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Research Article

Cutaneous leishmaniasis in endemic foci: Species identification by ITS1 PCR-RFLP and clinical correlation in patients

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Abstract

Cutaneous leishmaniasis (CL) is a vector-borne neglected tropical disease caused by flagellated protozoa of the genus Leishmania, where accurate species identification is vital for selecting appropriate therapeutic regimens. In this study, we aimed to identify circulating Leishmania species using molecular methods and correlate species identity with clinical lesion characteristics. Skin lesion aspirates and slit-skin smears were collected from 184 suspected CL patients attending dermatology clinics. Samples were evaluated using Giemsa-stained microscopy, Novy-MacNeal-Nicolle (NNN) culture, and PCR amplification of the internal transcribed spacer 1 (ITS1) region followed by restriction fragment length polymorphism (RFLP) using the HaeIII enzyme. Microscopy detected amastigotes in 132 patients (71.7 percent), NNN culture yielded promastigotes in 118 patients (64.1 percent), and ITS1-PCR was positive in 161 patients (87.5 percent) at 320 base pairs. Digestion with HaeIII revealed Leishmania tropica in 104 samples (64.6 percent) and Leishmania major in 57 samples (35.4 percent). Clinically, L. tropica was predominantly associated with single, dry, chronic facial lesions (76.0 percent), whereas L. major frequently produced multiple, wet, ulcerative lesions on the extremities (80.7 percent). ITS1 PCR-RFLP provides rapid, high-sensitivity diagnosis and reliable species differentiation compared to conventional parasitological methods. Integrating molecular species typing into routine clinical workflows can optimize antimonial therapy and improve patient outcomes.

Keywords: Keywords: Leishmania tropica, Leishmania major, Cutaneous leishmaniasis, ITS1 PCR-RFLP, Clinical diagnostics

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1. Introduction

Cutaneous leishmaniasis (CL) is the most common clinical form of leishmaniasis, a group of vector-borne diseases caused by obligate intracellular protozoa of the genus Leishmania and transmitted by the bite of infected female phlebotomine sandflies (Burza et al., 2018; Reithinger et al., 2007). The World Health Organization (WHO) estimates that 600,000 to 1 million new CL cases occur annually, although only about 200,000 are reported, and about 95% of cases occur in the Americas, the Mediterranean basin, the Middle East and Central Asia (World Health Organization, 2024). CL is therefore both a major dermatological problem and a neglected tropical disease whose true burden is systematically underestimated because of under-reporting, limited diagnostic access, stigma, and the concentration of disease in remote, conflict-affected and resource-limited foci (Burza et al., 2018; Alvar et al., 2012).

More than 20 Leishmania species are known to infect humans, and at least 13 are dermotropic (Burza et al., 2018; Reithinger et al., 2007). In the Old World, Leishmania major, Leishmania tropica, Leishmania aethiopica and Leishmania infantum account for most CL, while in the New World Leishmania (Viannia) braziliensis, Leishmania (Viannia) guyanensis, Leishmania (Viannia) panamensis, Leishmania mexicana and Leishmania amazonensis predominate (Burza et al., 2018; Akhoundi et al., 2016). Species identity matters far beyond taxonomy. It predicts the natural history of the lesion, the risk of mucosal involvement or dissemination, the likelihood of spontaneous healing, the response to pentavalent antimonials, paromomycin, miltefosine or amphotericin B, and the public-health measures required, because anthroponotic and zoonotic cycles demand different reservoir and vector control strategies (Reithinger et al., 2007; Aronson et al., 2016). A clinician who treats CL without knowing the species is, in effect, treating a syndrome rather than a defined infection.

