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Advantages and limits of real-time PCR assay and PCR-restriction fragment length polymorphism for the identification of cutaneous *Leishmania* species in Tunisia

Imène Ben Abda[a,b], Frédérique de Monbrison[b,*], Nadia Bousslimi[a], Karim Aoun[a], Aïda Bouratbine[a], Stéphane Picot[b]

[a] *Research Laboratory of Emerging Parasitosis (LR 05 SP 03), Pasteur Institute of Tunis,13, Place Pasteur. BP 74-1002 Tunis, Tunisia*
[b] *Service Paludisme, Parasites du Sang et Mycologie Médicale, Hospices Civils de Lyon, 103 Grande Rue de la Croix Rousse, 69317 Lyon Cedex 04, France*

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ABSTRACT

Cutaneous leishmaniasis (CL), a public health problem in Tunisia, is associated to three species: *Leishmania (L.) infantum*, *L. major* and *L. killicki*. Accurate and sensitive procedures for the diagnostic of *Leishmania* infection and for species identification are required to enable adequate treatment and appropriate control measures. Several PCR-methods are applied for the diagnosis and the identification of *Leishmania* parasites such as PCR-restriction fragment length polymorphism (PCR-RFLP), DNA sequencing, hybridization probes and real-time PCR (RT-PCR). In this study, PCR-RFLP and RT-PCR were performed on skin scrapings from 27 patients with confirmed CL by microscopic examination, in order to compare their usefulness and efficiency for identification of *Leishmania* species in routine diagnostic laboratories. Identification of *Leishmania* species was successfully achieved in 96.3% and 81.5% respectively. Agreement between using internal transcribed spacer 1 (ITS1)-PCR-RFLP and kDNA-RT-PCR assays was 70% (19/27). Characterization problems using RT-PCR were mainly due to the difficulties in analyzing the melting temperatures.

ITS1-PCR-RFLP and kDNA-RT-PCR presented an interesting alternative to conventional methods for the identification of *Leishmania* parasites from clinical samples. Both PCR assays can be used in a routine diagnostic, however, further prospective studies including largest sampling, are required to determine their performances in a routine use.

1. Introduction

Cutaneous Leishmaniasis (CL) is one of the most important resurgent parasitic vector-borne diseases endemic in many regions of the Old World: Central Asia, Middle East and the Mediterranean area.[1] It produces a broad spectrum of cutaneous lesions in humans, ranging from a small self-healing lesion to a disabling affliction.[2] Relapses and treatment failures, especially when *Leishmania tropica* is involved, are major issues of the disease.[3] CL is prevalent in many areas in Tunisia, related to three *Leishmania* species: (i) *L. infantum* associated to sporadic CL in the north of the country, (ii) *L. major* responsible for zoonotic CL in the centre and the south and (iii) *L. killicki* (syn. *L. tropica* MON-8)[4], the causative agent of chronic CL, in limited foci of the southeast and the centre of the country.[5]

In endemic areas, the presence of multiple *Leishmania* species with overlapping clinical features and geographical distribution requires the development of sensitive laboratory tests with *Leishmania* species identification in order to evaluate the prognosis of CL and to choose appropriate therapies.[6,7] Species identification will also contribute to a better understanding of CL epidemiology.[5]

* Corresponding author. Tel.: +33 4 72 00 15 17; fax: +33 4 72 00 15 21.
E-mail address: demonbrison@sfr.fr (F. de Monbrison).

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Currently, isoenzyme analysis is the gold standard technique for the characterisation and the classification of *Leishmania* parasites.[4] However, this assay is culture dependent, time-consuming and arduous, requiring the examination of 15 different enzymatic profiles. For this reason, isoenzyme analysis is not available in a routine diagnostic laboratory. Moreover, difficulties in cultivating some species like *L. infantum* and the risk of contamination can hamper the identification of the parasite.[8,9]

In the past decade, several molecular methods were employed for the diagnostic and epidemiological studies on leishmaniasis. PCR technology was successfully applied for *Leishmania* diagnosis and identification of the causative species directly from clinical samples avoiding the need of cultivation.[10–14] PCR-restriction fragment length polymorphism (RFLP) was widely used for species identification of *Leishmania* parasites.[15–19] Real-time (RT) PCR, however, improved molecular diagnostics by adding simplicity and sensitivity.[11,20–22]

In the present work, we applied two PCR assays, a PCR-RFLP and subsequently RT-PCR to identify *Leishmania* species in 27 skin samples collected from Tunisian patients with confirmed CL. Our aim was to evaluate their advantages and limits in the identification of *Leishmania* species in a routine diagnostic laboratory-use.

