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Development and Assessment of *Leishmania major* and *Leishmania tropica* Specific Loop-Mediated Isothermal Amplification Assays for the Diagnosis of Cutaneous Leishmaniasis in Tunisia

Melek Chaouch,[1,2]* Karim Aoun,[1,3] Souad Ben Othman,[1] Meriem Ben Abid,[1] Ines Ben Sghaier,[1] Aida Bouratbine,[1,3] and Souha Ben Abderrazak[1]

[1]*Laboratory of Medical Parasitology, Biotechnology and Biomolecules LR 11 IPT 06, Institut Pasteur de Tunis, Tunis, Tunisia;* [2]*Laboratory of Bioinformatics, Biomathematics and Biostatistics LR 16 IPT 09, Institut Pasteur de Tunis, Tunis, Tunisia;* [3]*Laboratory of Parasitology and Mycology, Institut Pasteur de Tunis, Tunis, Tunisia*

Abstract. Cutaneous leishmaniasis (CL) remains one of the world's most prevalent neglected diseases, particularly in developing countries. Identification of the involved *Leishmania* species is an important step in the diagnosis and case management process. In this study, we tested simple, rapid, and highly sensitive loop-mediated isothermal amplification (LAMP) assays for *Leishmania* DNA species-specific detection from cutaneous lesions. Two LAMP assays, targeting cysteine protease B (cpb) gene, were developed to detect and identify *Leishmania major* and *Leishmania tropica* species. Loop-mediated isothermal amplification specificity was examined using DNA samples from other *Leishmania* species and *Trypanosoma* species. No cross-reactions were detected. The developed LAMP assays exhibited sensitivity with a detection limit of 20 fg and 200 fg for *L. major* and *L. tropica*, respectively. Both tests were applied on clinical samples of CL suspected patients living in endemic Tunisian regions and compared with kinetoplast DNA quantitative PCR (qPCR), microscopic, and conventional cpb-based polymerase chain reaction (PCR) assays. Our LAMP tests were able to discriminate between *L. major* and *L. tropica* species and showed a sensitivity of 84% and a specificity of 100%. However, when compared with the performance of the diagnostic tests with latent class analysis (LCA), our LAMP assays show a sensitivity of 100%. These assays can be used as a first-line molecular test for early diagnosis and prompt management of CL cases in public health programs.

INTRODUCTION

Cutaneous leishmaniasis (CL) is a disease caused by flagellated protozoan parasites of the genus *Leishmania*, which belong to the order of Kinetoplastida. It causes single or multiple, sometimes persisting, often ulcerated skin lesions on the uncovered parts of the body. Cutaneous leishmaniasis is endemic in around 98 countries of the New and Old World. Between 0.7 and 1.2 million new cases are reported each year,[1] and 350 million people are at risk of contracting the disease. Three clinico-epidemiological CL forms occur in Tunisia: zoonotic CL (ZCL), chronic CL (CCL), and sporadic CL, caused by *Leishmania* (*L.*) *major*, *Leishmania tropica*, and *Leishmania infantum*, respectively.[2,3] Recently, *L. major* has largely spread to the central and southern parts of the country, whereas *L. tropica* distribution is no longer restricted to southeastern Tunisia.[4] This distribution has favored the development of CL mixed foci in which both *L. tropica* and *L. major* are transmitted especially in the Tataouine governorate, southeast Tunisia, where around 400 cases are reported yearly.[5]

Rapid and accurate diagnosis of leishmaniasis is crucial to initiate early management of infected cases and to control transmission according to the identified species. Laboratory diagnosis is based on parasitological methods (microscopic examination and culture), serological assays, and molecular tools (conventional PCR and quantitative PCR [qPCR]).[6–10] Microscopic examination is widely used and exhibits relatively low sensitivity, underestimating the infection rate and missing many cases. PCR techniques, especially qPCR technology, exhibit higher sensitivity but require costly equipment which are not available in rural areas, where most cases are observed. Because of the absence of a gold standard for CL diagnosis,[11–14] many in-house PCR protocols have been developed and used for *Leishmania* detection.[15]