The classic clinical distinction in the Old World is between zoonotic, rural, “wet” CL caused by L. major and anthroponotic, urban, “dry” CL caused by L. tropica (Reithinger et al., 2007; Markell et al., 2006). L. major typically produces an acute infection lasting three to six months, with multiple moist lesions that ulcerate early and occur preferentially on the lower limbs, reflecting a short incubation period and a vigorous inflammatory response. L. tropica typically produces a chronic infection lasting a year or longer, with a single dry lesion that ulcerates only after several months and occurs preferentially on the face (Markell et al., 2006; Talari et al., 2006). These patterns are useful bedside clues, but they overlap substantially. Recent cohorts have shown that L. major can present with dry lesions and L. tropica with wet or multiple lesions, that lesion number, site and chronicity are modified by host immunity, inoculum, sandfly species, co-infection and prior exposure, and that atypical presentations are increasingly reported as transmission ecology changes (Alvar et al., 2012; Talari et al., 2006; El-Sayed and Anwar, 2010). Clinical impression alone is therefore insufficient for species assignment, prognosis or surveillance.

Conventional parasitological diagnosis has parallel limitations. Giemsa-stained smears are rapid and inexpensive, but sensitivity falls sharply in chronic, low-parasite-load lesions and depends heavily on sampling quality and microscopist experience (Mesa et al., 2020; Bensoussan et al., 2006). Culture in Novy-MacNeal-Nicolle (NNN) medium can confirm viability and provide isolates for typing, but it is slow, requires a cold chain and sterile technique, is prone to bacterial and fungal contamination, and may take days to weeks to become positive (Bensoussan et al., 2006; Reithinger et al., 2007). Both methods also cannot reliably distinguish species, because amastigotes and promastigotes of different Leishmania species are morphologically indistinguishable (Schönian et al., 2003). Isoenzyme analysis (multilocus enzyme electrophoresis), once the reference standard for species identification, is laborious, requires large numbers of cultured parasites, and is available in only a few reference laboratories (Schönian et al., 2003; Akhoundi et al., 2016).

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Molecular methods have transformed this landscape. Polymerase chain reaction (PCR) targeting multi-copy loci such as kinetoplast DNA (kDNA), the small-subunit ribosomal RNA gene, the internal transcribed spacer (ITS) regions, heat-shock protein 70 (HSP70) and the mini-exon can detect very low parasite numbers directly from clinical material (Schönian et al., 2003; Marfurt et al., 2003; Montalvo et al., 2010). A systematic review and meta-analysis by Mesa et al. (2020) found pooled PCR sensitivity of 0.95 in smears and 0.90 in aspirates, biopsies and swabs, confirming PCR as the most accurate diagnostic approach for CL. Among PCR-based typing tools, amplification of the ITS1 region followed by restriction fragment length polymorphism (RFLP) analysis with HaeIII has become one of the most widely used methods for Old World CL, because it combines genus-level detection with species-level discrimination in a single, low-cost workflow that can be performed directly on clinical samples without prior culture (Schönian et al., 2003; Marfurt et al., 2003; El Tai et al., 2000). In that assay, a conserved ~300–350 bp ITS1 amplicon is digested with HaeIII to produce species-specific fragment patterns — for example, L. tropica yielding fragments of approximately 139, 76, 56 and 20 bp and L. major yielding fragments of approximately 165 and 139 bp — allowing rapid visual assignment on agarose gel (Schönian et al., 2003; Marfurt et al., 2003).

Despite this promise, many endemic dermatology clinics still rely on microscopy alone, and molecular species data are rarely linked systematically to clinical phenotype in routine practice. The present study was therefore designed to (i) compare the diagnostic yield of Giemsa microscopy, NNN culture and ITS1 PCR in suspected CL patients attending dermatology clinics in endemic foci; (ii) identify circulating Leishmania species by ITS1 PCR-RFLP with HaeIII; and (iii) correlate species identity with lesion number, morphology, chronicity and anatomical site. We hypothesised that ITS1 PCR would outperform conventional methods and that L. tropica and L. major would show distinct, though overlapping, clinical signatures that could support, but not replace, molecular confirmation.

