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Original Research Article

Identifying the Predominant *Leishmania* Species of Cutaneous Infections in Al-Diwaniyah Governorate / Iraq

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Abstract: ***Purpose***: This study aimed to identify the causative leishmania species of cutaneous leishmaniasis (CL) in Al-Diwaniyah Governorate, a central regin in Iraq using molecular technique. ***Methods*:** Twenty clinically suspected cases were collected from patients who visited Al-Diwaniyah Hospital during the period between January and April 2024. Initial diagnosis was performed using Giemsa stained microscopy and parasite culture on RPMI-1640 medium. Molecular characterization was performed using polymerase chain reaction (PCR) amplification targeting the mitochondrial cytochrome b (*Cyt b*) gene, followed by DNA sequencing and BLAST analysis to determine species identity and genetic relationships. ***Results***: All samples were confirmed to be infected with cutaneous leishmaniasis. Molecular analysis of the *cytochrome b* gene revealed that the isolates were *Leishmania tropica*. With limited nucleotide variation, it did reveal minor amino acid differences, leading to the classification of the isolates into three groups. ***Conclusion***: The results confirm that *Leishmania tropica* is the predominant causative agent of cutaneous leishmaniasis in the study area. The study highlights the importance of molecular diagnostics for accurate identification of the parasite.

Keywords: Cutaneous leishmaniasis, Leishmania tropica, Cytochrome b gene, PCR, Al-Diwaniyah, Molecular diagnosis.

INTRODUCTION

Leishmaniasis is a vector-borne ailment, where the causative agent is the protozoan parasite belonging to the genus Leishmania. The mode of transmission is via the bites of the female sand flies which are assigned to the genera Phlebotomus in the Old World and Lutzomyia in the New World. Clinically, leishmaniasis is characterized by a wide spectrum of manifestations and three distinctive syndromes: a) cutaneous leishmaniasis (CL), the most common form; b) visceral leishmaniasis (VL), the most serious fatal form; and c) mucosal leishmaniasis (ML) [1]. Approximately 350 million people are at risk of contracting the disease and an estimated 1.6 million new cases and 30,000 deaths occur annually [2, 3, 4, 5]. Leishmaniasis disproportionately affects populations living in low socioeconomic conditions, including those with malnutrition, poor housing, immunosuppression, and limited access to healthcare [6-8]. As of November 2024, the endemicity status for leishmaniasis in 2023 reported by the World Health Organization (WHO) Global Leishmaniasis Program indicated 56 CL-endemic countries (62%) and 53 VL-endemic countries (66%). Iraq reported endemic VL with 190 cases and 4,611 reported CL cases in 2023.

In addition to its widespread distribution, accurate diagnosis and species identification of *Leishmania* are critical for effective clinical management and epidemiological surveillance. Conventional diagnostic methods include microscopic examination of Giemsa-stained smears and in vitro parasite culture, which remain widely used due to their simplicity and cost-effectiveness. Microscopy allows visualization of amastigote forms within host macrophages and is considered a primary diagnostic tool; however, its sensitivity depends on parasite load and operator expertise [9, 10].

Despite their utility, conventional methods are limited in their ability to accurately differentiate between *Leishmania* species. Therefore, molecular diagnostic

techniques have become increasingly important. Among these, polymerase chain reaction (PCR)-based methods are considered the most sensitive and specific approaches for detecting and identifying *Leishmania* parasites directly from clinical samples [11, 12]. Molecular targets commonly used include the *cytochrome b* (*Cyt b*) gene, which provides a reliable tool for species identification and phylogenetic analysis [13, 14].

The use of molecular methods is particularly important in endemic regions where multiple *Leishmania* species coexist, such as *Leishmania tropica* and *Leishmania major*, which are the main causative agents of cutaneous leishmaniasis in the Old World. Accurate species identification is essential not only for appropriate treatment selection but also for understanding transmission dynamics and implementing effective control strategies [4].

The present study aimed to identify the causative *Leishmania* species in clinical cases of cutaneous leishmaniasis in Al-Diwaniyah Governorate via molecular methods based on *cytochrome b* (*Cyt b*) gene sequencing.

MATERIALS & METHODS

Culturing

The scraped tissue from each patient was inoculated on the liquid phase of RPMI-1640 (Roswell Park Memorial Institute) medium [15, 16]. The medium was supplemented with 1% glutamine, 10 % fetal calf serum, penicillin 1U/100 ml and streptomycin 1 mg/ 100 ml and 2 mg of nystatin [17]. The parasite growth in terms of promastigotes was visualized by the light microscope after 3 days of incubation.

