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Molecular Diagnosis of Human Cutaneous Leishmaniasis and Identification of the Causative *Leishmania* Species in Iran: A Narrative Review

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ABSTRACT

Cutaneous leishmaniasis is a common form of leishmaniasis in underdeveloped countries. Although CL tends to be self-limiting, it can cause significant scars and may progress to more severe manifestations. Additionally, *Leishmania* species vary in susceptibility to the available treatments. The selection of treatment and clinical outcome of CL depend on the accurate determination of the *Leishmania* species. This mini-review aims to provide an overview of the molecular diagnosis techniques such as PCR-based assays, NASBA, and LAMP utilized in the identification of *Leishmania* species in Iran. ***DOI: 10.61186/ibj.4239***

Keywords: Humans, Iran, Leishmaniasis, Molecular diagnostic techniques

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INTRODUCTION

Leishmaniasis generally manifests in four clinical forms: CL, DCL, MCL, and VL. Around 350 million people are estimated to be at the risk of leishmaniasis in nearly 100 countries. Annually, the incidence of VL ranges from 200,000 to 400,000 cases, while CL affects 700,000 to 1.2 million individuals, leading to an estimated 20,000 to 40,000 deaths[1].

Identification of the particular species of *Leishmania* is necessary for accurate diagnosis and treatment of CL, as well as for successful patient management. CL is diagnosed based on clinical presentation, supported by epidemiological data, and laboratory testing[2]. Furthermore, immune-compromised patients exhibit atypical manifestations with varying levels of metastatic dissemination of the pathogen, indicating that host immune status contributes to clinical outcomes[3]. Patients with localized CL also produce low levels of antibodies against the disease. However, immunocompromised patients, including those who are co-infected with the human immunodeficiency virus or are under chemotherapy, produce little or no antibodies, which results in negative serological tests[4,5]. Because of this, the patients may benefit from the enhanced sensitivity of molecular methods compared to serology tests[2,4,5]. Identification and differentiation of *Leishmania* species in various regions of the world have

List of Abbreviations:
AAP3: amino acid permease 3; **AFLP:** amplified fragment length polymorphism; **AI:** artificial intelligence; **CI:** confidence interval; **CL:** cutaneous leishmaniasis; **COII:** cytochrome oxidase II; ***cpb***: cysteine proteinase b; **DCL:** diffuse cutaneous leishmaniasis; **DIG:** digoxigenin; **DIG-dUTP:** digoxigenin-11-dUTP; **dNTP:** deoxynucleotide triphosphate; **ELISA:** enzyme-linked immunosorbent assay; ***gp63***: glycoprotein 63; **ITS:** internal transcribed spacer; **kDNA:** kinetoplast DNA; **LAMP:** loop-mediated isothermal amplification; **MCL:** mucocutaneous leishmaniasis; **NASBA:** nucleic acid sequence-based amplification; **OC:** oligochromatography; **PCR:** polymerase chain reaction; **QT-NASBA:** quantitative nucleic acid sequence-based amplification; **RFLP:** restriction fragment length polymorphism; **rRNA:** ribosomal RNA; **RT-PCR:** reverse transcription PCR; **SAG:** sodium antimony gluconate; **SL RNA:** spliced leader RNA; **ssDNA:** single-stranded DNA; **TD-LAMP:** tape-disc LAMP; **VL:** visceral leishmaniasis

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revealed new parasite-related disease manifestations. For instance, there is evidence that *L. donovani*, which is commonly associated with VL, may also present as CL disease. Moreover, the clinical overlap between VL and CL presentations has previously been described for *L. infantum*/*L. chagasi*[3].

There are several diagnostic methods available for CL, including microscopy, histopathology, culturing, and molecular methods, all with varying levels of accuracy[6]. Indeed, the infrastructure and resources of the facility rather than diagnostic accuracy, determine the type of diagnostic test. However, diagnosis is still contingent upon direct parasitological methods[3]. The present review aims to cover important molecular diagnostic techniques with particular attention to Iranian studies. Finally, we touch on the potential of AI as an emerging tool that could aid in diagnosing CL.

