen, specific to VL patients[59]. The test is easy, cost-effective and helpful for monitoring treatment progress as the antigen can be detected from one to six months after treatment. Besides, in immunocompromised cases, it exhibited high sensitivity (85–100%) and specificity (96–100%) values[57].

Enzyme linked immunosorbent assay (ELISA): It is one of the primary diagnostic approaches for the serodiagnosis of leishmaniasis, as it plays a crucial role in screening vast samples by using different antigens[60]. However, the type of antigen used designates the sensitivity of the assay. It showed high sensitivity (80-100%) and specificity (82-95%) values using crude soluble antigens[58]. However, cross-reactivity in some patients with tuberculosis, trypanosomiasis, and toxoplasmosis was observed[58,61]. Moreover, the ability to differentiate between present infection and possible recovery is still lacking. Monoclonal antibody-based multiplexed capture ELISA was developed for antigen detection, and showed a high sensitivity (93%). This test was designed for simultaneous detection of six *Leishmania* protein biomarkers (*Li*-isd1, *Li*-txn1, *Li*-ntf2, *Ld*-mao1, *Ld*-ppi1, and *Ld*-mad1) in urine samples collected from patients with VL. These biomarkers were iron superoxide dismutase 1 (ISD1), tryparedoxin (Txn), nuclear transport factor 2 (NTF 2) of *L. infantum*, and Maoc family dehydratase 1 (Mao 1), peptidyl-prolyl isomerase 1 (PPI 1), and malate dehydrogenase 1 (MAD 1) of *L. donovani*[62].

Immuno-chromatographic strip test (ICT): As a rapid screening test for VL, it is useful in field diagnosis due to its low cost, and short analysis time. In this strip test, a recombinant kinesin-39 antigen (rK39) is immobilized on nitro-cellulose paper along with colloidal gold and the suspected patient serum or blood added on the strip produces visible color development within 10–15 min[63]. Its sensitivity and specificity values revealed huge variation among the different epidemiological zones. In the Mediterranean region, its sensitivity recorded 78% in all cases of VL, and 67.3% in immunocompromised patients[55]. In another study performed in Colombia, 91,5% sensitivity was reported[61]. Numerous recombinant kinesins including rK9, rK26, and rK8.3 were developed and used for serodiagnosis of VL; however, the WHO reports considered that the rK39 is the most efficient ICT[58].

Interestingly, a laser direct-write technology was used to develop a new lateral flow device on a low-cost paper platform. The device was centered on a laser-patterned microfluidic apparatus employing two recombinant *Leishmania* proteins, β-tubulin and a hypothetical amastigote-specific *Leishmania* protein (*Li*Hyp1). It proved to be a rapid and accurate method for serodiagnosis of human VL, exhibiting promising sensitivity (95%) and specificity (95%)[64].

Comparative studies

A study was conducted in Morocco[65] to compare between usefulness of ICT rK39, ELISA, and IFA for the serodiagnosis of VL. The investigators reported the highest sensitivity of 95.5% for ICT strip, followed by IFA (87.5%) and ELISA (75%). Rezaei *et al*.[66] evaluated the diagnostic potential of Li-rK39 antigen of Iranian *L. infantum* (MCAN14/47) in an ELISA, using sera from VL patients. The results revealed 96% sensitivity, and 93.8% specificity. A commercial rK39 based ICT was applied simultaneously on the same samples, revealing 90% sensitivity and 100% specificity[66].

Later, two studies[67,68] evaluated two commercial ICTs (TruQuick IgG/IgM®, and LEISH®) in comparison to other commercial kits. For serodiagnosis of VL in Mediterranean regions, it was concluded that TruQuick® is the most efficient kit having 90.1% sensitivity, and 95.7% specificity[67]. The second study, conducted in South France, proved that IFAT and TruQuick® exhibited the highest diagnostic performance. While IFAT showed 100% sensitivity and specificity, TruQuick had 96% sensitivity and 100% specificity. Both tests showed high accuracy (100% for IFAT, and 98% for TruQuick)[68].