2. Materials and methods

2.1. Samples

2.1.1. Clinical specimens

Samples of dermal scrapings were collected from 27 patients with CL referred to the Parasitology Laboratory in the Pasteur Institute of Tunis (PIT), Tunisia, for parasite diagnosis or during epidemiologic investigations conducted in Tunisia from 2005 to 2009. All patients or their legal guardians enrolled in the study provided free and verbal consent.

All patients were Tunisian nationals originated from 10 governorates of Tunisia: three patients from the north, 3 from the centre and 21 from the south. After cleaning with alcohol, scrapings were obtained from the active edge of the lesion using sterile lancets. The diagnosis of CL was confirmed in all patients by direct examination of *Leishmania* amastigotes in Giemsa-stained skin slit smears. *Leishmania* promastigotes were isolated in 14 out of 21 cultures, performed in Novy-MacNeal-Nicolle medium.[23] The 14 isolates were typed by isoenzyme analysis, carried out in the Reference Center for *Leishmania* typing, Pasteur Institute of Algiers (Algeria).

All samples were subjected to a DNA extraction and submitted to DNA amplification for *Leishmania* species identification. PCR-RFLP was performed in the parasitology laboratory of PIT and RT-PCR in Service Paludisme, Parasites du Sang et Mycologie Médicale, Lyon, France.

2.2. Leishmania reference strains

Six reference strains were used as positive controls: *L. infantum* MHOM/TN/2005/LC11, *L. major* MHOM/TN/2003/LCT4, *L. killicki* MHOM/TN/2003/LCK in PCR-RFLP and *L. infantum* MHOM/TN/80/IPT1, *L. major* MHOM/SU/73/5-ASKH, *L. tropica* MHOM/SU/74/SAF -K27 in RT-PCR.

L. infantum MHOM/TN/80/IPT1, *L. major* MHOM/SU/73/5-ASKH and *L. tropica* MHOM/SU/74/SAF-K27 were kindly provided by M. Gramiccia, Istituto Superiore di Sanita in Roma, Italy.

L. infantum MHOM/TN/2005/LC11, *L. major* MHOM/TN/2003/LCT4 and *L. killicki* MHOM/TN/2003/LCK were gifted from Pasteur Institute of Algiers (Algeria).

The six reference strains were isoenzyme typed using the technique described by Rioux et al.[4]

Reference strains were cultivated at 25 °C in RPMI medium supplemented with 15% foetal calf serum and penicillin. Parasites were harvested at a density of 10^4 parasites/ml. They were washed twice with PBS buffer, pelleted with centrifugation (5900 × *g* for 3 min) and stored at −20 °C until DNA extraction.

3. Molecular diagnosis

3.1. DNA extraction

3.1.1. Clinical samples

DNA extraction was performed on scrapped products in all patients in PIT using QiAamp DNA Blood Mini Kit (Qiagen®, Hilden, Germany) according to the manufacturer's instructions. DNA was eluted from the column with 100 μl of TE Buffer. Samples were kept at −20 °C until used.

3.1.2. Leishmania reference strains

DNA was isolated from promastigote forms using QiAamp DNA mini kit following the manufacturer's instructions, with minor modifications. The incubation time with proteinase K at 56 °C was increased to 1 h and DNA was eluted from the column with 50 μl of PCR grade H20.

3.2. Molecular analysis

3.2.1. PCR-RFLP

This assay was carried out first in PIT, as previously described by Schönian et al.[15] LITSR (5′-CTGGATCATTTTCCGATG-3′) and L5.8S (5′-TGATACCACTTATCGCACTTA-3′) primers were used for amplification of ribosomal internal transcribed spacer (ITS) 1 region. Amplification reactions were performed in a 50 μl volume. The extracted DNA (5 μl) was added to 45 μl of reaction mixture containing 50 mM of MgCl2, 25 μM of each primer (LITSR and L5,8S),10 mM dNTP's and 5 U Taq polymerase. Conditions for cycling were 94 °C for 4 min, followed by 36 cycles of 95 °C for 40 s, 53 °C for 30 s, 72 °C for 60 s and 72 °C for 6 min. *Leishmania* DNA from three reference strains and water were used as positive and negative controls in each PCR experiment.