PCR-based methods

Conventional PCR and PCR-RFLP

In epidemiological studies, PCR is often used along with other methodologies, such as RFLP analysis and gene sequencing to confirm different *Leishmania* species[6,7]. These techniques are of value in monitoring the progression of the disease, assessing the efficacy of antileishmanial treatment, discerning species-level differences, and identifying drug resistance[8]. Various target sequences, such as kDNA, small subunit rRNA, ITS of the ribosomal DNA, the mini-exon gene or the SL RNA gene, the tubulin gene, *gp63* gene, and repetitive genomic sequences, have been well described[9,10]. A high abundance of minicircles in the pathogen makes kDNA an attractive PCR target[10]. According to one study, the PCR assay based on kDNA, exhibited a high sensitivity (93.6%) in the diagnosis and identification of CL caused by *L. major*, *L. tropica*, and *L. infantum* in Shiraz City. This sensitivity was significantly higher than that of direct microscopy (76.7%) and culture methods (50.9%)[11]. Another study demonstrated that ITS-PCR had a higher level of sensitivity (98.8%) compared to microscopy (79.3%) and culturing assay (86.2%) for detecting *L. tropica* and *L. major* in Mashhad City[12]. ITS-PCR, followed by RFLP, was also successfully used to detect *L. major* in CL patients from Ilam Province, Iran[13]. Teimouri et al. utilized the same method to detect *L. tropica* and *L. major* in CL patients across different areas of Iran. Based on their results, *L. tropica* isolates exhibited a higher level of heterogeneity compared to *L. major* and *L. infantum* isolates[14]. By employing kDNA PCR-RFLP, Ghatee et al. observed a high diversity among *L. tropica* strains isolated from CL cases in Kerman and Shiraz cities[15]. Darudi et al. recommended kDNA PCR as an appropriate diagnostic tool for CL in endemic areas and suggested ITS-PCR RFLP for the rapid characterization of *Leishmania* species[16]. A simple smear sample is sufficient to attain reliable results for PCR. The sensitivity of PCR in smear samples is 95% (95% CI: 90%-98%), while its specificity is 91% (95% CI: 70%-98%). However, its sensitivity and specificity are lower for aspirates, skin biopsies, and swab samples[17].

Nested and semi-nested PCR

Nested PCR minimizes non-specific amplification of DNA template. When a gene is present in low abundance, nested PCR could be a suitable technique for its amplification. The nested PCR technique allows to achieve results that are largely devoid of impurities due to primer dimers, alternative primer targets, and hairpins[18]. In a study by Feiz Haddad et al., the specificity and sensitivity of the nested PCR for detecting kDNA in CL patients were 92% and 100%, respectively[19]. Also, similar results were observed by Shirian et al. in patients with localized CL[20]. Similarly, Khosravi et al. detected *L. major* and *L. tropica* among CL patients in Kerman province, Iran[21]. The differentiation between *L. tropica* and *L. major* in the suspected cases of CL was also achieved using nested PCR in a study conducted in Ahvaz City, Iran[22]. Maraghi and co-workers successfully utilized nested PCR to differentiate *L. tropica* and *L. major* in lesions of patients with CL[23]. Several investigators also used semi-nested PCR to detect different *Leishmania* species in sandflies[24-26] and lesions of patients with CL[27,28] based on the primers targeting kDNA or ITS regions. For instance, in a study performed by Badirzadeh et al., the application of semi-nested PCR based on kDNA was effective in identifying *L. infantum* and *L. major* in Iranian patients with CL[29]. To reduce the risk of carryover in nested PCR, it is essential to implement physical separation of equipment and designated areas during various stages of the process[7].

