J Vector Borne Dis 57, March 2020, pp. 71–77

Molecular identification of *Leishmania tropica* and *L. infantum* isolated from cutaneous human leishmaniasis samples in central Morocco

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ABSTRACT

Background & objectives: Cutaneous leishmaniasis (CL) in Marrakesh-Safi region located in the central-south part of Morocco is a public health problem. This study assessed the efficiency of a microscopic examination method in establishing the diagnosis of CL and PCR for the characterization and identification of the circulating *Leishmania* strains in different CL foci of the study area.

Methods: A total of 297 smears obtained from cutaneous lesions of suspected patients with CL were stained with May-Grünwald Giemsa (MGG) for microscopic examination. For each positive smear, genomic DNA was extracted and PCR-analysed, targeting the small subunit ribosomal ribonucleic acid (*ssu rRNA*) gene to detect *Leishmania* DNA. Then, the internal transcribed spacer 1 (ITS1) was amplified and sequenced in order to identify the *Leishmania* species. The sensitivity and specificity of the conventional microscopy with *ssu rRNA* gene were compared by *Leishmania* nested PCR (LnPCR) and *ITS1* gene by ITS-PCR.

Results: A total of 257 smears were positive in the microscopic examination, *i.e*. the detection rate of amastigotes by optical microscopy was 86.53% (257/297). The LnPCR was found to have a specificity and a sensitivity of 100%, each. Interestingly, the sequencing results showed that 99.61% (256/257) of the isolates had *Leishmania tropica* and 0.39% (1/257) had *L. infantum* infection.

Interpretation & conclusion: Though, classical microscopic examination is useful and economical, it is not sensitive enough, especially in endemic regions where several *Leishmania* species coexist. In such situations, PCR constitutes a complementary method for the identification of the causal species. The results indicate that both the *L. tropica* (dominant) and *L. infantum* are the causative agents of CL in the Marrakesh-Safi region. The rate of CL infection is high in Imintanout, and Chichaoua provinces. Hence, early diagnosis and prompt treatment of CL patients is necessary to prevent its extension to neighboring localities.

Key words Cutaneous leishmaniasis; ITS-PCR; *Leishmania infantum; L. tropica;* LnPCR; microscopic examination; Morocco

INTRODUCTION

Cutaneous leishmaniasis (CL) is an endemic parasitic disease that occurs in 87 tropical and subtropical countries (20 in the new world and 67 in the old world); some 95% of the cases occur in the Americas, the Middle East, Central Asia, and the Mediterranean Basin. An estimated 500,000 to 1,000,000 new cases occur worldwide each year[1].

In Morocco, *Leishmania tropica, L. major*, and *L. infantum* may all cause CL[2], which is recognized as a major public health problem. Diagnosis is mostly based on clinical features and the detection of amastigotes in May-Grünwald Giemsa (MGG)-stained slit-skin smears. However, amastigote form is morphologically similar for all *Leishmania* species; making the microscopic examination method insufficient to identify the causative agent of the disease[3–4], while, the species identification is crucial for prognostic, epidemiological, and therapeutic reasons[5].

In fact, clinicians are commonly reduced to using the geographic distributions of the different species for making identifications. Unfortunately, this method is becoming ever more unreliable as the ranges of *L. tropica* and *L. infantum* in Morocco have expanded over recent decades. For example, in Chichaoua, Al Haouz and Essaouira, the localities in the Marrakesh-Safi region of central Morocco, are known to be endemic for *L. tropica*-induced CL in humans[6–8], but *L. infantum* has now been reported to cause sporadic cases of visceral leishmaniasis (VL)[9–10]. In the same area, *L. infantum*-induced canine leishmaniasis was reported[11], while *L. tropica* and *L.*

infantum infections were identified in rodents[12]. It is, therefore, necessary to determine whether the latter species is also responsible for CL in this region.

When available, molecular techniques are the gold standard for identifying *Leishmania* species[13], and the small subunit ribosomal ribonucleic acid *(ssu rRNA)* and internal transcribed spacer 1 *(ITS 1)* genes have been successfully used as the amplification targets[14–16]. The aim of the present work was to determine, *via* the use of PCR methods, whether *L. infantum* is also now responsible for cases of CL in the Marrakesh-Safi region of Morocco.