Correct CL diagnosis and species identification would require a highly sensitive, specific, and easy-to-use diagnostic tool (or a combination of tools).[16] DNA loop-mediated isothermal amplification (LAMP) could meet such criteria.[17] The process has the potential to combine the high sensitivity and specificity of a molecular diagnostic test with the ability to operate the test under the limited resources of field conditions. Indeed, the LAMP has been used as a powerful diagnostic tool and successfully used for the diagnosis of several protozoan parasitic diseases, such as malaria,[18,19] trypanosomiasis,[20–22] and leishmaniasis.[23–25]

In the presented study, we developed two species-specific LAMP assays for the diagnosis of CL due to *L. major* and *L. tropica*, targeting the cysteine protease B (cpb) multi-copy gene. The multi-copy nature of the cpb genes and their polymorphism allow major structural change between *Leishmania* species and strains,[26] and make it an excellent target gene for the development of both species-sensitive and specific LAMP assays.[27] Whereas *L. tropica* contains an unknown number of cpb copy genes, *L. major* contains eight tandemly arranged cpb genes present in two variants: variant 1 (1,047 bp), present in five copies, and variant 2 (1,332 bp), present in three copies.[28] *Leishmania chagasi*, *Leishmania donovani*/*L. infantum*, and *Leishmania mexicana* present five, six, and 19 tandemly arranged cpb genes, respectively.[29,30]

To assess our developed LAMP assay, we locally collected skin samples from CL suspected patients living in endemic regions of Tunisia. Sensitivity and specificity of our LAMP assay were compared with those of qPCR, cpb PCR, and microscopic techniques.

* Address correspondence to Melek Chaouch, Laboratory of Medical Parasitology, Biotechnology and Biomolecules LR 11 IPT 06, Institut Pasteur de Tunis, Tunis 1002, Tunisia. E-mail: mcmelek@msn.com

MATERIALS AND METHODS

Study sites. The study was carried out between September 2015 and July 2016 at three endemic Tunisian governorates: two in central (Sidi Bouzid and Kairouan) and one in southern (Tataouine) Tunisia.

Study participants. Samples were collected from patients with suggestive CL symptoms, referred to public health centers by their physicians as part of the CL diagnostic procedure. In total, 81 patients were recruited.

Ethical concerns. Informed consent was requested from all adults recruited for the study. Consent was obtained from parents or guardians for the inclusion of young children. Study procedures were approved by the Committee of Bio-Medical Ethics of Institut Pasteur de Tunis (Ref 2015/06/I/LR11IPT06/V2).

Sample collection. The tissue material was aspirated after actively scraping the skin lesions' peripheral edge. Smears were prepared and stained on the site for microscopic examination. Part of the collected serosities was placed in an Eppendorf tube containing phosphate-buffered saline, coded and enclosed in a self-sealing bag, and stored at 4°C for further molecular testing.

Microscopic confirmation. A positive test was based on the microscopic demonstration of *Leishmania* amastigotes on smear slides stained by May-Grunwald Giemsa stain and screened at ×100 objective.

Culture. A total of 26 cultures were carried out on Novy, McNeal, Nicolle (NNN) media, prepared according to Nicolle and Berrebi recommendations.[31,32] Inoculation of dermal scraping was immediately performed after sampling. The NNN tubes were incubated between 22°C and 26°C.[31] Microscopic examination and subculturing were performed weekly for 5 weeks. Positive cultures were conserved for isoenzyme typing.

DNA extraction. DNA was extracted from tissue materials, aspirated from skin lesions using the Wizard Genomic DNA Purification kit (Promega, Madison, WI) according to the manufacturer's instructions. Each DNA sample was investigated with different molecular tools.