Multiplex PCR

This method enables various DNA targets to be amplified concurrently, allowing multiple PCR reactions to be run at the same time. By using numerous primers in one PCR mixture, amplicons of specific DNA targets with varying sizes can be generated[7,9]. For leishmaniasis diagnosis, various markers, such as kDNA and SL RNA, have been employed[30-33]. In this context, Nateghi Rostami et al. designed a kDNA-based multiplex PCR for the simultaneous detection of *L. major*, *L. tropica*, and *L. infantum* in CL patients. They showed 100% sensitivity and 100 the isolates from lesion scrapings, exudates, and biopsy samples[34]. The effectiveness of multiplex PCR to detect *L. major* and *L.*

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tropica in CL patients, was also demonstrated in another study performed in Tehran, Iran[35].

Real-time PCR

The application of real-time PCR provides benefits in diagnosing, monitoring clinical treatments, following up of patients, drug development, and drug efficacy evaluation[8,10,36]. Researchers have designed various real-time PCR protocols targeting *kDNA*, *tryparedoxin peroxidase* gene, and *ITS1*[8]. A number of internal controls, such as *GAPDH*, *β-microglobulin*, *β-actin*, and *18S rRNA* genes, have been used to determine optimal nucleic acid extraction and sample quality[9]. In Iran, the implementation of real-time PCR has proven to be successful in detecting various *Leishmania* species in CL patients. In a study conducted in the southwest of Iran, real-time PCR targeting kDNA on Giemsa-stained smears of the cutaneous lesions, which were collected from CL patients, was found to have a sensitivity of 98%[37]. In a separate work, the detection of *L. major* and *L. tropica* in CL patients was performed using real-time PCR targeting the *tryparedoxine peroxidase* gene. The sensitivity and specificity of this method were found to be 98.7% and 59.8%, respectively[38]. Real-time PCR based on ITS was also used to detect *L. tropica* in paraffin-embedded tissue samples and monitor the parasite load during treatment and following up in a study from Kerman, Iran[39]. Recently, Fotouhi-Ardakani et al. have devised a novel TaqMan real-time PCR technique, targeting two gene regions, AAP3 and COII, to identify *L. major* and *L. tropica* in CL samples collected from various regions of Iran. They achieved an outstanding sensitivity of 98.1% and a specificity of 100% for the method mentioned above in their study[40]. Overall, real-time PCR appears to be a promising technique, but its application is restricted by the high expenses and also the necessity for an expert to interpret the results.

PCR-ELISA

In PCR-ELISA, PCR products are detected and quantified directly following the immobilization of biotinylated DNA on a microtiter plate using an immunological technique (ELISA)[41-43]. The method was successfully used for the diagnosis of CL using lesion scrapings, biopsies, and exudates[41-43]. In many studies, kDNA was employed as a target for PCR-ELISA[41,44]. In Iran, few studies applied PCR-ELISA for detecting *Leishmania* among patients. In a study conducted by Samimi et al., PCR-ELISA was utilized to detect *L. major* in patients diagnosed with CL in Golestan Province, Iran[42]. In another research work, the detection of *L. infantum* in blood samples of healthy individuals who reside in endemic regions was conducted using PCR-ELISA targeting kDNA. The study indicated that the kDNA-based PCR-ELISA test cannot be considered a reliable method for diagnosing active VL in endemic areas, primarily due to the high prevalence of positive samples[44].

AFLP

AFLP is a multiplex PCR-based method that enables the observation of fragments produced by restriction endonucleases locating at random sites across the genome. This method allows researchers to gain a complete understanding of the genetic variations present in an organism's genome[45]. A comparative analysis of *L. donovani*, *L. major*, and *L. tropica* was performed using AFLP to search for markers that can differentiate visceral from cutaneous manifestations[46]. As for Iran, there are limited data on the use of the AFLP method for studying genetic variations in *Leishmania*. In one study, authors attempted to develop a cDNA-AFLP method confirmed by the real-time PCR, for identifying target genes associated with antimony resistance in clinical isolates of *L. tropica*[47]. A similar approach was also utilized for the identification of Glucantime® resistance markers in *L. infantum*[48].