MATERIAL & METHODS

Patients and samples

In three provinces of the Marrakesh-Safi region, central Morocco, Al Haouz, Chichaoua, and Essaouira, a total of 297 smears were obtained from skin lesions of the suspicious patients with CL. Samples were collected (between March 2013 and June 2015) from patients consulting regional health centers for diagnosis and treatment of skin lesions suggestive of CL; those were collected by specialized nurses for parasitological diagnosis and molecular identification of the species. Diagnosis primarily was based on clinical signs and microscopic observation of parasites on direct stained smears. A simple direct questionnaire about clinical and epidemiological information (including age, gender, time of lesion evolution, kind of lesion/sign, clinical symptoms, travel outside the area of residence in the last 6 months, insect's and animal's presence/exposure) was filled out for each patient. The patients were notified of all the procedures, and signed informed consent was obtained from them.

In this study, we selected only the stained slide smears showing a positive result for *Leishmania* amastigote for confirmatory diagnosis by *Leishmania*-nested PCR (LnPCR) and characterization and identification of species by ITS1-PCR.

Direct microscopic examination (ME)

The serosity of cutaneous lesion aspirated by the syringe was spread on glass slides; air-dried and fixed with absolute methanol, allowed to dry, and then stained with Giemsa. All the Giemsa colored slides were observed under an optical microscope with high magnification (100 ×) and with immersion oil in search of amastigotes intracellular and extracellular (Fig. 1). Amastigotes were identified as having round to ovoid shape and characterized by a distinctive nucleus and adjacent kinetoplast. The results of the microscopic test were recorded. The slides were kept in the boxes for DNA extraction.

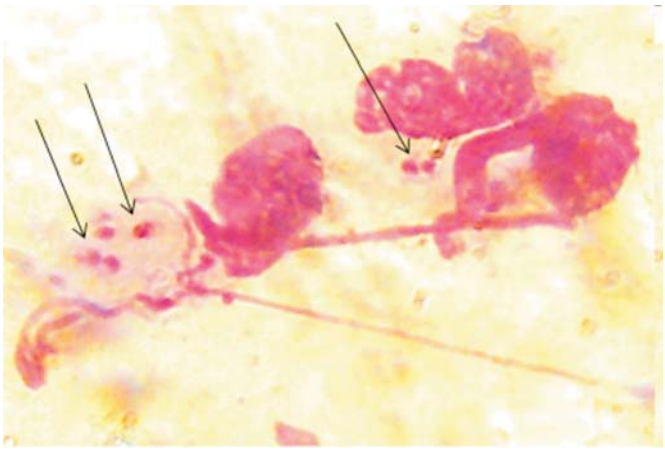

Fig. 1: Presence of extracellular and intracellular *Leishmania* amastigotes in Giemsa-stained smears obtained from a skin lesion of an infected patient (100 ×).

Extraction of Leishmania DNA

The DNA was extracted from human lesion smear samples positive for *Leishmania*. The slides were first cleaned with xylol to remove oil. Genomic DNA was extracted from the positive slides by using the Speed Tools DNA extraction Kit (Biotools, Madrid, Spain) following the manufacturer's recommendations, and was eluted in a final volume of 200 µl of PCR-grade water. The extracts were stored at 4°C until PCR analysis.

Detection of Leishmania DNA

Leishmania DNA was detected by nested PCR (LnPCR), amplifying the *ssu rRNA* gene, which lies within a region highly conserved across *Leishmania* species[17–18]. In this way, LnPCR allows amplifying all species of *Leishmania* in a specific way.

The first PCR reaction was performed using primers R221 (5´- GGT TCC TTT CCT GAT TTA CG - 3´), specific for Order of Kinetoplastida protozoa, and R332 (5´- GGC CGG TAA AGG CCG AAT AG - 3´), specific for the genus *Leishmania* and *Crithidia*[17]. Amplification reactions were performed in volumes of 50 µl[12], including 5 µl (2 mM) $MgCl_2$ standard reaction buffer, 1 µl (0.2 mM) of each dNTP, 1.4 µl (1U/µl) of *Thermus* spp DNA polymerase (*Tth*) (Biotools, B&M Labs, SA, Madrid, Spain), 1 µl (15 pmol) of primers R221 and R332, and 30.6 µl of sterile, distilled water.