Cysteine protease B conventional PCR amplification. Different cpb gene copies were amplified using three different species-specific PCR assays for the *L. major*, *L. tropica*, and *L. infantum* complex. Amplification sizes were 1,176 bp for *L. major*, 600 bp for *L. tropica*, and 325 bp for *L. infantum*.[33]

Polymerase chain reactions targeting the cpb gene were performed following the modified protocol described by Chaouch et al.[23,33] using a 25-μL final volume, containing 5 μL of the sample, 1.5 mM of $MgCl_2$, 0.2 μM of each deoxynucleotide, 50 pmol of each primer, and 1.25 U of Taq DNA polymerase. Reactions were conducted in a TECHNE TC512 machine following the cycling conditions: initial denaturation step at 94°C for 5 minutes, followed by 35 cycles at 94°C for 1 minute, 62°C for 1 minute, and 72°C for 1 minute, with a final elongation step at 72°C for 10 minutes. The PCR products were electrophoresed on 1% agarose gels, stained with ethidium bromide, and then visualized under UV light. A 1-kb ladder (Fermentas, Germany) was used to size amplicons.

Quantitative real-time PCR assay. A qPCR was conducted according Mary et al.[34] and as described by Chouihi et al.[35] Each sample was tested in duplicate. A distilled water sample and DNA extract from *L. infantum* promastigotes were included as negative and positive controls, respectively. Amplification was conducted in an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA).

Loop-mediated isothermal amplification. *Primer design.* The LAMP primer sets were designed using the Primer Explorer ver.4 software (Eiken Chemical, Japan [http://primerexplorer.jp/lamp4.0.0/index.html]), using *L. major* and *L. tropica* consensus sequences of the cpb multi-copy gene, accessible on the GenBank database.[33] For each species, a set of four LAMP primers, recognizing six specific sections of *L. major* and *L. tropica* cpb genes, were designed (Table 1). Two additional loop primers, loop forward (LF) and loop backward (LoR),[15] were manually designed. The loop primers were added to increase the number of loops in the reaction, thereby increasing the reaction speed. Final specificity verification was performed using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis against human DNA and any other organisms included in leishmaniasis differential diagnosis.

Loop-mediated isothermal amplification reactions. Leishmania major and *L. tropica* specific LAMP reactions were

TABLE 1
Species-specific LAMP primer sequences used in the study

Species	Oligo name	Sequence (5′–3)
Leishmania tropica	Tropica-F3	GCAGGCGTTCGAGTGG
	Tropica-B3	CAATCGAGATGGGGCCATT
	Tropica-LF	GGACACGTAGGGGTAGCTGTCC
	Tropica-LB	GACGATCGAAAGCAGCGAAA
	Tropica-FIP	CGGGCACATAACCGCTGCTGAACGGGACCATGTTCACG
	Tropica-BIP	AGTGCTCGAACAGCAGTCAACTTAACCGTTTCGCTGCTTTCG
Leishmania major	Major-F3	GTGCATGTGTCGCAGAGC
	Major-B3	TCAGCAACTTGTGCAGACTG
	Major-FIP	TCGTGCAGCACATGTCGCTTGCTAGCACGGAGAGCGAAGA
	Major-BIP	TTCTCACCGCCAACAAGTGTGCCACTTGGTCCTAGAGGAGCC
	Major-LF	TCTCCTCCATCGTCGCCCG
	Major-LB	TGCTACGAGAACCGGGGGC
Leishmania infantum[33]	Infantum-F3	GCGATGACAAAGACAATGGC
	Infantum-B3	TCATCACGTAGCCGTCGA
	Infantum-FIP	TCTCCGTGAACACGATCCCGTCTGATGCTGCAGGCGTTC
	Infantum-BIP	AGAGCTACCCCTACACGTCCGTTTGCGCGCCGGGAAC
	Infantum-LF	ACATGTGTCGCAGCAGCCAC
	Infantum-LB	GGTGATGTGGCCGAGTGCTT

LF = loop forward.