After the amplification, PCR product was analysed on 2% agarose gel by electrophoresis and visualized under ultraviolet light after staining in ethidium bromide. The reference strains gave a PCR product lying from 300–350 base pairs (bp). Secondly, amplicons were subjected to a digestion with the endonuclease HaeIII (Hybaid GmbH, Heidelberg, Germany) at 37 °C for 1 hour following the manufacturer's

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instructions. Fragment length was analyzed to characterize *Leishmania* species, after agarose electrophoresis (2%) and ethidium bromide staining.[15] Band patterns obtained after digestion, were compared to the patterns obtained with the reference strains. The digestion with the restriction enzyme HaeIII revealed three bands for *L. infantum* MHOM/TN/2005/LC11 (200, 100, and 50 bp), two bands for *L. tropica/killicki* MHOM/TN/2003/LCK (220 and 50 bp), and two for *L. major* MHOM/TN/2003/LCT4 (220 and 127 bp).

3.2.2. Real-time PCR

The extracted DNA from the 27 samples was subjected to RT-PCR amplification, as previously described.[19,20] Primers JW13 and JW14, designed on the conserved region of *Leishmania* kDNA minicircle, were used for the amplification of *L. infantum*, *L. major* and *L. tropica*.[21] RT-PCR technology was performed by hot-start PCR using LightCycler FastStart DNA Master SYBR Green I Kit (Roche Diagnostics®, Meylan, France) in a LightCycler™ (Roche Diagnostics®).

The extracted DNA (5 μl) was added to 15 μl of reaction mixture containing 4 mM of MgCl2, 0.5 μM of each primer (JW13 and JW14), 2 μl of LightCycler FastStart DNA Master SybrGreen I buffer (Roche Diagnostics®). Conditions for cycling were similar as previously mentioned,[21] and PCR product identity was confirmed with melting curve analysis.

To ensure the reproducibility of the assay, three positive controls (DNA from *L. major, L. infantum* and *L. tropica* reference strains), were included in each run. A negative control (uninfected sample), and a contamination control (water) were also added.

4. Results

4.1. ITS1-PCR-RFLP

DNA from 26 out of the 27 examined samples (96.3%) was amplified and presented restriction band patterns correlated to reference strains. *L. major* was identified in 15 samples, *L. tropica/killicki* in six samples and *L. infantum* in five. PCR-RFLP identification correlated with that of isoenzyme analysis and DNA sequencing performed respectively in 13 cases (eight characterized as *L. major*, three as *L. killicki* and two as *L. infantum*) and three cases (two as *L. killicki* and one as *L. infantum*).

4.2. kDNA-RT-PCR

The post-amplification melting temperature (Tm), related to the sequence of the PCR products, allows the discrimination between the three positive controls included in each run. *L. major*, *L. infantum* and *L. tropica* could be easily differentiated by their Tm values. Their Tms were, respectively, $87 \pm 0.5\,^{\circ}C$ for *L. major* (MHOM/ SU/73/5-ASKH), $89.3 \pm 0.3\,^{\circ}C$ for *L. infantum* (MHOM/TN/80/IPT) and $88.5 \pm 0.2\,^{\circ}C$ for *L. tropica* (MHOM/SU/74/K27). The variability between different runs did not exceed 0.57%. The identification of *Leishmania* species from clinical samples was realised by comparison to the Tm values of the positive controls.

The specificity of the RT-PCR was confirmed by the lack of Tm with water control and DNA extracted from human healthy skin tissue (data not shown). Eighteen out of the 27 examined samples had Tms conforming to that of reference strains; 17 corresponded to *L. major* and one to *L. tropica/ killicki* (Table 1). Four samples, presenting a Tm at $84.3 \pm 0.3\,^{\circ}C$ different from reference strains Tm values, were associated to *L. infantum* as previously described[20] (Table 1). One out of those four samples was tested using isoenzyme analysis and/or DNA sequencing and confirmed as *L. infantum* species (Table 1). Overall, the identification of *Leishmania* species by kDNA-RT-PCR was achieved in 22 out of the 27 examined samples (81.5%). Two of the five non-identified samples were undetermined exhibiting an overlapping Tm with that of reference strains, and DNA amplification failed in three cases.