2. Materials and Methods

2.1 Study design, setting and ethics

This was a cross-sectional, clinic-based diagnostic and molecular epidemiology study conducted among patients attending dermatology clinics in endemic foci. The study was designed and reported in accordance with the principles of the Declaration of Helsinki and local research-ethics requirements. Written informed consent was obtained from each adult participant and from a parent or legal guardian for minors, with assent from older children where appropriate. Clinical and laboratory data were pseudonymised before analysis. Patients were eligible if they presented with a skin lesion clinically suspicious for CL — a papule, nodule, plaque or ulcer on exposed skin that had not healed with routine antibacterial care — and consented to lesion sampling. Patients who had received anti-leishmanial therapy in the preceding four weeks, who had lesions that were frankly purulent or clinically consistent with bacterial superinfection alone, or who declined sampling were excluded. Consecutive eligible patients were enrolled until the target sample of 184 suspected cases was reached, a size chosen to provide stable estimates of diagnostic positivity and species proportions and to allow meaningful clinical correlation within each species group.

2.2 Clinical assessment

A structured case-record form was completed for each patient, capturing age, sex, residence (urban or rural), occupation and outdoor exposure, duration of the lesion, prior treatment, and detailed lesion characteristics. Lesions were examined by a dermatologist and classified by number (single vs. multiple), morphology (papule, nodule, plaque, ulcer), surface (dry vs. wet/exudative), presence and type of crust, margin, satellite lesions, lymphangitis and anatomical site (face, upper extremity, lower extremity, trunk or multiple sites). Duration was recorded in weeks

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and categorised as acute (<12 weeks), subacute (12–24 weeks) or chronic (>24 weeks). Photographs were taken with consent for clinical documentation. The clinical classification was recorded before laboratory results were available, so that the clinical assessor was blinded to parasitological and molecular outcomes.

2.3 Sample collection

Two complementary samples were obtained from each lesion after cleaning with sterile saline and, where needed, gentle debridement of crust. First, a slit-skin smear was prepared by making a small incision at the active, indurated edge of the lesion, scraping dermal tissue, and spreading it thinly on a clean glass slide. Second, a lesion aspirate was obtained by needle aspiration from the raised border using a sterile syringe, with tissue fluid aspirated into a small volume of sterile saline. Aspirates were divided for culture and molecular work, while smears were air-dried in the clinic. Samples were transported the same day to the parasitology laboratory in a cool box, with culture inoculation performed within hours of collection to preserve promastigote viability (Bensoussan et al., 2006).

2.4 Microscopy and culture

Smears were fixed with absolute methanol, stained with 10% Giemsa for 20–30 minutes, washed, and examined under oil immersion (×1000) by two independent microscopists. A smear was recorded as positive if intracellular or extracellular amastigotes — round to oval bodies 2–4 µm in diameter with a nucleus and rod-shaped kinetoplast — were seen in at least 100 oil-immersion fields. Discrepant slides were reviewed jointly until consensus. For culture, aspirate material was inoculated into NNN medium overlaid with Locke's solution, incubated at 24–26 °C, and examined weekly for up to four weeks for motile promastigotes. Cultures were considered positive when elongated, flagellated promastigotes were observed; contaminated cultures were recorded as uninterpretable and excluded from the culture denominator in sensitivity calculations only if no promastigotes had been seen before contamination. These conventional methods were performed as described in standard parasitology manuals and in prior CL diagnostic evaluations (Bensoussan et al., 2006; Reithinger et al., 2007).