standardized for optimal temperature after gradient analysis (64–68°C) and time (45–50 minutes) using *L. major* and *L. tropica* reference strains. Fifty-one different parasite strains (including *L. infantum*, *L. donovani*, *L. major*, *L. tropica*, *Leishmania turanica*, *Leishmania gerbilii*, *Leishmania tarentolea*, and *Trypanosoma cruzi*) were used as references to test the LAMP assay specificity. The analytical sensitivity of the LAMP assays was evaluated after serial dilutions (10-fold serial dilutions from 0.00002 ng to 20 ng) of reference DNA samples. Briefly, the LAMP assay was achieved in a 25-μL reaction mixture containing in-house buffer (2.5 mM), forward inner primer (FIP) and backward inner primer (BIP) (1.6 mM), loop-F and loop-B (0.8 μM), F3 and B3 primers (0.2 mM), Bst DNA polymerase (8 U, GeneON, Germany), betaine (10 μM), deoxynucleotide triphosphates (2.5 mM), $MgSO_4$ (10 mM), ddH_2O, and DNA template (2.5 μL). The LAMP test was performed for 50 minutes at 67°C and completed by increasing the temperature to 80°C for 5 minutes in a heat block. To confirm positivity, amplicons were electrophoresed in 1% agarose gels stained with ethidium bromide and visualized under UV light.

Statistical analysis. Data were captured anonymously. The sensitivity and specificity of the techniques used were estimated with 2 × 2 contingency tables using qPCR as the gold standard. Sensitivity and specificity were also estimated by using the latent class analysis (LCA),[36] without considering a gold standard. In this LCA, unobserved or latent *Leishmania* infection is used to explain dependencies between the observed diagnostics. In basic latent class models, the observed variables are assumed to be independent conditions of latent class; that is, there are no associations between the observed variables within each category of the latent variable. The latent variable is the true status of the disease, and the hypothesis is that there are two latent classes (presence or absence of disease). Molecular tests and microscopic examination are assumed to be conditionally independent, given the true unobserved infection status. Latent class analysis was performed using the LEM package "(Vermunt, unpublished data)", including the latent variable "Cutaneous Leishmaniasis disease" (X) and four observed diagnostic test variables: q-PCR, LAMP, cpb PCR, and microcopy.

RESULTS

Detection threshold and analytical specificity of species-specific LAMPs. A set of specific oligonucleotide primers were designed to amplify the sequences of the target cpb genes from *L. major* and *L. tropica* genomic DNA. Serially diluted samples (20 ng–20 fg) of *L. major* and *L. tropica* promastigote DNA were used as references. Loop-mediated isothermal amplification was able to detect 20 fg and 200 fg of *L. major* and *L. tropica* DNA, respectively (Figure 1A). Loop-mediated isothermal amplification reaction specificity was next evaluated using DNA samples from other *Trypanosoma* genera. No amplicon was detected using up to 20 ng of DNA and no cross-reactions were observed, particularly between endemic *Leishmania* species in Tunisia. Our results showed that LAMP cpb is species specific for *L. major* and *L. tropica* detection (Figure 1B).

Diagnostics evaluation. A total of 81 suspected CL patients were sampled for this study. Figure 2 shows the workflow applied for the tested samples. Seventy-two of the 81 samples were tested using either microscopy or molecular tools, whereas nine samples were excluded because of the absence of slides (four samples) or poor DNA quality (five samples).

Microscopy and culture. Leishmania amastigotes were detected by microscopy in 43 cases (59.7%) (29 slides were considered as negative). Among the microscopy-positive samples, seven showed positive cultures and were identified by isoenzyme typing as *L. major* (six isolates) and *L. tropica* (one isolate).