Utilizing bioinformatic analysis, a recent study identified a new recombinant kinesin candidate from *L. infantum* (K*Li*8.3). The study evaluated the diagnostic performance of rK*Li*8.3 using ELISA, and lateral flow test (LFT) on a panel of human sera collected from Sudan, India, and South America. Samples were obtained from patients diagnosed with VL, or other diseases, including tuberculosis, malaria, and trypanosomiasis. The diagnostic accuracy of rK*Li*8.3 was compared with rK39 and *L. donovani* K8 (rK*Ld*8). Results revealed that both rK*Li*8.3-based ELISA and LFT showed improved sensitivity with no cross-reactivity with other endemic diseases. It was concluded that rK*Li*8.3-based serodiagnostic tests performed efficiently in the diagnosis of VL in areas with high endemicity[69]. Unfortunately, almost all commercial kits commonly used in serodiagosis of leishmaniasis are unable to discriminate between current infection and cured cases, which makes its use as prognostic assay doubtful.

For the diagnosis of canine leishmaniasis, five recent comparative studies[70-74] were conducted during two years (2024 and 2025). In comparison to three commercial kits; ICT (FASTest LEISH®), IFAT (FLUO LEISH®), and ELISA (ELISA LEISH®), the investigators evaluated the performance of in-house ELISA in 215

canine serum samples. All methods recorded sensitivity that ranged between 98.5% for commercial ELISA and 99.4% for ICT. Similarly, specificity ranged from 91.9% for commercial IFAT and ELISA to 98.4% for ICT. Among commercial serodiagnostic tests, the study recommended the use of ICT due to its highest sensitivity and specificity[70]. The second study was conducted at seven veterinary diagnostic centers in southern Europe. The investigators compared sensitivity, specificity, and diagnostic accuracy of in-house ELISA, and commercial IFAT kit using 272 canine serum samples. While sensitivity and specificity for ELISA recorded a range of 95-99%, and 92-97%, respectively, those of IFAT were 89-99% and 83-94%, respectively. Test agreement was 78% with a pair-wise agreement between 65 and 89%. Of note, seroprevalence of *L. infantum* differs according to environmental variables across southern Europe, and sand fly density. Due to high diagnostic agreement among several geographic centers, the study validated using both methods[71].

Teixeira *et al*.,[72] utilized r*KLi*8.3 in the diagnosis of canine leishmaniasis. The investigators tested dogs' sera using r*KLi*8.3-ELISA as well as two ICTs (r*KLi*8.3-LFT) and rK28-dual path platform (DPP®). The results revealed similar diagnostic accuracies of ELISA, LFT, and DPP, missing several asymptomatic dogs. Meanwhile, the addition of a secondary, amplifying anti-dog IgG antibody in the r*KLi*8.3-ELISA allowed the detection of nearly all asymptomatic dogs without compromising its specificity[72]. The fourth study was conducted to evaluate two commercial IFAT and ELISA kits (VetLine®, GSD Frankfurt, Germany) that utilized whole *Leishmania* antigens (WLAs). For comparison, the study employed three serodiagnostic methods, ELISA, and LFT that utilized r*KLi*8.3, and immunoblot utilizing WLAs. Results revealed that r*KLi*8.3 antigens are superior to WLAs irrespective of the endemic area. Additionally, LFT is inexpensive, does not require professional technicians, nor equipment, *i.e.*, laboratory-independent[73].

In the year 2025, a study[74] was performed to compare the diagnostic performance of 3 ELISA commercial kits (CIVTEST®, *Leishmania*-ELISA®, and ELISA/S7®), and IFAT (MegaFLUO®LEISH). The study included canine samples from seropositive infected dogs, and three groups of apparently healthy dogs, seropositive, and seronegative from high and low endemic areas. Results analysis, using positive and negative percents of agreement, demonstrated that the diagnostic performance of the commercially available ELISA assays for canine leishmaniasis widely varied, however; CIVTEST® and IFAT showed the highest results for reliability in different clinical and epidemiological settings[74].