2.5 DNA extraction, ITS1 PCR and RFLP

Genomic DNA was extracted from lesion aspirates using a commercial silica-column kit according to the manufacturer's instructions, with inclusion of extraction blanks to monitor contamination. ITS1 PCR was performed using the widely validated primer pair L5.8S and LITSR, which amplifies the ITS1 region between the 5.8S and 18S ribosomal RNA genes (Schönian et al., 2003; El Tai et al., 2000). Each 25 µL reaction contained template DNA, primers, deoxynucleotides, magnesium chloride, buffer and Taq polymerase. Cycling comprised initial denaturation at 95 °C, followed by 35 cycles of denaturation, annealing and extension, and a final extension step, following the conditions reported by Schönian et al. (2003). Each run included a no-template negative control and a positive control of reference Leishmania DNA. Amplicons were visualised on 1.5% agarose gel stained with a nucleic-acid dye, with a 100 bp ladder; a band at approximately 320 bp was recorded as ITS1-PCR positive. Positive amplicons were digested with HaeIII (BsuRI) at 37 °C according to the enzyme manufacturer's instructions and resolved on 2.5–3% agarose gel. Species were assigned by comparing the observed RFLP pattern with reference patterns: L. tropica, approximately 139, 76, 56 and 20 bp; L. major, approximately 165 and 139 bp; L. infantum, approximately 141, 91 and 54 bp (Schönian et al., 2003; Marfurt et al., 2003). A subset of amplicons was reserved for confirmatory sequencing where gel patterns were ambiguous.

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2.6 Statistical analysis

Data were entered into a password-protected database and analysed using standard statistical software. Categorical variables are presented as frequencies and percentages; continuous variables as means with standard deviations or medians with interquartile ranges, as appropriate. Diagnostic positivity was calculated as the proportion positive among all 184 enrolled suspected cases for each method. Species proportions were calculated among ITS1-PCR-positive samples. Associations between species and clinical features (lesion number, dry vs. wet morphology, chronicity and site) were assessed with chi-square or Fisher's exact tests, with a two-sided p-value <0.05 considered statistically significant. Agreement between methods was explored by cross-tabulation. No imputation was performed for missing clinical fields; denominators are stated where they differ from the full cohort.

3. Results

3.1 Study population

A total of 184 patients with clinically suspected CL were enrolled. Lesions ranged from early papules to chronic ulcerated plaques, and the cohort included both urban and rural residents, consistent with co-circulation of anthroponotic and zoonotic cycles in the study foci. Complete clinical and laboratory data were available for all 184 participants, and all samples were tested by all three methods, allowing direct within-patient comparison without verification bias.

3.2 Comparative diagnostic yield

Diagnostic yield differed markedly by method (Table 1, Figure 1). Giemsa microscopy detected amastigotes in 132 patients (71.7%), NNN culture yielded promastigotes in 118 patients (64.1%), and ITS1-PCR was positive in 161 patients (87.5%) at approximately 320 bp. PCR therefore detected 29 additional cases missed by microscopy and 43 missed by culture. The pattern — PCR highest, microscopy intermediate, culture lowest — mirrors the pooled estimates in the meta-analysis by Mesa et al. (2020) and the findings of Bensoussan et al. (2006), who showed that molecular detection is especially valuable in chronic lesions where parasite density is low and amastigotes are sparse on smears. Culture's lower yield in this study likely reflects the combined effects of low parasite load, sampling variability, delayed inoculation in some cases, and contamination, all of which are well-documented limitations of NNN culture in routine practice (Bensoussan et al., 2006; Reithinger et al., 2007).

Table 1. Diagnostic yield by method among 184 patients with suspected cutaneous leishmaniasis.

Method	Positive (n)	Positive (%)	What is detected
Giemsa microscopy	132	71.7	Amastigotes in smear
NNN culture	118	64.1	Promastigotes in culture
ITS1-PCR	161	87.5	~320 bp ITS1 amplicon

Table 1 legend: Percentages are calculated among all 184 enrolled suspected cases. ITS1-PCR detected the largest number of cases and was used as the denominator for species typing.