For the second reaction, we used a nested PCR with specific primers (R223 and R333) of genus *Leishmania*[17, 19]. The amplification was performed in an Applied Biosystems 2720 programmable thermocycler (Applied Biosystems, Forst City, CA, USA) as follows: Initial denaturation for 5 min at 94°C, 30 cycles at 94°C for 30 sec, 60°C for 30 sec, plus 72°C for 30 sec, and a final extension step at 72°C for 5 min.

A re-amplification reaction was then performed in a 25 µl final volume, involving 10 µl of a 1/40 dilution of the first PCR amplicons as the template, 2.5 µl (2 mM) $MgCl_2$ standard reaction buffer, 0.5 µl (0.2 mM) of each dNTP, 0.7 µl (0.5 U/ µl) of *Tth* DNA polymerase (Biotools, B & M Labs, SA, Madrid, Spain), 0.5 µl (7.5 pmol) of primer R233 (5´- TCC CAT CGC AAC CTC GGT T - 3´), 0.25 µl (3.75 pmol) of primer R333 (5´- AAA GCG GGC GCG GTG CTG - 3´), and 10.55 µl sterile, distilled water[12]. The following amplification protocol was followed: Initial denaturation for 5 min at 94°C, 30 cycles at 94°C for 30 sec, 65°C for 30 sec, plus 72°C for 30 sec, and a final extension step at 72°C for 5 min.

The amplification products were resolved in a 1.5% agarose gel, stained with Gel Red Nucleic Acid stain (Biotium, Fremont, CA, USA), and visualized under UV light. Samples yielding a PCR product of 603 bp and 358 bp, respectively by first and second amplification reaction were deemed positive for *Leishmania* DNA (Fig. 2). Negative controls without DNA were employed in all assays. DNA from the reference *L. infantum* strain MHOM/FR/78/LEM75 was used as a positive control (Figs. 2–4).

Identification of Leishmania species

Samples positive by LnPCR were further analyzed to identify the *Leishmania* species by nested amplification of the ribosomal ITS1. The first PCR reaction was performed using the primers LITSR/L5.8S[12, 20–21]. A 10 µl of DNA solution was added to 40 µl of PCR mix containing 1 µl (15 pmol) of the primers LITSR (5´-CTGGATCATTTTCCGATG-3´) and 1 µl (15 pmol) of L5.8S (5´-TGATACCACTTATCGCACTT-3´), 5 µl standard reaction buffer 2 mM $MgCl_2$, 1 µl (0.2 mM) of each dNTP, 1.4 U of *Tth* polymerase (Biotools, B&M Labs, SA, Madrid, Spain), and 30.6 µl of sterile, distilled water[12]. Amplification was performed in initial denaturation for 5 min at 94°C, 30 cycles at 94°C for 30 sec, 53°C for 30 sec, plus 72°C for 30 sec, and a final extension at 72°C for 5 min.

For the second reaction, we used the primers SAC (5'-CATTTTCCGATGATTACACC-3') and VAN2 (5´-CGTTCTTCAACGAAATAGG-3´)[22]. A re-amplification reaction was then performed in a 25 µl final volume, involving 10 µl of a 1/40 dilution of the first PCR amplicons[12]. The annealing temperature was 57°C and all other conditions were the same as in the first amplification step.

Negative (without DNA) and positive (with DNA of the reference *L. infantum* strain MHOM/FR/78/LEM75) controls were also included in this assay. All reactives