NASBA

NASBA is a transcription-based amplification and isothermal complex method for determining RNA targets. By detecting RNA, NASBA is able to determine the viability of parasites[7]. By targeting RNA, a significantly higher number of template molecules will be available, leading to the enhanced sensitivity of the assay and a reduced sample volume. QT-NASBA could detect the levels of parasites 100-fold lower than those detected by conventional PCR[49]. The lower detection limit of QT-NASBA (based on 18S rRNA) was two parasites per skin biopsy sample or 10^2 parasites/mL (corresponds to 0.1 parasites per μL) of blood sample[49,50]. In Iran, limited efforts have been made to develop NASBA to detect *Leishmania*. Niazi et al. showed a sensitivity of 81% and a specificity of 100% for the NASBA method in detecting *L. major* in wound samples, while the sensitivity and specificity of RT-PCR were 51% and 100%, respectively[51]. One study also attempted to standardize 18S rRNA-based NASBA for the detection of *L. major*[52]. Similarly, another study developed a colorimetric assay based on NASBA-GNR (18S rRNA-based NASBA assay combined with gold nanorods) for identifying *L. major* in skin biopsy samples. The results confirmed 100% sensitivity for NASBA-GNR assays compared to RT-PCR and also the specificity of the colorimetric assay was 80% in comparison with RT-PCR[53].

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LAMP

The isothermal nature of the LAMP reaction obviates the requirement for a thermal cycler[9,54]. LAMP uses a strand-displacing DNA polymerase and often comprises four to six primers recognizing six to eight distinct regions of target DNA. The presence of amplified targets by LAMP can visually be identified as either a white precipitate or a solution with a yellow-green color upon the introduction of SYBR green dye. This combination of features renders the technique suitable for being used under field conditions[7]. Different targets such as kDNA, *18S rRNA* gene, *cpb* gene, *ITS1* DNA sequences, *k26* or *hydrophilic surface protein B* gene, and *histone H3* gene were detected by LAMP assay[55]. LAMP can detect as low as 0.01 parasites/mL, whereas reverse transcription LAMP has a detection limit of 10–100 parasites/mL based on serial dilutions of extracted *Leishmania* DNA[54]. In one study, the LAMP assay based on *18S rRNA* gene, was able to accurately detect *Leishmania* species in patients with CL in Khuzestan Province, Iran. The LAMP assay achieved a sensitivity of 98% and a specificity of 100%, whereas kDNA-based nested PCR yielded 100% sensitivity and specificity[56]. By contrast, another study showed that both real-time PCR and LAMP methods had 100% sensitivity and specificity in detecting *Leishmania* species in stained smears of cutaneous lesions[57]. Recently, Taslimi et al. successfully utilized TD-LAMP for diagnosing CL. Their findings revealed a sensitivity of 97% and a specificity of 100% in detecting *L. tropica* infection. The detection limit of the TD-LAMP assay was 1 fg[58]. Although the LAMP assay offers considerable ease and simplicity, there is a significant risk of cross-contamination.

AI in CL diagnosis

AI can enable us to identify specific morphological features and patterns with exceptional accuracy. By integrating AI with telemedicine platforms, there has been an improvement in efficient patient management, follow-up, and tailored treatment plans, marking a new era in medical care. Recently, a new AI-driven system has been developed in Iran to identify *Leishmania* parasites in microscopic images. Notably, the system achieved a specificity of 52%, a sensitivity of 71%, and an accuracy of 70% in the identification of the parasites[59]. Another study utilized a machine learning-based approach to identify unresponsive cases of CL caused by *L. tropica*. Among the various classifier models, the multilayer perceptron classifier showed promising results, achieving an accuracy of 87.8%, a sensitivity of 90.3%, and a specificity of 86%. The study highlighted the potential of AI in disease prognosis and treatment selection, particularly in CL cases [60].