DNA Extraction

The genomic DNA of the parasite was isolated from the cultured promastigotes of the parasite using Genomic DNA Mini Kit (Blood/Cultured Cell) (Geneaid., Taiwan) according to the instructions of the manufacturers. The purity and concentration of the isolated genomic DNA was evaluated using Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer.

PCR Partial Amplification of *Cyt b* Gene

The *cytochrome b* (*Cyt b*) gene (866 base pairs) was partially amplified in the large circle using polymerase chain reaction (PCR) with the primer set: LCBF1 (5′-GGT GTA GGT TTT AGT TTA GG-3′) and LCBR2 (5′-CTA CAA TAA ACA AAT CAT AAT ATA CAA TT-3′) [18, 19]. The PCR involved 3 µL of each primer, 25 µL of 2X master reaction mix (Geneaid, Taiwan), and 4 µL of genomic DNA, with the final volume completed to 50 µL using nuclease-free water. The conditions of the thermal cycler (Biometra, Analytik Jena, Germany) were programmed as follows: initial separation of the strips at 95°C for 5 minutes, followed by 35 cycles, each consisting of 30 seconds at 95°C, 30 seconds at 52°C, and 45 seconds at 72°C, and then a final extension at 72°C for 7 minutes. Electrophoresis and imaging of the polymerase chain reaction (PCR) product were performed using 1% agarose gel electrophoresis and visualized under UV light using a UV light source (Clever, UK).

Bioinformatics Analysis

The *Cyt b* gene sequence was subjected to the BLASTN similarity search sequence analysis algorithm. Subsequently, some nucleotide sequences that showed a high match with the target nucleotide sequences were selected for multiple sequence alignment (MSA) using MEGA 12.0 software. A phylogenetic tree of the *Cyt b* gene sequences was then constructed.

RESULTS

Detection of Promastigote Form

After 72 hours, a sample of the culture medium was examined microscopically and the presence of the promastigotes stage of the parasite were observed, which characterized by elongated spindle shape, with progressively tapered ends, its length…...µm, the parasites appeared transparent and colorless; however, their distinctive morphology remained clearly visible under routine light microscopy, (Figure 1).

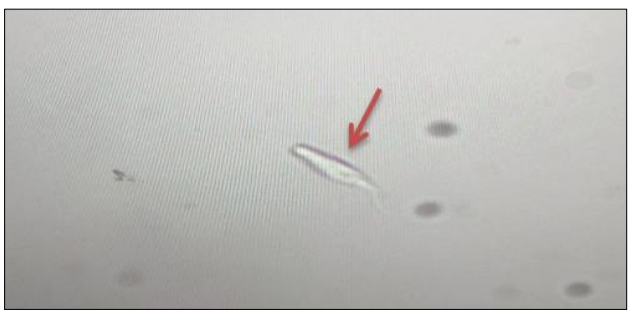

Figure1: Unstained *Leishmania* spp promastigotes under light microscope

Conventional PCR Detection of *Leishmania* spp

The *cytochrome b* (*Cyt b*) gene of all twenty cultured samples of leishmania infected cases included in this study was successfully partially amplified to the expected size of 866 base pairs (Figure 2).

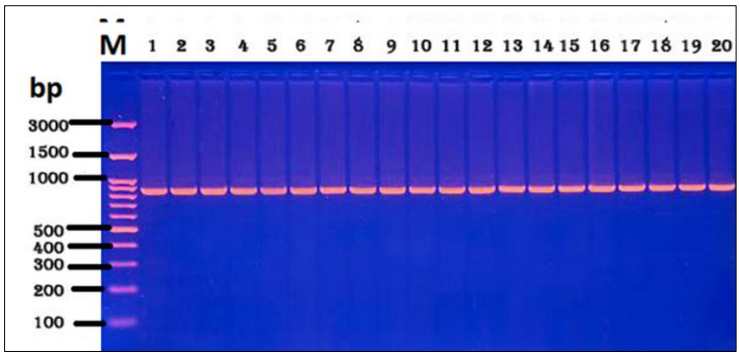

Figure 2: 1.5% agarose gel electrophoresis showing the amplification of *Cyt b* gene. M: DNA marker. Lanes 1-20: PCR products of successfully amplified *Cyt b* gene of *Leishmania* spp.