Molecular methods

Assays based on PCR have been considered the main methods for diagnosis and species identification, particularly for CL induced by variable species. In general, PCR-based methods are characterized by their feasibility, safety, and reliability for application in a routine laboratory[46]. The PCR-based approaches do not require parasite cultivation and can be directly applied to clinical samples. The PCR product, an amplification of the target DNA, is manipulated in gel electrophoresis, followed by downstream analysis through the use of restriction endonucleases, hybridization, DNA sequencing, or detection and analysis of fluorescent signals. Besides detection and typing, a quantitative PCR is useful for monitoring clinical cure and follow-up of patients[57].

Real-time PCR: It is a PCR variety that measures the amount of DNA generated by monitoring the amplification of a specific target during each PCR cycle. Several methods have been implemented for detection, quantification of *Leishmania* burden and species typing, using different targets and protocols[57]. It possesses higher sensitivity, with a simpler standardization procedure, compared to the standard PCR protocols. Furthermore, there is no need for PCR product manipulation through gel electrophoresis.

Multiplex PCR: This PCR variety was generated to use a panel of multiple primers optimized for identifying different *Leishmania* species simultaneously in a single reaction. A recent protocol was successfully designed, employing species-specific primers for *L. amazonensis*, *L. braziliensis*, *L. donovani*, *L. infantum*, *L. mexicana* and for the *L. guyanensis* complex with a very low detection limit[75].

Sequencing techniques: This process determines the order of succession of nucleotide bases that make up a specific fragment of DNA. The sequencing of PCR-amplified products has been applied to several targets of the genome of *Leishmania* for species identification in human samples derived from patients with VL[76]. The whole-genome sequencing was applied for diagnosis of VL in a bone marrow aspirate from an immunosuppressed patient with identification of *L. infantum*[77]. This mode may also be valuable for disease surveillance. It provides epidemiological data of importance to public health, such as characterization of transmission cycles, detection of variants of the parasite with possible new clinical features, and identification of genetic markers related to drug resistance and virulence[78].

Restriction fragment length polymorphism (PCR-RFLP): It is a relatively simple technique based on the pattern of DNA fragments after digestion with one or more restriction enzymes and then evaluated in gel electrophoresis. Gene encoding HSP70 was validated as a target for species discrimination by the PCR-RFLP approach, with misidentification of some species only in the subgenus *Viannia* possessing the same profile of restriction with the HaeIII enzyme[79]. This method was applied to clinical samples and specifically identified the species by the kinetoplast cytochrome b (*cyt b*) gene[80]. Moreover, rDNA locus (internal transcribed spacer; ITS1 and ITS2), miniexon for nuclear DNA, and kinetoplast DNA (kDNA) were considered promising *Leishmania* diagnostic biomarkers[81].

Al-Fahdawi *et al*.,[82] conducted a study to investigate the best method for the diagnosis of CL and to detect the genotypes of *L. tropica* and *L. major* in human cases from Ramadi (Iraq). The study identified the gene encoding ITS1 by conventional PCR and PCR-RFLP techniques. Microscopic examination of smears from cutaneous lesions revealed parasites in 41% of the involved patients, while PCR was positive in 51% of them. The RFLP, adopted on ITS1-PCR product for positive samples, yielded two fragments of 60 and 200 bp for *L. tropica*, and two fragments of 140 and 210 bp were identified for *L. major*[82]. Recently, a study used RFLP for molecular identification of *Leishmania* spp. in samples of skin and lymph nodes taken from suspected dogs and humans, targeting ITS1 gene. Two fragments of 101 and 140 bp for *L. infantum*, and 108 and 157 bp for *L. major* were reported. This denotes that RFLP method proved to be a reliable tool for species identification[83].

High resolution melting (HRM): This is a scheme centered on variations in DNA sequences. It employs double-stranded DNA binding dyes to determine the intensity of fluorescence during dissociation of double-stranded to single-stranded DNA amplicons generated from a real-time PCR assay. It was applied for identification and diagnosis of leishmaniasis in the Americas, Europe and Asia, using amino acid permease 3 gene as a target[84]. Targeting the gene encoding 7SL RNA, PCR-HRM identified the species responsible for CL in Iran[85]. It was highly efficient for discriminating the main species responsible for CL , VL and PKDL in the Indian subcontinent, employing two multi-copy targets (ITS-1 and 7SL-RNA genes)[86]. It is a highly promising method but requires specific PCR-HRM equipment or an adapted real-time PCR instrument and trained professionals.