Molecular tools. Fifty of the 72 (69.4%) samples were tested positive using the qPCR assays targeting the kinetoplast *Leishmania* genome, and were thus considered as confirmed CL cases. Among those, 42 were also found to be positive

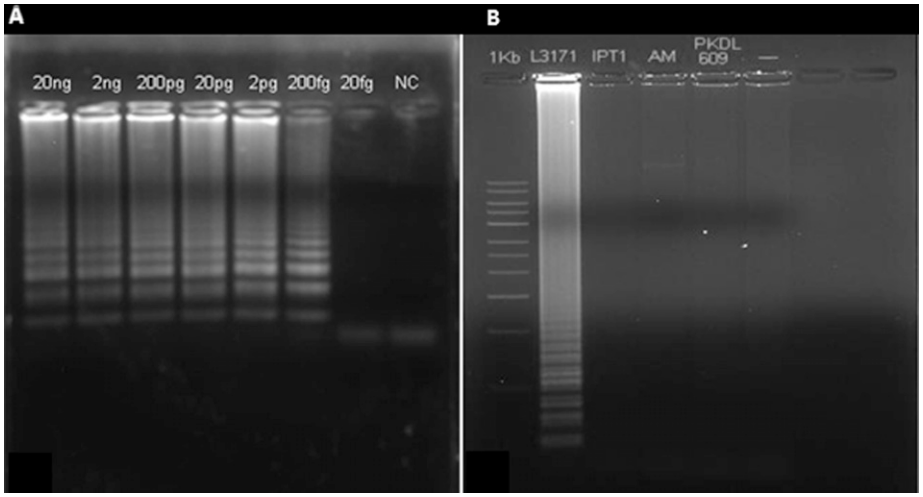

FIGURE 1. (**A**) Sensitivity of LAMP assays for the detection of *Leishmania* DNA using serial dilutions of *Leishmania tropica*. After incubation at 67°C for 50 minutes, LAMP reactions were inspected by agarose gel electrophoresis of LAMP products. Lanes 1–6 show the typical ladder-shaped pattern of a positive reaction. (**B**) Specificity of *Leishmania major* LAMP assay tested on different species: lane 1: 1-kb ladder, lane 2: *L. major* (L3171), lane 3: *Leishmania infantum* (MHOM/TN/1980/IPT1), lane 4: *L. tropica* (AM), lane 5: *Leishmania donovani* (PKDL306), and lane 6: negative control.

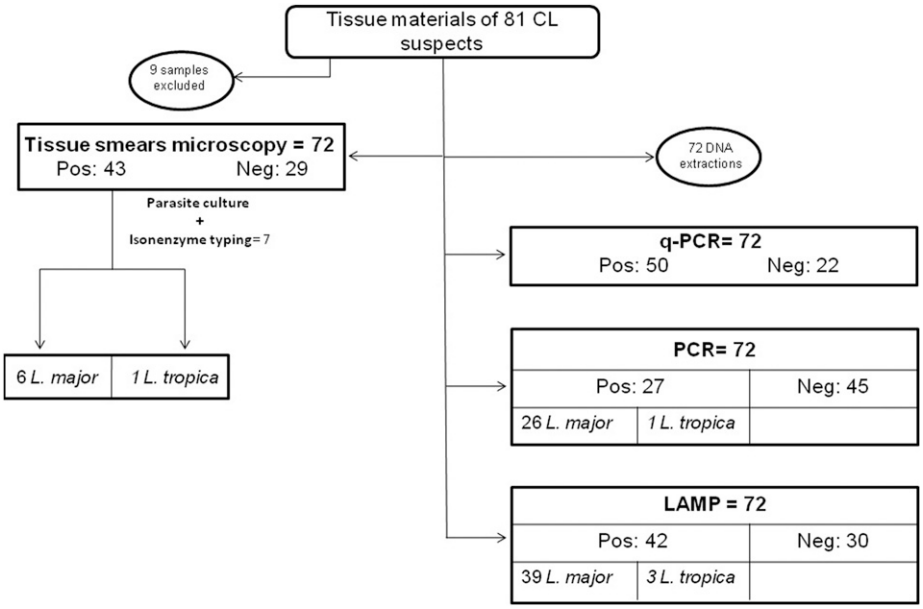

FIGURE 2. Diagram describing the workflow of samples from cutaneous leishmaniasis suspects in the study.

using the LAMP assay, but only 27 were found to be positive by cpb PCR. Thus, 15 samples were positive by LAMP and negative by cpb PCR, and eight samples were positive by qPCR and negative using LAMP. This low sensitivity of PCR can be explained by the large size of the amplified fragment.