The obtained 20 nucleotide sequences of *Cyt b* gene PCR products were subjected to BLASTX (program version 2.17.0) similarity sequence search analysis against the non-redundant protein sequences (nr) separately. The output of BLASTX revealed that all isolates nucleotide sequences matched with the protein subject sequence cytochrome b of *Leishmania tropica* with identity ranged from 95.82-96.58%, query coverage of 91%, and e-value ranged from 5.0 X 10-107 – 2.0 X 10-76. Based on the BLASTX outputs, the twenty *Cyt b* gene nucleotide sequences were deposited in the GenBank database of NCBI under accession numbers from PX437961 to PX437972 and from PX437973 to PX437980. Therefore, *Cyt b* nucleotide sequences of the twenty *Leishmania* strains enrolled in this study greatly reinforced the affiliation of these strains to *Leishmania tropica*.

To better understand the phylogenetic relatedness of the twenty *L. tropica* strains in this study, a multiple sequence alignment of their *Cyt b* amino acid sequences was conducted. This analysis included additional selected *Cyt b* protein sequences retrieved from the GenPept database and was performed using the MUSCLE algorithm in MEGA 12.0 software (Figure 3A). Amino acid variations were noted in different sites allover these amino acids sequences. At most, these amino acid variations are allocated at two main types of sites: a) minor single site variations and b) major sequence divergence or frameshifts. The minor single site variations encompassed the amino acid sequences RNQVP1 through RNQVM11 and RNQVM13 through RNQVM19). However, the major sequence divergence or frameshifts included the amino acid sequences RNQVM12 and RNQVM20. For the minor single site variations, for instance, around the positions 24 and 47, there is an amino acid substitution as follow: 24F (phenylalanine) to 24 Y (tyrosine) in *L. tropica* strain RNQVP3 and 47 V (valine) to 47E (glutamic acid) in *L. tropica* strain RNQVP2 (Figure 3A). In stark contradict, the amino acid sequences RNQVM12 and RNQVM20 exhibited a high degree of divergence along the whole length of the two sequences compared to the remaining sequences RNQVP1 through RNQVP7 and RNQVM8-RNQVM11, RNQVM13 through RNQVM19 (Figure 3A). These amino acids variations in RNQVM12 and RNQVM20 appeared earlier in the alignment around positions 20-30. Notably, the two amino acid sequences RNQVM12 and RNQVM20 showed a degree of similarity. In summary, the majority of twenty strains enrolled in this study are nearly identical, but two sequences exhibit a large, localized change, suggesting a translational error or sequencing anomaly.

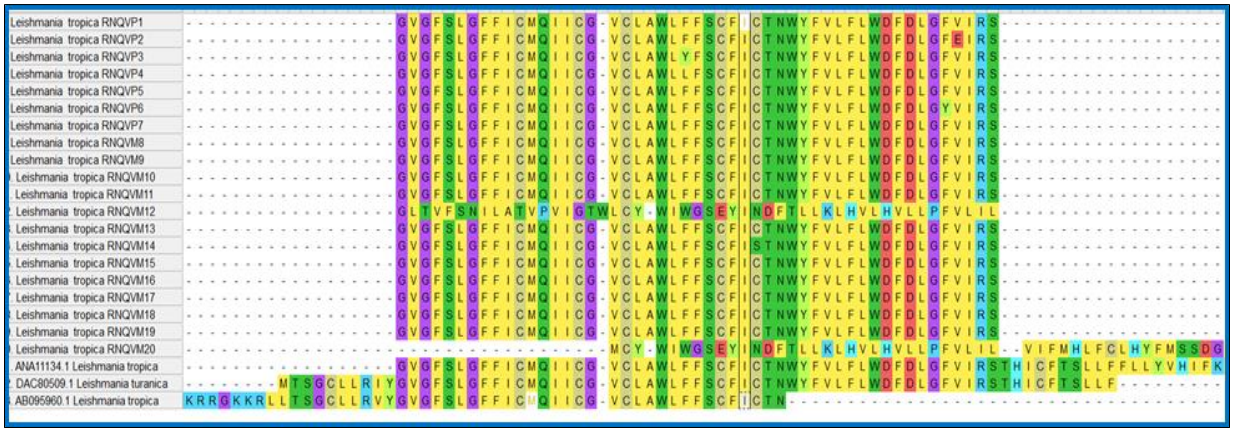

Figure 3A: Multiple amino acid sequence alignment, based on cytochrome b protein sequences, was performed on 20 *L. tropica* strains included in this study and three closely related strains retrieved from the GenPept database. The amino acid sequence alignment reveals amino acid variations (single nucleotide mutations) across 89 sites in all aligned protein sequences. Conserved sites appear in the same color along the sequences, while sites exhibiting amino acid variation appear in a different color.