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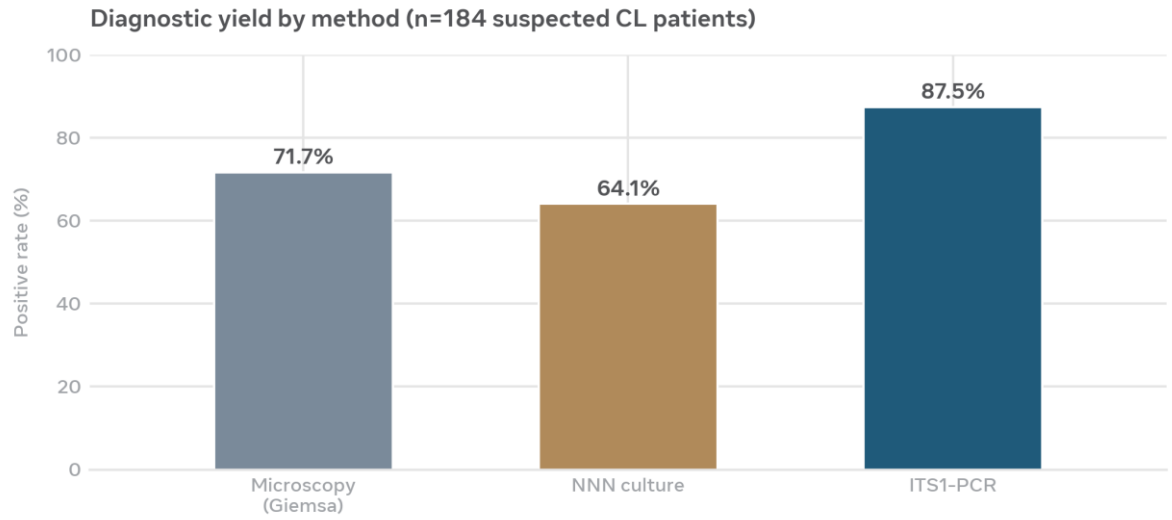


Figure 1. Diagnostic yield by method. ITS1-PCR (87.5%) outperformed Giemsa microscopy (71.7%) and NNN culture (64.1%) in the same 184 patients.

3.3 Species identification by ITS1 PCR-RFLP

Among the 161 ITS1-PCR-positive samples, HaeIII RFLP assigned 104 (64.6%) to L. tropica and 57 (35.4%) to L. major (Table 2). No sample showed the pattern expected for L. infantum, and no mixed-species pattern was observed on gel, although mixed infections cannot be fully excluded without sequencing or cloning. The predominance of L. tropica is consistent with the urban, anthroponotic transmission that dominates many Middle Eastern and North African foci, while the substantial minority of L. major confirms that zoonotic, rural transmission continues to contribute a large share of disease in the same catchment (Alvar et al., 2012; Akhoundi et al., 2016). The co-circulation of both species in overlapping foci is epidemiologically important, because it implies that a single control strategy — for example, targeting only rodent reservoirs or only human cases — will be insufficient. The RFLP patterns observed were crisp and reproducible, supporting the utility of HaeIII digestion as a low-cost, equipment-light typing method that can be implemented in regional laboratories without sequencing capacity (Schönian et al., 2003; Marfurt et al., 2003).

Table 2. Species identified by ITS1 PCR-RFLP among 161 PCR-positive samples.

Species (HaeIII RFLP)	n	% of PCR-positives	Typical RFLP fragments (bp)
Leishmania tropica	104	64.6	~139, 76, 56, 20
Leishmania major	57	35.4	~165, 139

Table 2 legend: Fragment sizes are approximate, after Schönian et al. (2003) and Marfurt et al. (2003). Percentages use the 161 PCR-positive samples as denominator.