Among the 27 samples (37.5%) found to be positive by species-specific applied cpb PCRs, 26 corresponded to *L. major*, one amplification corresponded to *L. tropica*, and none corresponded to *L. infantum* PCR. No mixed amplifications were observed using species-specific PCR.

The species-specific LAMPs showed 42 positive reactions (58.3%), with 39 *L. major* and three *L. tropica*. Isoenzyme typing results were concordant with those of LAMP and cpb PCR identifications, except for one sample which was identified as *L. major* by isoenzyme typing but amplified with an *L. tropica* LAMP.

Statistics. Performance of the techniques was assessed using a statistical 2 × 2 contingency table, assuming qPCR as the gold standard. Microscopy, LAMP, and cpb PCR showed sensitivities of 82%, 84%, and 54%, respectively, and specificities of 90.9%, 100%, and 100%, respectively (Table 2). The lowest negative likelihood ratio (LR–) was achieved by the LAMP with 0.16 (a LR– below 0.1 virtually rules out the chance that a patient has the disease) (Table 2). Moreover, we compared the results of the 2 × 2 contingency tables with those of the LCA. Loop-mediated isothermal amplification sensitivity and specificity estimated from the 2 × 2 contingency tables were broadly corroborated by the LCA (LAMP: Se 100%–Sp 100%) (Table 3).

DISCUSSION

With an increased number of cases and geographical distribution, CL is becoming an important health concern in endemic regions, especially in Maghreb countries.[37] Moreover, conventional diagnostic methods (microscopy and PCRs) require expertise and advanced facilities, and are time-consuming. Therefore, simplifying and improving CL diagnostic modalities is becoming critical, especially in low-resource areas that are most affected by the disease. A reliable, rapid, and cost-effective diagnostic tool is required for effective CL control and efficient case management, especially when more than one *Leishmania* species is present in one region. This is indeed the case in different countries, including Tunisia, where the spreading of the parasite leads to the coexistence in the same region, for example, of *L. major* and *L. tropica* in the southeast[37]; therefore, species characterization is crucial for case management and epidemiological purposes. Accordingly, LAMP assays targeting the cpb multicopy gene were developed to complement the CL diagnosis process and reinforce epidemiological studies. This was tested on skin samples collected from 72 patients with suspected CL. The results were compared with those obtained by microscopic, qPCR (targeting kDNA), and conventional cpb PCR analyses. The developed LAMP systems exhibited great sensitivity by detecting very low *Leishmania* DNA concentrations (20 fg and 200 fg for *L. major* and *L. tropica*, respectively). The threshold variations were likely due to the difference in the cpb gene copy number present in the studied species' genomes.[38] Adams et al.[39] stated that the parasite load of skin

TABLE 2
Performance of the different assays for the diagnosis of cutaneous leishmaniasis by classical validation (2 × 2) (95% CI) (qPCR as gold standard)

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR+	LR–	Kappa (κ)
Microscopy	82 (70–90)	90.9 (70–98)	95.3 (89–100)	69 (52.1–85.8)	9 (2.3–43)	0.19 (0.1–0.3)	0.66
LAMP	84 (71.1–91.8)	100 (82–100)	100 (100–100)	73.3 (57.5–89.2)	–	0.16 (0.08–0.30)	0.76
PCR	54 (40.4–67)	100 (82.1–100)	100 (100–100)	48.9 (34.3–63.5)	–	0.46 (0.3–0.6)	0.41

LAMP = loop-mediated isothermal amplification; LR = likelihood ratio; NPV = negative predictive value; PPV = positive predictive value.