The multiple sequence alignment was conducted as a base to construct a UPGAMA phylogenetic tree (Figure 3B) not only to unravel the genetic relatedness of the 20 *L. tropica* strains enrolled in this study but also to unveil the evolutionary relationship between the present 20 strains and other nationally and globally *L. tropica* strains. The phylogenetic tree could assign the twenty *L. tropica* strains, enrolled in this study, into 3 clades: clade I-clade III as inferred from Figure 4B. Clade I encompassed *L. tropica* RNQVP2, *L. tropica* RNQVP3, *L. tropica* RNQVP4, *L. tropica* RNQVM12, *L. tropica* RNQVM14, and *L. tropica* RNQVM20. Clade II encompassed *L. tropica* RNQVP5, *L. tropica* RNQVM8, *L. tropica* RNQVM10, *L. tropica* RNQVM13, *L. tropica* RNQVM16, and *L. tropica* RNQVM18. The last clade III involved *L. tropica* RNQVP1, *L. tropica* RNQVP7, *L. tropica* RNQVM9, *L. tropica* RNQVM11, *L. tropica* RNQVM15, *L. tropica* RNQVM107, and *L. tropica* RNQVM19.

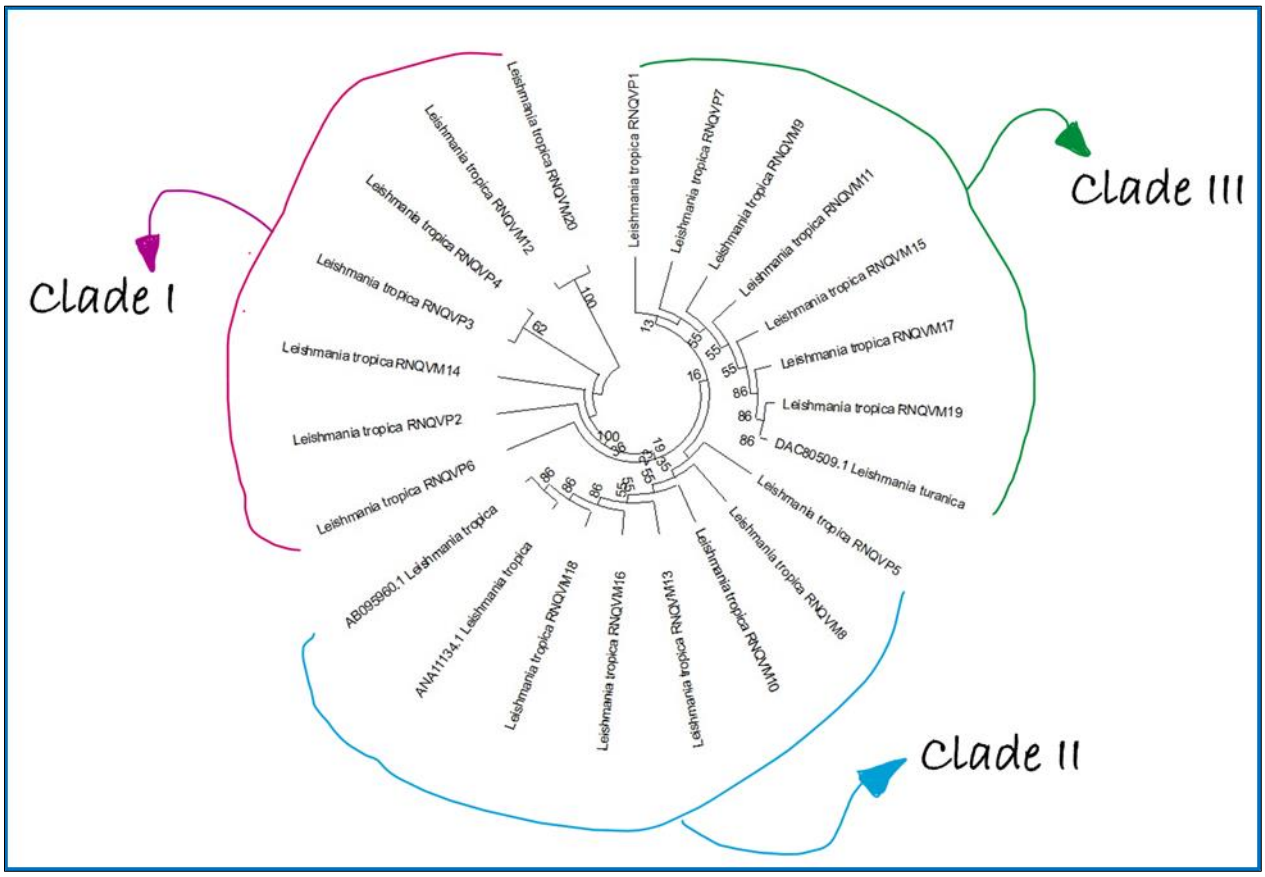

Figure 3B: A phylogenetic tree, based on *cytochrome b* (*Cyt b*) amino acid sequences, was constructed to illustrate the evolutionary relationship of 20 *Leishmania tropica* strains isolated in this study from 20 clinically symptomatic cohorts of cutaneous leishmaniasis, as well as other closely related *Leishmania* strains. The numbers shown on the arms represent the regrouping values of 1000 resampling's. The optimal tree is shown with a sum of branch lengths of 1.572. Evolutionary distances were calculated using Poisson's correction, expressed in units of the number of amino acid substitutions per locus. The analytical procedure included 23 amino acid sequences. The pairwise deletion option was applied to all ambiguous loci in each pair of sequences, resulting in a final dataset of 89 loci. The phylogenetic analyses were performed in MEGA12 software using up to 7 parallel computing threads.

DISCUSSION

In general, polymerase chain reaction (PCR)-based techniques are more sensitive than traditional in vitro culture methods [20, 11]. The studies included in this review have addressed several molecular approaches for identifying the causative agent of leishmaniasis from clinical specimens, particularly cutaneous leishmaniasis, such as *Cyt b* gene sequencing to identify the causative agent in skin lesions from patients suspected of having leishmaniasis. We specifically chose *Cyt b* for this study, although many other molecular methods are available. Identifying the causative *Leishmania* species, down to the genus and species level, is crucial for prescribing appropriate medications, understanding epidemiological issues, and achieving accurate disease diagnosis [14, 12].