DISCUSSION

CL manifests as a complex disease, influenced by the intricate interaction among parasite reservoirs, parasites, vectors, climate, ecology, political instability, poverty, and socioeconomic status. The accurate, rapid, and sensitive diagnosis of leishmaniasis heavily relies on the detection of *Leishmania* species. This matter is crucial for the successful implementation of treatment and control strategies[6]. Owing to the unavailability of molecular techniques in resource-poor areas, species identification has become unfeasible. As a result, data on the burden of CL are primarily derived from microscopy or clinical descriptions of lesions, such as dry or wet cutaneous lesions[2].

In areas affected by endemic diseases, where the implementation of sophisticated techniques is impractical, a demand arises for a molecular test which is expeditious, while also maintaining a high level of sensitivity and specificity. Moreover, the molecular tests should be designed for laboratory personnel without specialized expertise to ensure their applicability under the demanding conditions[3]. The main molecular methods currently employed are outlined in Table 1, highlighting their benefits and drawbacks.

PCR-based assays are widely utilized as the primary method for detecting and typing *Leishmania* species. In this context, kDNA and ITS fragments are frequently utilized in genomic diagnostic protocols, with kDNA being a commonly targeted region for PCR amplification[10]. The application of PCR is dependent on having the necessary infrastructure and skilled operators. To address this issue, isothermal diagnostic amplification techniques such as NASBA and LAMP have been developed in recent years as an alternative solution[3]. We believe that the use of isothermal diagnostic amplification techniques has the potential to revolutionize the detection of infectious agents in developing countries such as Iran.

CONCLUSION

Diagnosis of leishmaniasis has gained significant advancements with the introduction of numerous molecular methods. Nevertheless, the practical utilization of these techniques in clinical settings and healthcare facilities still remains limited. Emphasis should be placed on initiatives that improve the usability and cost-effectiveness of molecular diagnostic techniques, as well as the development of AI-based platforms in developing countries.

Table 1. An overview of the pros and cons of various molecular diagnostic techniques

Molecular technique	Pros	Cons
Conventional PCR	- High level of accuracy and reliability - High specificity and sensitivity - Clear and straightforward diagnostic analyses	- Time consuming - Qualitative approach - Limited detection range
Nested and semi-nested PCR	- Higher sensitivity and specificity in comparison to conventional PCR - An advantageous approach for studying molecular epidemiology within the field	- More time-consuming and expensive than conventional PCR - Qualitative approach
Multiplex PCR	- Simultaneous detection of target genes - Cost effective (lower amounts of dNTPs, enzymes, and other consumables)	- Reduced sensitivity compared to conventional PCR - Accurate design of primers - Extensive optimization tests
Real-time PCR	- Higher levels of specificity and sensitivity compared to conventional PCR - Numerical potential and rapid results - Differentiation of species by analysis of melting temperature	- Complexity in explaining the results - Requires a highly-trained laboratory staff - Requires expensive equipment and reagents
PCR-ELISA	- Higher levels of specificity and sensitivity compared to conventional PCR - Shorter analytical time and lower detection limit	- Lower accuracy in quantification compared to real-time PCR - Requires sophisticated equipment such as an ELISA plate reader -Expensive reagents
AFLP	- Highly reproducible - No sequence data for primer construction are required - High-resolution genotyping of fingerprinting quality	- Requires pure and high molecular weight DNA - Markers usually scored as dominant - Technically demanding
NASBA	- Higher specificity and rapid results - Isothermal nature - Lower detection limits compared to other molecular techniques - Not required expensive equipment	- Susceptible to degradation by RNases - Sensitive to temperature fluctuations
LAMP	- Rapid, simple, and cost-effective - Isothermal nature - Not required expensive equipment	- Sensitive to cross-contamination - False-positive binding and non-specific binding

DECLARATIONS

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Ethical approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

SEE: conceptualization, data curation, formal analysis, and supervision; MM: data curation, formal analysis, and writing original draft; HM; writing original draft; MMO: review and editing; AK: review and editing.

Data availability

All relevant data can be found within the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Supplementary information

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