TABLE 3
Performance of the different assays for the diagnosis of CL by classical validation (2 × 2) and through LCA (95% CI)

	Sensitivity (%)		Specificity (%)	
	2 × 2 analysis	LCA	2 × 2 analysis	LCA
qPCR	100*	100 (95.2–99.9)	100*	73.3 (65.3–81.3)
Microscopy	82 (70–90)	88.1 (83.1–93.1)	90.9 (70–98)	80 (68.9–84.4)
LAMP	84 (71.1–91.8)	100 (98–100)	100 (82–100)	100 (96–100)
PCR	54 (40.4–67)	64.3 (57.0–71.6)	100 (82.1–100)	100 (89–100)

LAMP = loop-mediated isothermal amplification; LCA = latent class analysis; qPCR = quantitative PCR.
* By definition (qPCR reference test).

lesion swab samples taken from CL patients should be high enough to achieve direct gene amplification, whereas van der Meide et al.[40] reported that a 2-mm-diameter skin biopsy sample from a CL patient contains 6,100–94,100 parasites.

When compared with qPCR, the LAMP assay exhibits high sensitivity (84%) and specificity (100%), with an interesting positive predictive value (PPV = 100) and acceptable negative predictive value (NPV = 73.3). The sensitivity and specificity of the LAMP were greatly improved after applying the LCA statistical test (Table 3). The positive likelihood ratio (LR+) confirms that the patients diagnosed as positive using the LAMP are potentially true positives. The negative likelihood ratio (LR–) is greater than 0.1 (0.16) and lower than 1, supporting the possibility of low false-negative results. Using Kappa, the LAMP exhibited a higher agreement degree (0.76) than microscopy (0.66) and conventional cpb PCR (0.41), although it is simpler and less expensive as reported in the literature.[41] By comparing the positive results of all these techniques, we noted good concordance with a reduced number of false positives and false negatives.

Concerning species identification ($n = 42$), the majority of our strains corresponded to *L. major* (92.9%), confirming the large predominance of this species throughout the Tunisian territory. In fact, ZCL due to *L. major* is responsible for more than 90% of CL cases in Tunisia, with an annual incidence exceeding 5,000 cases during epidemic years, especially in the center and the south.[37] Our specific LAMPs identified more strains than conventional cpb PCR for either *L. major* (39 versus 26) or *L. tropica* (3 versus 1). No *L. infantum* strains, which are endemic predominantly in the northern parts of the country, were detected.

The three *L. tropica* strains were observed in the historical focus of Tataouine, southeast Tunisia, where CCL is most prevalent.[37]

Only one discrepancy over seven identified strains was observed between the LAMP and isoenzyme typing: The DNA extracted from the concerned sample was amplified and identified as *L. tropica* with the LAMP, whereas isoenzyme electrophoresis pointed it as *L. major*. This discrepancy could be due to a possible contamination during the culture process needed for the isoenzyme typing.

Compared with other molecular techniques, the LAMP assay has many advantages. The amplification is fast and implementable with basic equipment and post-amplification handling.[42,43] In fact, the average time taken for the LAMP assay was 50 minutes, regardless of DNA extraction that could be avoided, whereas the qPCR and conventional PCR take approximately 3 hours. Moreover, the LAMP reaction is unaffected by PCR inhibitors found in biological components.[39,44,45] The use of agarose gel migration can also be avoided and the result assessed by staining with different stains such as malachite green,[25] FDR,[46] and SYBR green.[47] Furthermore, the stability of the LAMP reagents facilitates the field deployment of the technique.[48]

LAMP tests have been developed and applied to different cases of leishmaniasis, including *Leishmania* infection screening in dogs and detection of human asymptomatic subjects.[23,47,49] Loop-mediated isothermal amplification tests targeting the kDNA minicircles (10^4 copies) showed improved performance compared with microscopy for CL diagnosis in Sri Lanka and Colombia.[50,51] Similar results were obtained by Verma et al. who used the LAMP test targeting the 18S rRNA gene ($10^{4.8}$ copies) for CL and post-kala-azar dermal leishmaniasis (PKDL) diagnosis in India.[49,52] Despite targeting the cpb gene that is present in reduced number of copies compared with the kDNA and the 18S rRNA genes, our LAMP assay showed promising performance.