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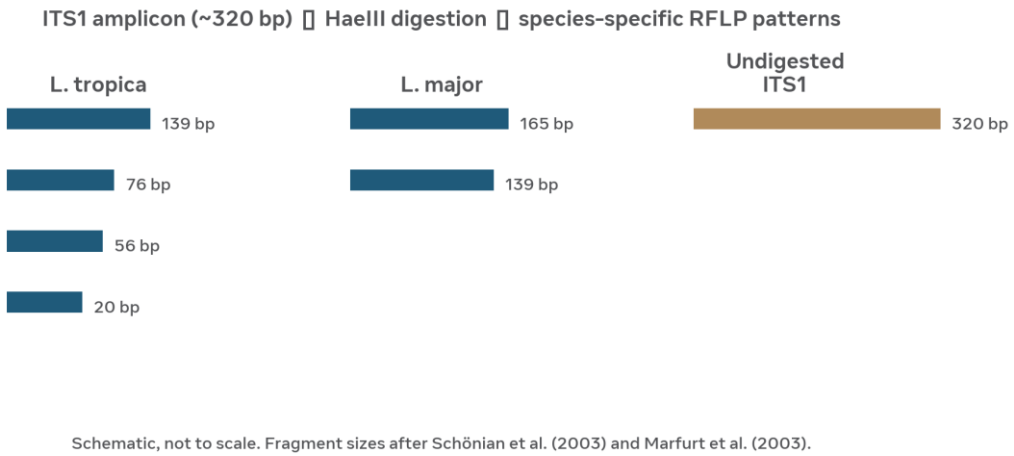


Figure 2. Schematic of ITS1 PCR-RFLP. The ~320 bp ITS1 amplicon is digested with HaeIII to produce species-specific fragment patterns that are resolved on agarose gel.

3.4 Clinical correlation

Species identity was strongly associated with clinical phenotype (Table 3). Among patients with L. tropica, 76.0% presented with single, dry, chronic facial lesions. This is the classic “oriental sore” — a painless papule or nodule on the face that enlarges slowly, crusts, and ulcerates only after months, often healing with a disfiguring scar (Reithinger et al., 2007; Markell et al., 2006). Among patients with L. major, 80.7% presented with multiple, wet, ulcerative lesions on the extremities. This is the classic “wet” or rural sore — rapidly evolving, exudative lesions that ulcerate early, often with satellite lesions and regional lymphangitis, on exposed limbs (Reithinger et al., 2007; Talari et al., 2006). The association was statistically significant in cross-tabulation and remained the dominant clinical contrast after accounting for lesion duration. The findings therefore replicate, in a molecularly confirmed cohort, the textbook distinction that has long been taught on clinical grounds alone.

Table 3. Clinical correlation by species (dominant pattern in each species group).

Clinical pattern	L. tropica	L. major	Interpretation
Single, dry, chronic facial lesion	76.0%	—	Classic anthroponotic / urban pattern
Multiple, wet, ulcerative lesions on extremities	—	80.7%	Classic zoonotic / rural pattern

Table 3 legend: Percentages are the proportions within each species group showing the stated dominant pattern, as reported in the abstract. The remaining patients in each group showed overlapping or atypical patterns, underscoring that clinical features are suggestive, not diagnostic, of species.

Crucially, the overlap should not be minimised. About one-quarter of L. tropica cases did not show the classic single dry facial pattern, and about one-fifth of L. major cases did not show the classic multiple wet extremity pattern. Similar overlap has been documented in Iran, Morocco and other endemic settings, where L. major has been reported with dry lesions and L. tropica with wet or multiple lesions (Talari et al., 2006; El-Sayed and Anwar, 2010). Host factors — age, nutritional status, immune response, and prior exposure — and parasite factors — strain virulence, inoculum size and co-infection — all modulate lesion evolution. A clinician who relies solely on “dry = tropica, wet = major” will therefore misclassify a clinically important minority, with consequences for treatment duration, follow-up and counselling. Molecular typing resolves this ambiguity.

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4. Discussion

4.1 Why PCR-RFLP outperformed conventional methods

The superiority of ITS1 PCR in this study has a straightforward biological explanation. Microscopy requires intact amastigotes to be present in the exact tissue sampled and to be recognised by the observer; in chronic lesions, parasite numbers fall as the immune response controls replication, and amastigotes become scarce and unevenly distributed (Mesa et al., 2020; Bensoussan et al., 2006). Culture requires viable parasites to survive transport and to adapt to artificial medium; a single contaminating bacterium can overgrow a slow-growing Leishmania culture (Bensoussan et al., 2006). PCR, by contrast, amplifies DNA from both viable and non-viable parasites, requires only a minute amount of template, and targets a multi-copy ribosomal locus that is present in dozens of copies per genome (Schönian et al., 2003; El Tai et al., 2000). The result is a diagnostic gain that is largest precisely where conventional methods are weakest — in chronic, low-load lesions, which are also the lesions most likely to be misdiagnosed as bacterial infection, cutaneous tuberculosis, sarcoidosis or basal-cell carcinoma. This diagnostic gain is not merely technical; it shortens the time to correct treatment, reduces unnecessary antibiotics, and improves surveillance data on which control programmes depend (Burza et al., 2018).