Loop-mediated isothermal amplification (LAMP): It relies on the amplification of DNA in less than one hour without the use of a thermocycler. The amplified products can be detected visually by their turbidity, fluorescence, and color by the naked eye and/or UV light[87]. It showed higher sensitivity than conventional PCR in the diagnosis of CL and VL, with no post-amplification processing and it can be implemented in endemic regions with limited facilities. The gene encoding 18S rRNA was the main target due to its high conservation in *Leishmania* genus, and elevated copy number which enhanced the sensitivity of detection[87]. Interestingly, LAMP assay using primer sets targeting kinetoplast DNA (kDNA) of *L. tropica* was successfully applied for species-specific diagnosis of CL in Iran[88].

Proteomics-based techniques

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS): Since protein content is commonly analyzed using mass spectrometry, this allows the analysis of protein abundance, post-translational modifications, different protein interactions, and structure. Proteomics analysis involves extraction, separation, identification, and precise protein quantification. Separated proteins or peptides are subsequently ionized by matrix-assisted laser desorption ionization. The time-of-flight value for particular ions corresponds to the molecular weight of the ionized molecule[89]. Accordingly, a unique characteristic pattern is generated and compared with an existing mass spectra database for identification. It was implemented for species identification of 33 species of 10 *Leishmania* complexes. Among these isolates, only one was misidentified at the complex level (typed *L. guyanensis* instead of *L. braziliensis*) [90]. Although this method was faster than other molecular methods unfortunately, it requires a fully-equipped laboratory containing costly equipment and well-trained professionals. It also necessitates isolation and cultivation of the parasite before species identification. It is a promising method that offers both rapidity and efficiency for the identification and phylogenetic analysis of *Leishmania* spp.[91]. All strains isolated from CL patients in Bolivia, were correctly identified by MALDI-TOF MS at the subgenus, genus, and complex level[92].

Major liquid chromatography-mass spectrometry: This is another proteomics-based technique that efficiently identified and quantified specific glycated protein, plasminogen and vitronectin proteins biomarkers for the diagnosis of PKDL[93]. Immuno-proteomics is an approach applied to detect proteins associated with immune responses induced by leishmaniasis. It involves protein extraction and separation by 2-D electrophoresis, western blotting of the segregated proteins, application of the patient serum to the blot, and a specific antigen-antibody reaction. This is followed by mass spectrometry to analyze the formed fragments and peptide fingerprints for immunogenic proteins. This immune-proteomics assay was successfully employed in identification of *L. infantum* proteins for the diagnosis of VL in immunocompromised patients[94]. Furthermore, an acyl transporter protein (protein 3-oxoacil reductase) was identified from *L. panamensis*, and explored as a potential disease-specific diagnostic marker with promising results[89].

Nanotechnology

Because of their unique optical and physicochemical properties and high surface area to volume ratio, nanosized particles possess the ability to bind and interact specifically with individual biomolecules such as lipids, DNA, and proteins. Biomolecular detection platforms based on nanoparticles are cost-effective, rapid, and culture non-dependent. These provide fast, one-step, and reliable results with acceptable sensitivity and specificity.

Welearegay *et al*.,[95] presented a simple-to-use, noninvasive approach for diagnosis of human CL, based on the analysis of volatile organic compounds in exhaled breath of patients using specifically-designed chemical gas sensors. They developed metallic nanoparticle-based sensors. One sensor

was based on copper nanoparticles functionalized with 2-mercaptobenzoxazole, which yielded 100% accuracy, 100% sensitivity, and 100% specificity for CL diagnosis. Another one was based on gold nanoparticles, and achieved 98.2% accuracy, 96.4% sensitivity, and 100% specificity[95]. Another approach was designed for the detection of *Leishmania* infection in asymptomatic HIV patients using dual indicators (SYBR safe and gold-nanoparticle probe; AuNP-probe) in one-step LAMP assay. It achieved promising sensitivity (94.1%), and specificity (97.1%)[96]. One year later, a study developed a simplified, and non-expensive distance-based paper device combined with fluorescent SYBR safe and gold-nanoparticle probe LAMP assay. This design allowed rapid screening of infection as well as semi-quantification of *Leishmania* in the buffy coat collected from patients with *Leishmania*/HIV co-infection within a few minutes. It achieved sensitivity and specificity as high as 95.5% with a detection limit of 10^2 parasites/ml, which was 10 times more sensitive than other related studies[97].