This study demonstrated complete agreement with the cytochrome b gene sequence analysis. Specifically, the molecular method confirmed that all 20 isolates were belonged to *L. tropica*. Despite the importance of this step and the availability of numerous molecular techniques, no single approach is currently considered the gold standard for *Leishmania* species identification [21]. The lack of a definitive gold standard for *Leishmania* species classification stems from

insufficient standardization among laboratories, which negatively impacts the interpretation of results. This challenge is further complicated by the critical need to accurately distinguish between *Leishmania* hybrids, both between and within species [22]. The use of the BLAST algorithm for *Leishmania* species identification places a significant burden on inter-species and intra-species differentiation. The algorithm may classify a query sequence into a specific genus and species, but this classification may change if the researcher modifies settings, such as the critical E value. A low E value restricts the definition to a narrow set of species, and the chosen database also influences the outcome. Ultimately, due to factors such as algorithm settings, database selection, and variations in DNA sequence quality, the *Leishmania* definition based on BLAST research is considered dynamic or mosaic [23].

Although *Cyt b* gene sequencing unanimously identified all 20 strains as belonging to the *Leishmania tropica* species, it could not differentiate between them. While *Cyt b* is a well-known polymorphic marker used for species differentiation and some intraspecies differentiation, nucleotide sequencing failed to differentiate the 20 *Leishmania tropica* strains in this study (Figure 2). However, amino acid sequence analysis of the *Cyt b* gene showed slight discriminatory power, grouping the strains into three distinct groups (Figure 3b). Nevertheless, *Cyt b* remains an excellent and proven method for separating the 13 human *Leishmania* species found worldwide [18].

CONCLUSION

This study confirmed that *Leishmania tropica* is the predominant species causing cutaneous leishmaniasis in the studied region of Iraq. Molecular methods, including *Cyt b* gene analysis, proved to be a reliable tool for accurately identifying the parasite and its evolutionary classification.