4.3. ITS1-PCR-RFLP and kDNA-RT-PCR

The agreement between the ITS1-PCR-RFLP and the kDNA-RT-PCR for the identification of *Leishmania* species was 70% (19/27): 14 samples corresponding to *L. major*, one to *L. tropica/ killicki* and four to *L. infantum* (Table 1). Identification correlated with isoenzyme or molecular typing when performed (Table 1). Using ITS1-PCR-RFLP, identification failed in one sample, corresponding to *L. major* by kDNA-RT-PCR and isoenzyme analysis (Table 1). Using kDNA-RT-PCR, identification failed in five samples. The three non-amplified samples were identified by PCR-RFLP and isoenzyme typing as *L. major*, *L. tropica/killicki* and *L. infantum* respectively.

The two undetermined samples were characterized as *L. tropica killicki* by ITS1-PCR-RFLP and confirmed by DNA sequencing and/or isoenzyme typing. Two samples were mis-identified, associated to *L. major* species by melting curve analysis but identified as *L. killicki* using isoenzyme analysis and/or DNA sequencing.

5. Discussion

With the advent of the PCR technology, several PCR based assays for species differentiation were developed.[14–19] Many different PCR techniques and targets were used for this purpose, complicating the comparison between PCR identification results. Most molecular diagnostic methods applied for species identification were based on PCR-RFLP and RT-PCR. Several studies reported the efficiency of PCR-RFLP assay in identifying *Leishmania* species directly from clinical material.[14–19] PCR assay is clearly less laborious than an isoenzyme analysis, the gold standard method. It avoids the need of parasite isolation, but this assay is less reproducible and provides complex band patterns not easily computerizable, exchangeable and comparable between different laboratories.

Several DNA targets were used for DNA amplification, such as the SSU rRNA gene[24], the ITS regions[15], the microsatellite DNA[25] or extrachromosomal DNA, such as the repetitive kinetoplast DNA (kDNA) minicircles.[11] Related to the sequence variation in the first part of the spacer, the ITS1-DNA target allows the identification of almost all medically relevant *Leishmania* parasites with the

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Table 1
ITS1-PCR-RFLP and kDNA-RT-PCR results for the identification of *Leishmania* species in 27 skin-lesions.

Sample no.	PCR-RFLP result	RT-PCR result	Isoenzyme analysis[a]	DNA sequencing[a]
1–3, 6, 7, 11, 12, 14	*L. major*	*L. major*	*L. major* MON-25	NA
4, 5, 8–10, 13	*L. major*	*L. major*	NA	NA
15	Non amplified	*L. major*	*L. major* MON-25	NA
16	*L. major*	non amplified[b]	NA	NA
17	*L. tropica*	*L. tropica*	NA	NA
18	*L. tropica*	*L. major*[c]	*L. killicki* MON-8	*L. killicki*
19	*L. tropica*	*L. major*[c]	*L. killicki* MON-8	NA
20	*L. tropica*	undetermined[d]	*L. killicki* MON-8	*L. killicki*
21	*L. tropica*	undetermined[d]	NA	*L. killicki*
22	*L. tropica*	non amplified[b]	NA	NA
23–25	*L. infantum*	*L. infantum*[e]	NA	NA
26	*L. infantum*	non amplified[b]	*L. infantum* MON-24	NA
27	*L. infantum*	*L. infantum*[e]	*L. infantum* MON-24	*L. infantum*

ITS1: internal transcribed spacer 1; PCR: polymerase chain reaction; RFLP: restriction fragment length polymorphism; RT: real-time
[a] Isoenzyme analysis and DNA sequencing were not performed on all samples; NA: Not performed
[b] Non amplified samples: samples presenting no melting temperature (Tm) on RT-PCR
[c] Mis-identified samples on RT-PCR (related to isoenzyme and/or DNA sequencing results)
[d] Undetermined samples: samples exhibiting an overlapping Tm with that of reference strains on RT-PCR
[e] samples presenting a Tm value at 83.4+/−0.3 °C on RT-PCR, associated to *L. infantum* species as previously described[21] or after DNA sequencing and isoenzyme analysis.
The total of non amplified samples ([b]) and undetermined samples ([d]) corresponds to non-identified samples on RT-PCR.

use of only one restriction enzyme (HaeIII) for amplicon digestion.[15] However, ITS1-PCR-RFLP is less sensitive compared to kDNA minicircles since the copy number of rDNA (less than 200) is lower than the copy number of kDNA minicircles (tens of thousands).