TABLE 4
Details and summary of the diagnostic performance of different studies using LAMP for CL diagnosis compared with our study

	Kothalawala et al.[50]	Adams et al.[51]	Verma et al.[49]	Verma et al.[52]	This study: 3 different species-specific LAMP
Country	Suriname	Colombia	India	India	Tunisia
LAMP target	18S rRNA	18S rRNA	kDNA	kDNA	cpb
Primer specificity	*L. donovani*	*Leishmania* genus	*L. donovani*, *L. major*, *L. tropica*	*L. donovani*	*L. major*, *L. tropica*, *L. infantum*
Study population	17 CL	105 CL	10 CL	62 PKDL	82 CL suspects
Clinical specimen tested	Lesion aspirate	Lesion swab	Skin biopsy	Skin biopsy	Lesion aspirate
DNA purification method	Boom method	Qiagen DNAeasy blood and tissue kit	QIAamp Mini Kit (QIAGEN, Hilden, Germany)	QIAamp Mini Kit (QIAGEN)	Wizard® Genomic DNA Purification kit (Promega)
Reference test	Microscopy	Microscopy and/or culture	rK39 RDT-positive qPCR	rK39 RDT-positive qPCR	qPCR
Sensitivity (%)	82.6	95	80	97	84
Specificity (%)	100	86	100	100	100

CL = cutaneous leishmaniasis; cpb = cysteine protease B; *L.* = *Leishmania*; LAMP = loop-mediated isothermal amplification; qPCR = quantitative PCR.

Otherwise, our methodology permitted, for the first time, to our knowledge, the identification of *Leishmania* species using a non-invasive technique and a fairly representative sampling (Table 4).

CONCLUSION

Our developed LAMP assay targeting the multi-copy cpb gene clearly allows the discrimination between different *Leishmania* parasite species causing CL in north Africa. It is more convenient and less expensive than qPCR[41] or PCR[53] for the detection and identification of *Leishmania* species.

Our results demonstrate the high performance of this technique, and the relevance of its implementation, especially in endemic CL countries, such as Tunisia, where more than one species are transmitted. It could help achieve early diagnosis and prompt case management and adapt control programs.

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Authors' addresses: Melek Chaouch, Laboratory of Medical Parasitology, Biotechnology and Biomolecules LR 11 IPT 06, Institut Pasteur de Tunis, Tunis, Tunisia, and Laboratory of Bioinformatics, Biomathematics and Biostatistics LR 16 IPT 09, Institut Pasteur de Tunis, Tunis, Tunisia, E-mail: mcmelek@msn.com. Karim Aoun and Aida Bouratbine, Laboratory of Medical Parasitology, Biotechnology and Biomolecules LR 11 IPT 06, Institut Pasteur de Tunis, Tunis, Tunisia, and Laboratory of Parasitology and Mycology, Institut Pasteur de Tunis, Tunis, Tunisia, E-mails: karim.aoun@pasteur.rns.tn and aida.bouratbine@pasteur.rns.tn. Souad Ben Othman, Meriem Ben Abid, Ines Ben Sghaier, and Souha Ben Abderrazak, Laboratory of Medical Parasitology, Biotechnology and Biomolecules LR 11 IPT 06, Institut Pasteur de Tunis, Tunis, Tunisia, E-mails: souaad.bo@gmail.com, meriem_benabid@yahoo.fr, ines.bensghaier@gmail.com, and souha.benabderrazak@pasteur.rns.tn.

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