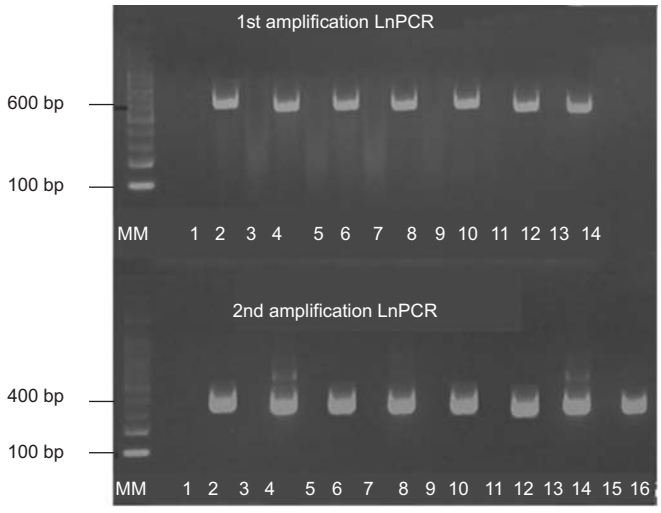

Fig. 2: Identification of *Leishmania* spp. from CL infected patients. LnPCR (1st amplification and 2nd amplification) was used to amplify part of the *ssu rRNA* gene for diagnosis. Reactions were run on a 1.5% agarose gel. MM: 100 bp DNA ladder; Wells 1, 3, 5, 7, 9, 11, 13, 15: Negative controls [no DNA]; Wells 14 and 16: Positive control, MHOM/FR/78/LEM75; and Wells 2, 4, 6, 8, 10, 12: DNA extracted from the positive slides.

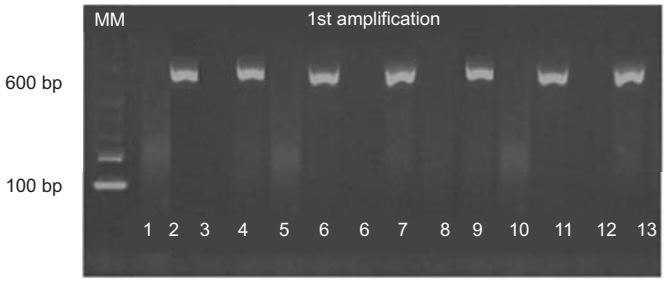

Fig. 3: Profile of agarose (1.5%) gel for characterization of *Leishmania* species in DNA extracted from the positive slides with LnPCR positive result, using ITS1-PCR to amplify the ITS1 region. MM: 100 bp molecular marker (DNA ladder); Lanes 1, 3, 5, 7, 9, 11, 13, 15: Negative control [no DNA PCR]; Lanes 14, 16: Positive control, MHOM/FR/78/LEM75; Lanes 2, 4, 6, 8, 10, 12: DNA extracted from the positive slides.

Fig. 4: Profile of agarose (1.5%) gel for characterization of *Leishmania* species in DNA extracted from the positive slides with LnPCR positive result, using ITS1-PCR to amplify the ITS1 region. MM: 100 bp molecular marker (DNA ladder); Lanes 1, 3, 5, 7, 9, 11, 13, 15: Negative control [no DNA PCR]; Lanes 14, 16: Positive control, MHOM/FR/78/LEM75; Lanes 2, 4, 6, 8, 10, 12: DNA extracted from the positive slides. ITS1-PCR was used for second amplification using SAC/VAN2 primers.

were synthesized commercially (Biotools, B&M Labs, SA, Madrid, Spain).

All the PCR products were visualized in 1.5% agarose gel (Figs. 2–4). Samples yielding 300–350 bp (Fig. 2) and 280–330 bp (Fig. 4), respectively, were deemed positive. The ITS1-PCR products were excised from the agarose gels and purified using the QIA quick extraction kit (QIAGEN). They were then sequenced which allows to correctly identify all the species of the genus *Leishmania* of the Old World[23], using the Big-Dye Terminator Cycle Sequencing Ready Reaction kit v3.1 and an automated ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA). These sequences were edited using BioEdit Sequence Alignment Editor Software[24] ver. 7.0.9.0 and compared with others in the GenBank database using BLAST software (*http://www.ncbi.nlm.nih.gov/genbank/*).

Ethical statement

This study was part of a project approved by the Ethical Hospital-University Committee (Faculty of Medicine and Pharmacy, University of Cadi Ayyad, Marrakesh, Morocco) with approval number 020/2016, to carry out an epidemiological study on leishmaniasis in the Marrakesh-Safi region. The authorization to examine files, interview, and microscopic examination of the stained smears prepared from the skin lesions, was obtained from the Regional Health Directorate, Marrakesh. A consent form was explained to and signed by all the participants.