REFERENCES

1. Murray, H. W., Berman, J. D., Davies, C. R., & Saravia, N. G. (2005). Advances in leishmaniasis. The Lancet, 366, 1561–1577.
2. Alvar J, Yactayo S, Bern C. Leishmaniasis and poverty. Trends Parasitol. 2006; 22:552–7. doi: 10.1016/j.pt.2006.09.004.
3. Mathers, C. D., Ezzati, M., & Lopez, A. D. (2007). Measuring the burden of neglected tropical diseases. PLoS Neglected Tropical Diseases.
4. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. PLoS One. 2012;7: e35671. doi: 10.1371/journal.pone.0035671.
5. Scheufele C. J., Giesey R. L., Delost G. R. The Global, Regional, and National Burden of Leishmaniasis: An Ecologic Analysis from the Global Burden of Disease Study 1990-2017. Journal of the American Academy of Dermatology. 2021;84(4):1203–1205.
6. Cerf, B. J., Jones, T. C., Badaro, R., Sampaio, D., Teixeira, R., & Johnson, W. D. (1987). Malnutrition as a risk factor for severe visceral leishmaniasis. The Journal of infectious diseases, 156(6), 1030-1033.
7. Antinori, S., Cascio, A., Parravicini, C., Bianchi, R., & Corbellino, M. (2008). Leishmaniasis among organ transplant recipients. The Lancet infectious diseases, 8(3), 191-199.
8. Georgiadou SP, Makaritsis KP, Dalekos GN. Leishmaniasis revisited: Current aspects on epidemiology, diagnosis and treatment. J Transl Int Med. 2015 Apr-Jun;3(2):43-50. doi: 10.1515/jtim-2015-0002.
9. Sundar, S., & Rai, M. (2002),Laboratory diagnosis of visceral leishmaniasis. Clinical and Diagnostic Laboratory Immunology, 9(5), 951–958.
10. de Vries, H. J. C., Reedijk, S. H., & Schallig, H. D. F. H. (2015),Cutaneous leishmaniasis: Recent developments in diagnosis and management. American Journal of Clinical Dermatology, 16(2), 99–109.
11. Lachôd, J., et al. (2002). Comparison of PCR-based methods for detection of *Leishmania* parasites.
12. Galluzzi, L., Ceccarelli, M., Diotallevi, A., Menotta, M., Magnani, M., & Diotallevi, A. (2018). Real-time PCR applications for diagnosis of leishmaniasis. Parasites & Vectors, 11, 273.
13. Schönian, G., Nasereddin, A., Dinse, N., Schweynoch, C., Schallig, H. D. F. H., Presber, W., & Jaffe, C. L. (2003),PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. Diagnostic Microbiology and Infectious Disease, 47(1), 349–358.
14. Satow, M. M., Yamashiro-Kanashiro, E. H., Rocha, M. C., Oyafuso, L. K., Soler, R. C., Cotrim, P. C., & Lindoso, J. A. (2013). Applicability of *cytochrome b* gene sequencing for identification of *Leishmania* species. PLoS ONE, 8(2), e55968.
15. Moore, G.E. and Woods L.K., (1976). Culture Media for Human Cells- RPMI 1603, RPMI 1634, RPMI 1640 and GEM 1717. Tissue Culture Association Manual. 3, 503-508.
16. Boggild, A. K., Valencia, B. M., Veland, N., Ramos, A. P., Calderon, F., Arevalo, J., Low, D. E., Llanos-Cuentas, A., & Kain, K. C. (2010). Detection and species identification of *Leishmania* parasites from clinical specimens using molecular methods. Journal of Clinical Microbiology, 48(7), 2575–2581.
17. Paun, A. (2022). Culture of cutaneous *Leishmania* from skin biopsy specimens. Current Protocols, 2(2), e367.
18. Luyo-Acero, G., Uezato, H., Oshiro, M., Kariya, K., Katakura, K., Gomez-Landires, E., Hashiguchi, Y., & Nonaka, S. (2004). Sequence variation of the cytochrome b gene of *Leishmania* species and its application for species identification. Parasitology, 128(5), 483–491.
19. Mohammedpour, G., et al. (2019). Molecular identification and phylogenetic analysis of

Leishmania species using the cytochrome b gene. Iranian Journal of Parasitology.
20. Costa, J. M., Durand, R., Deniau, M., Rivollet, D., Izri, A., Houin, R., Vidaud, M., & Bretagne, S. (1996). PCR enzyme-linked immunosorbent assay for diagnosis of leishmaniasis. Journal of Clinical Microbiology, 34(7), 1831–1833.
21. Gow, I., et al. (2019). Molecular approaches for the identification and classification of *Leishmania* species.
22. Brodskyn, C., et al. (2019). Advances in the understanding of *Leishmania* biology and host–parasite interactions. Frontiers in Cellular and Infection Microbiology.
23. van der Auwera, G., & Dujardin, J. C. (2016). Species typing in dermal leishmaniasis. Clinical Microbiology Reviews, 29(4), 816–845.