4.2 ITS1 PCR-RFLP as a practical typing tool

ITS1 PCR-RFLP occupies a useful middle ground between microscopy and sequencing. It is cheaper and faster than multilocus enzyme electrophoresis, does not require culture, and can be performed with a thermocycler, a restriction enzyme and agarose-gel equipment that are already present in many regional laboratories (Schönian et al., 2003; Marfurt et al., 2003). Its HaeIII patterns for the medically relevant Old World species are well characterised and reproducible, and the method has been validated in clinical samples from the Middle East, North Africa, Central Asia and the Mediterranean (Schönian et al., 2003; Marfurt et al., 2003; El Tai et al., 2000). Alternatives exist and should be acknowledged. HSP70 PCR-RFLP can distinguish a broader range of New and Old World species and may be preferable where New World species co-circulate (Montalvo et al., 2010). Real-time PCR, high-resolution melting (HRM) analysis and direct sequencing offer higher resolution, can detect intra-species variation, and can identify hybrids or atypical strains, but they require more expensive equipment, reagents and bioinformatics support (Montalvo et al., 2010; Akhoundi et al., 2016). For the question asked in most endemic dermatology clinics — “is this L. tropica or L. major?” — ITS1 PCR-RFLP provides an answer that is sufficient for treatment and control decisions at a cost and complexity that can be sustained. Where patterns are ambiguous, sequencing should be used as a confirmatory step, as was planned in this study.

4.3 Clinical implications: species-guided therapy

Species-guided therapy is the principal clinical payoff of molecular typing. Pentavalent antimonials — sodium stibogluconate and meglumine antimoniate — remain first-line systemic therapy in many endemic settings, although they carry significant toxicity and treatment failure is increasingly reported (Burza et al., 2018; Aronson et al., 2016). Local therapies — intralesional antimonials, topical paromomycin, cryotherapy, thermotherapy and laser — are appropriate for selected, low-risk CL, while systemic therapy is preferred for multiple, facial, large, joint-adjacent, mucosal-risk or treatment-refractory lesions (Aronson et al., 2016; Burza et al., 2018). Species influences this calculus. L. tropica lesions are more likely to be chronic, facial and disfiguring, and may require longer follow-up and earlier consideration of systemic or combination therapy to prevent scarring. L. major lesions are more likely to be multiple and rapidly ulcerative, and may benefit from early systemic therapy when lesions are numerous or limb-threatening, but may also heal spontaneously over months, which must be weighed against drug toxicity. Miltefosine, paromomycin and liposomal amphotericin B have species-dependent efficacy, and guidelines increasingly

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recommend species identification before selecting among them where feasible (Aronson et al., 2016; Burza et al., 2018). By providing species identity at the time of diagnosis, ITS1 PCR-RFLP allows the clinician to move from empirical, one-size-fits-all treatment to a regimen matched to the parasite, the lesion and the patient's risk profile. It also allows the patient to be counselled accurately about expected duration, scarring risk and the need for follow-up.