RESULTS

Demographic and clinical presentation of the patients

Of the 257 CL cases, 139 (56.3%) were males and 118 (43.8%) were females, with a sex ratio of 1.17. The patients were in the age range from 6 months to >50 yr (Table 1). Most of the patients are residents of urban areas. The lesions were mainly located on the facial region 70.03% (180/257) compared with the upper limb 25.3% (65/257) and on the inferior limb 4.67% (12/257). We noticed different types of CL lesions, varied from ulcer-crusted nodules, erythematous crusted, nodule, papulous lesion, and lupoid (Table 2). All the patients were examined in health centers, and none had been out of the residential area during the six months preceding the onset of lesions, suggesting that these cases are autochthonous. The time to observe lesions varied from <2 to >8 months, the majority of which were observed from 2–4 months (54.47%), followed by 4–8 months (27.24%), >8 months (10.51%), and <2 months (7.78%) (Table 3).

Table 1. Distribution of patients by gender and age groups in the Marrakesh-Safi region

Age	Female	Male	Total
6–24 months	80	69	149
25 months–5 yr	15	26	41
6–10 yr	10	18	28
11–25 yr	5	7	12
26–50 yr	6	12	18
>50 yr	2	7	9

Table 2. Type of skin lesion in CL patients

Type of skin lesion	No. of cases	Percentage
Ulcer-crusted nodular	134	52
Nodular	50	20
Erythematous crusted	18	7
Papulous lesion	47	18
Lupoid	8	3
Total	257	100

Table 3. Time of evolution of skin lesions prior to sampling

Duration (months)	No. of patients	Percentage
<2	20	7.78
2–4	140	54.47
4–8	70	27.24
>8	27	10.51

Microscopic confirmation

Out of the 297 diagnosed patients, 257 were positive by direct examination with the presence of *Leishmania* amastigote form (Fig.1). The detection rate of amastigotes by optical microscopy was 86.53% (257/297) for the smears performed from the cutaneous lesions (Table 4).

Table 4. Results of the classical method of microscopic examination

Classical method	No. of (+) ve	No. of (–) ve	Sensitivity (%)	Specificity (%)
Microscopic examination	257	40	86.53	100

Molecular confirmation and identification of species

Out of 257 positive slides stained with MGG, confirmation of molecular diagnosis by LnPCR was obtained in 100% (Table 5; Fig. 2). The sequencing of the product obtained by ITS1-PCR (Fig. 4) showed *L. tropica* in 256 patients and *L. infantum* in one patient (Table 5). According to the locality, the province of Chichaoua is the most affected in the Marrakesh-Safi region (166/257) (Table 5).

Table 5. Results of the molecular diagnostic methods and species sequences

Provinces	Total of patients	LnPCR	ITS1-PCR	Sensitivity (%)	Species identification (n)	Species sequences
Al Haouz	36				*L. tropica* (36)	'CTGGATCATTTTCCGATGATTACACCAAAAAACATATACAAAAC
Chichaoua	166				*L. tropica* (166)	TCGGGGAGGCCTATATATTATACATTATATAGGCCTTTCCCACAC
						ATACACAGCAAACTTGTATACTCGAAGTTTGCAGTAAACACAAAAG
						GCCGATCGACGTTATAACGCACCGCCTATACACAAAAGCAAAAA
						TGTCCGTTTATACAAATATACGGCGTTTCGGTTTTGTTGGCGGGGG
						GTGCGTGTGTGTGGATAACGGCTCACATAACGTGTCGCGATGGAT
					L. tropica (54)	GACTTGGCTTCCTATTTCGTTGAAGAACGCAGTAAAGTGCGATAA
		Positive	Positive			GTGGTATCA'
Essaouira	55			100		'CTGGATCATTTTCCGATGATTACACCCAAAAAACATATACAACTCG
						GGGAGACCTATGTATATATATGTAGGCCTTTCCCACATACACAGCA
					L. infantum (1)	AAGTTTTGTACTCAAAATTTGCAGTAAAAAAAAGGCCGATCGACGT
						TATAACGCACCGCCTATACAAAAGCAAAAATGTCCGTTTATACAAA
						AAATATACGGCGTTTCGGTTTTTGGCGGGGTGGGTGCGTGTGTGGAT
						AACGGCTCACATAACGTGTCGCGATGGATGACTTGGCTTCCTATTTC
						GTTGAAGAACGCAGTAAAGTGCGATAAGTGGTATCA'