RT-PCR technology presents some advantages in terms of sensitivity and reliability, reduces the risk of DNA contamination and avoids the use of the carcinogen, ethidium bromide. Moreover, this assay offers accurate parasite detection, species identification with melting curve analysis of fluorescent PCR products and specific DNA quantification.[20–22] Related to its heterogeneity, in terms of size and sequence, the kinetoplast DNA (kDNA) is a good target for the detection and the identification of *Leishmania* parasites.[26–28]

According to the species previously reported in Tunisia and to their geographical distribution, the three endemic species, *L. major*, *L. infantum* and *L. killicki* were identified.[29,30] ITS1-PCR-RFLP and kDNA-RT-PCR allowed species identification in, respectively, 96.3% (26/27) and 81.5% (22/27) of the 27 analyzed samples. RT-PCR succeeded to amplify and identify *L. major* in one sample missed by PCR-RFLP.

Although increased sensitivity of kDNA-RT-PCR was previously reported,[11] this assay succeeded in our study to amplify and to identify only one sample missed by PCR-RFLP. This could be related to the sample's conservation and the quite lengthy delay between the applications of both PCR assays. Since RT-PCR is an expensive method compared to RFLP-PCR, we suggest its use when the latter fails to amplify *Leishmania* DNA.

Nevertheless, the differentiation of the parasite species by PCR is not always obvious. The difficulty in separating *L. major* from *L. tropica* using PCR-RFLP has been previously reported[31] and the melting temperature analysis could sometimes be difficult in RT-PCR assay. In our study, in comparison to PCR-RFLP and/or isoenzyme analysis, kDNA-RT-PCR failed in identifying the Tunisian *L. tropica* MON-8 (*L. killicki*) in five samples: two samples undetermined presenting a Tm overlapping with that of reference strains, two samples presenting a Tm corresponding to *L. major* and one was non amplified. A recent method based on High Resolution Melting Analysis allows the differentiation between *L. tropica* and *L. major* and should be an interesting alternative for a better species analysis.[22]

According to the results of DNA sequencing and a previous report,[21] four samples, showing a Tm value at 84.3 ± 0.3 °C different from the references strains, were considered as *L. infantum*. The shifted and the overlapping Tm values may account for kinetoplast DNA polymorphism among *Leishmania* strains, which causes a variation of the amplified sequence and consequently of the corresponding melting temperature. Moreover, kDNA genotypes are dependent on the experimental conditions[32] and standardization of kDNA RT PCR assay is needed to allow interlaboratory comparisons and maximize repeatability.

The genetic heterogeneity was reported in *L. infantum* kDNA,[33] however, among *L. tropica* strains, it was only reported in Rdna and microsatellites.[34,35] Additional investigations on *L. tropica* kDNA polymorphism will be required to clarify those findings. To overcome this difficulty, we suggest the use of more appropriate reference strains in each RT-PCR run. Finally, the absence of DNA amplification observed for three samples using RT-PCR may be due to deterioration of extracted DNA related to time, storage and carriage. In fact, samples were first tested in Tunisia using PCR-RFLP and sent many months after to France for RT-PCR identification. These conditions interfere with PCR output (amplification curve) and with reproducibility of results (melting curve differences) and consequently may explain the difference in DNA amplification between the two PCR assays.[36] A prospective study could avoid this problem.

In conclusion, PCR-RFLP and RT-PCR represent an interesting alternative to isoenzyme analysis for the identification of CL species directly from clinical samples. To our knowledge, this is the first study describing the

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simultaneous application of molecular diagnostic tools for *Leishmania* species identification in patients with cutaneous leishmaniasis. Even if conducted on a small sampling, such study is required to evaluate the best assay performance for implementation in areas where the disease is endemic. However, a prospective study conducted on larger sampling would be necessary to assess the real efficiency and usefulness of ITS1-RFLP-PCR and kDNA-RT-PCR for the identification of cutaneous *Leishmania* species.

Authors' contributions: AB, KA and SP contributed to the design of the study. KA collected the samples. IBA carried out the RT-PCR. FDM and IBA analysed and interpreted the RT-PCR data. NB carried out the RFLP-PCR. AB and NB analysed and interpreted the RFLP-PCR data. FDM and IBA drafted the manuscript. All authors revised, read and approved the final manuscript. FDM is guarantor of the paper.

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