Pedro *et al*.,[98] illustrated the use of nanomaterials as nanoquenchers for nucleic acid fluorescent sensing platforms. These nanostructures interacted with the fluorophore of the labeled *L. infantum* DNA probe through electron transfer progressions which resulted in quenching of the fluorescence emission. They showed a high sensitivity, with respective low detection limits of 1.1 nM and 1.3 nM[98]. A procedure was developed for optical biosensing of *Leishmania* spp. sequence in clinical samples. It was based on hybridization of citrate-capped silver nanoparticles bound to specific single-stranded DNA probe of the parasite. It achieved a very low detection limit with a quick bioanalysis, helping in rapid specific diagnosis[99]. Therefore, metal nanoparticles were employed for the development of biosensors because of their benefits including low toxicity, biocompatibility, chemical inactivity, and water solubility, as well as their abundant and low-cost resources[100].

Later, the efficacy of a nano-biosensor designed from gold-nanoparticle probe conjugate was assessed in non-amplification and amplification assays for the diagnosis of leishmaniasis. The first method was conducted by DNA hybridization, while the second assay was followed by PCR amplification. The results were valuable; the sensitivity of non-amplification and amplification methods for the diagnosis of VL was 96%, and 100%, respectively, and for CL, it was 98% and 100%, respectively[101].

A commercial ICT (FASTest LEISH®), composed a combination of monoclonal antibodies conjugated with colloidal gold particles and recombinant *L. infantum* antigens, was developed. For comparison, the investigators used IFAT (MegaFLUO LEISH test®), and an in-house ELISA assay as references. Results revealed 100% sensitivity, 99.1.% specificity, and 99.6% diagnostic accuracy. The study concluded that FASTest LEISH® was a rapid screening test with high diagnostic accuracy[102]. Recently, lateral flow strips were designed using antibody-conjugated gold nanoparticles for rapid detection of *Leishmania* antigen in skin biopsy specimens obtained from CL patients. This method demonstrated promising accuracy, with high sensitivity (92%) and reasonable specificity (90%)[103].

CONCLUDING REMARKS

1. The presence of multiple epidemiological and clinical forms, socioeconomic factors, several *Leishmania* spp., various sand fly vectors, and the complicated life cycle, eventually mark leishmaniasis as a unique and sophisticated tropical disease.
2. Various challenges impede leishmaniasis control, and hinder the efficiency of preventive approaches. Late diagnosis, costly and prolonged therapeutic regimens, drug resistance, lack of efficient vaccine, and infeasibility of control measures against vectors, and reservoirs are the major challenges.
3. The ICTs, and LF devices are rapid, cost-effective, commercially-available, screening tests for leishmaniasis. Kinesins and their recombinant forms incorporated in immunological tests proved to be valuable biomarkers in the accurate diagnosis of leishmaniasis.
4. Modifications of commercial kits utilizing novel diagnostic biomarkers remarkably improved their sensitivity and specificity. Scientists should be encouraged to identify novel diagnostic candidates
5. Molecular techniques, such as real-time PCR, multiplex PCR, PCR-RFLP, PCR-HRM, LAMP, and DNA sequencing exhibited promising advances to achieve early and accurate diagnosis of leishmaniasis.
6. Proteomic-based approaches such as MALDI-TOF MS, and major liquid chromatography-MS proved potential efficacy in leishmaniasis diagnosis and species identification.
7. Advanced nanotechnology allows the scientists to develop biosensors incorporating gold, silver or copper nanoparticles in different platforms for detection of *Leishmania* specific antigens.

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