DISCUSSION

In this study, a molecular assay was performed for the diagnosis and characterization of *Leishmania* in positive Giemsa-stained slides. The diagnosis of leishmaniasis in Morocco is mostly based on clinical features and direct observation of the amastigotes stage in clinical materials. Microscopic examination has been the gold standard for CL diagnosis for the last 100 yr[25–26]. Several earlier studies have mentioned that the conventional methods are not able to differentiate between *Leishmania* species due to their homogeneous morphologies[3–4, 14, 27] with reported sensitivity ranging from 42–83%[13, 28–30]. Similar results were observed in this study regarding the sensitivity of microscopic examination for CL diagnosis (86.53%).

The performance of the parasitological diagnosis by the classical method of the microscopic examination depends on several factors, namely the quantity and quality of specimen obtained from the lesion, the technique of preparation and staining to provide a good smear, the experience of microscopist, the quality of microscopic resolution and field, the parasite load, the sampling site and the evolution of skin lesions at the time of the clinical examination. In this study, 140/257 samples (54.47%) were associated with the long time evolution of skin lesions before sampling, >2 months (2–4 months). These skin lesions are characterized by erythematous crusted, nodular, papulous, lupoid, and the majority are ulcer-crusted nodular 52% (134/257). The variability of these types of lesions may be explained by a high genetic diversity of Moroccan strains of *L. tropica*[31–34] and correlating with anthroponotic and zoonotic transmission cycles of the parasites present in the same CL foci[8, 12, 35].

The present study used PCR method for diagnosis and characterization of *Leishmania* species by extracted genomic DNA obtained from positive Giemsa-stained smears. It is an accurate technique for use in *Leishmania*-endemic areas[34, 36–40]. The PCR is very promising for CL diagnosis, and potentially becoming the gold standard due to its high sensitivity[25, 41]. Many researchers have reported 100% specificity with increasing sensitivity which overall is between 92 and 98%, appearing to be the most sensitive single diagnostic test for each form of leishmaniasis[42]. Our results showed that the greatest sensitivity was obtained when a combination of microscopic and molecular tests (PCR) was performed on positive slides colored with MGG from cutaneous lesions (100%).

It was shown for the first time that CL in the Essaouira region, an endemic focus of *L. tropica* anthroponotic cu-

taneous leishmaniasis (ACL), is caused also by *L. infantum*. Though both *L. tropica* and *L. infantum* were isolated from Essaouira Province, but we believe that the dominant and original species of *Leishmania* is *L. tropica* with sporadic cases of *L. infantum*[12]. The patient infected with *L. infantum* is a resident and never traveled outside.

In the present study, all the samples of skin lesions obtained from positive direct smears were positive by LnPCR (used for diagnostic) and ITS1-PCR (for characterization), which confirm the higher sensitivity of direct stained smear by microscopic examination.

CONCLUSION

Microscopic examination is very useful for the diagnosis of direct stained smears and is economical and fast. It is currently the most useful method in poor countries. However, this method remains limited especially in endemic regions where several *Leishmania* species coexist. In such situations, PCR constitutes a complementary method for the identification of the causal species. The study data indicate that both *L. tropica* and *L. infantum* are the causative agents of CL in the Marrakesh-Safi region; *L. tropica* being the dominant species.

The rate of CL is high in Imintanout, Chichaoua province. Hence, early diagnosis and prompt treatment of CL patients is necessary to prevent its extension to neighboring localities. The control of CL for reducing the risk of a disease requires close cooperation between different sectors, namely health centers, the research centers of medical sciences, local stakeholders as well as the government.

ACKNOWLEDGEMENTS

The authors would like to thank the Regional Director, the provincial delegates, and the officials of the Regional Health Centers in the Marrakesh-Safi region, Morocco, for help and useful collaboration. The authors are also thankful to the WHO Collaborating Centre for Leishmaniasis, Parasitology Service, National Center of Microbiology Institute of Health Carlos III, Majadahonda, Madrid, Spain for hosting the molecular section of the study.

Conflict of interest

The authors declare that they have no conflict of interest.

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Received: 19 July 2018 *Accepted in revised form:* 1 October 2018