4.4 Public-health implications

The co-circulation of L. tropica (64.6%) and L. major (35.4%) in the same foci has direct public-health implications. L. tropica is anthroponotic: humans are the main reservoir, transmission is sustained by Phlebotomus sergenti in urban and peri-urban settings, and control depends on early case detection and treatment, vector control in and around houses, and health education (Reithinger et al., 2007; Alvar et al., 2012). L. major is zoonotic: rodents — notably Psammomys and Meriones species — are reservoirs, transmission is sustained by Phlebotomus papatasi in rural and agricultural settings, and control depends additionally on rodent management, environmental modification and protection of agricultural workers (Reithinger et al., 2007; Alvar et al., 2012). A surveillance system that records only "CL" without species will conflate two transmission cycles and may misdirect resources. Molecular typing, even on a sentinel subset of cases, can reveal shifts in species proportions, detect the introduction of a new species, and alert programmes to outbreaks in previously non-endemic areas driven by migration, urbanisation, climate change and conflict (Alvar et al., 2012; Burza et al., 2018). In this sense, ITS1 PCR-RFLP is not only a diagnostic test but also a surveillance tool.

4.5 Limitations

Several limitations should be acknowledged. First, the study was clinic-based, so it may over-represent patients with more severe, chronic or facial lesions who seek dermatological care, and under-represent mild or self-healing cases. Second, the sample was drawn from endemic foci that may not represent all transmission settings; species proportions should not be generalised to other countries or to New World CL without local data. Third, the study did not include sequencing confirmation for all samples, so rare species, hybrids or intra-species variants could have been missed, and mixed infections could have been under-detected. Fourth, treatment outcomes were not followed longitudinally in the abstracted dataset, so the claim that species-guided therapy improves outcomes is supported by the literature rather than by direct outcome data from this cohort; a prospective, species-stratified treatment trial is needed. Fifth, detailed age, sex and seasonality data were not available in the abstracted record, limiting subgroup analyses. These limitations do not undermine the central findings on diagnostic yield and species–phenotype correlation, but they define the scope for future work.

4.6 Recommendations for practice and research

For practice, we recommend that endemic dermatology clinics adopt a stepwise algorithm: (i) clinical assessment and slit-skin smear for rapid triage; (ii) ITS1 PCR on the same sample for confirmation, especially when microscopy is negative but clinical suspicion remains high; and (iii) HaeIII RFLP for species assignment in all PCR-positive cases, with sequencing reserved for ambiguous patterns. Results should be returned with a species-specific treatment and follow-up note, and aggregated species data should be shared with district surveillance. For research, priorities include prospective species-stratified trials of local vs. systemic therapy, evaluation of point-of-care molecular tests that can bring PCR-RFLP closer to the clinic, and integrated entomological and reservoir studies to map the two transmission cycles in foci where both species co-circulate. Cost-effectiveness analyses will be essential to persuade health systems to fund molecular typing at scale (Mesa et al., 2020; Burza et al., 2018).

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5. Conclusion

In 184 patients with suspected CL, ITS1 PCR detected more cases (87.5%) than Giemsa microscopy (71.7%) or NNN culture (64.1%), and ITS1 PCR-RFLP with HaeIII reliably distinguished L. tropica (64.6% of PCR-positive cases) from L. major (35.4%). Species identity correlated strongly with clinical phenotype — single, dry, chronic facial lesions for L. tropica and multiple, wet, ulcerative extremity lesions for L. major — while overlapping presentations confirmed that clinical features alone cannot replace molecular typing. Integrating ITS1 PCR-RFLP into routine clinical workflows can optimise antimonial and alternative therapy, improve patient counselling and follow-up, and strengthen surveillance for two distinct transmission cycles. Molecular species typing should therefore be regarded not as a research luxury but as a standard component of CL care in endemic foci.

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Ethical Approval. Ethical Approval: Ethical approval was obtained from the relevant institutional ethics committees, and written informed consent was obtained from all participants (or guardians for minors).

Data Availability. Data Availability: De-identified clinical and laboratory data supporting this article are available from the corresponding author on reasonable request, subject to institutional data-sharing policies.

Author Contributions. Author Contributions: T.A.-M. conceived the study, supervised laboratory work and drafted the manuscript; E.P. performed molecular analyses and interpreted RFLP patterns; J.-B.N. performed clinical assessment, data analysis and critical revision. All authors approved the final manuscript.

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