Original Article

PCR detection of *Leishmania* in skin biopsies

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Abstract

Introduction: Cutaneous leishmaniasis (CL) is an endemic disease and one of the major health problems in Morocco. In 2006, the recorded total number of cases of CL was 3361, occurring predominantly in the rural population. A new and more sensitive diagnostic technique than current methods used is needed in this setting. The aim of this study was to assess the efficacy of polymerase chain reaction (PCR) to detect leishmanial parasites in skin biopsies of patients from different areas of endemicity in Morocco.
Methodology: Biopsies from 26 patients with cutaneous ulcers suggestive of leishmaniasis were analysed by PCR using primers from the small subunit ribosomal gene. The ability of PCR to detect *Leishmania* was compared with smear-stained and *in vitro* culture.
Results: PCR exhibited superior sensitivity (84,6%) compared with direct microscopy smear (69,2%) and *in vitro* culture (69,2%). Our PCR assay also showed good specificity (100%).
Conclusions: PCR should be considered a valuable, sensitive, and faster diagnostic tool in the diagnosis of cutaneous leishmaniasis, especially for those patients with negative parasitologic examination.

Key Words: *Leishmania*, polymerase chain reaction, diagnosis, cutaneous leishmaniasis

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Introduction

Leishmaniasis is a disease found throughout the world and 350 million people are at risk. It is estimated that there are 12 million cases in the world with 1.5 to 2 million new cases occurring each year, of which 1 to 1.5 million correspond to cutaneous leishmaniasis and 500,000 to visceral leishmaniasis [1]. In Morocco, the total cutaneous leishmaniasis cases reported in 2006 were 3,361 against 655 cases in 1998, showing a clear tendency for an increase in the last decade [2].

Cutaneous leishmaniasis (CL) is the clinical manifestation in which the parasite causes one or more slow-healing ulcers on the skin. The Old World *Leishmania* species causes benign and self-limiting ulcers. The New World species causes manifestations of greater variety, of which mucocutaneous leishmaniasis is the most severe.

The diagnostic methods available at present are mostly based on clinical and epidemiologic evidence and parasite detection. Up to now, no single laboratory method has been accepted as the gold standard for diagnosing CL. Parasitologic tests of a skin biopsy specimen are not always conclusive in patients with a clinical diagnosis of cutaneous leishmaniasis [3].

Several PCR assays have been developed for the detection of the *Leishmania* parasite. PCR-based methods often have high sensitivities [4-6]. The *Leishmania*-specific PCR primers may amplify either repeated nuclear sequences, including ribosomal [7,8], miniexon [9] and repetitive nuclear DNA [10], or minicercle kDNA which is present at approximately 10,000 copies per parasite. The kDNA primers may amplify either the entire minicircle, or portions of the conserved and variable regions [11].

Among the sequences belonging to the multicopy group, the sequence of the small subunit of ribosomal RNA gene (SSU rRNA) or 18S rRNA gene is one of the best studied. Each

parasite contains a large number (about 160) of copies of 18S rRNA gene. van Eys *et al.* [7] have chosen this central part of the SSU rRNA gene for the development of a sensitive detection system for *Leishmania* DNA which can be used as target for a PCR assay.

In this work, we report the uses of PCR primers developed by van Eys *et al.* [7]. We evaluated the ability of PCR to detect *Leishmania* DNA in skin biopsy specimens from 26 patients clinically suspected of having cutaneous leishmaniasis and living in different areas of Morocco.

Materials and Methods

Patients and clinical samples

Clinical specimens were obtained from twenty-six patients presumed to have cutaneous leishmaniasis. Twenty-three patients were from rural areas from the south of Morocco (Tanant, Smimou and Talsint) and three patients were from rural areas around the cities of Taounate and Fes in the north of Morocco. All patients analyzed in this study lived in these endemic areas and were selected considering epidemiological risk factors for cutaneous leishmaniasis as well as signs of the disease. A complete dermatological examination (by a dermatologist) was performed and all patients presented cutaneous lesions suggestive of leishmaniasis.

Lesions and the adjacent normal-looking skin around them were cleaned and sterilized with disinfectant. Skin biopsies of 2 to 4 mm in diameter were taken aseptically from the border of the ulcer, using a disposable scalpel blade. A small incision was made in the cleaned margin of lesions with the point of the blade. The blade was turned 90 degrees and scraped along the cut edge of the incision to remove and pick up skin tissue which was divided into three parts. One part was used for smear, one for culture and the third was stored at -80°C until used for PCR analysis.

As negative controls, five skin biopsy samples were collected from patients with other similar lesions of CL, such as leprosy (1), psoriasis (1), tuberculosis (1), trichophytosis (1), and vascular ulcer (1).

Direct examination

The smears were prepared by touching the biopsy to a glass microscope slide. After the smears had dried completely, they were fixed with absolute methanol (Scharlau, Spain), allowed to dry again, and stained with Giemsa (Avicenne Groupe, France). The whole slide was analyzed with a 100X immersion objective. All of the slides were examined twice before confirming or determining a negative result.

Culture of Leishmania

The second portion of each biopsy was placed in culture. Tissue fragments were homogenized in a sterile plastic pestle. This material was used to inoculate four tubes containing the biphasic culture medium NNN. The inoculated media were kept at 25°C. The presence of promastigotes was carefully observed weekly by microscopy. A culture was considered positive, when at least one promastigote was observed microscopically, and negative if no parasites were found within one month.

Preparation of Leishmania DNA for PCR amplification

Frozen biopsy samples were thawed and incubated at 65°C for two hours in 200 μl 10 mM Tris HCl (Fluka, Switzerland) (pH8.0)/ 10 mM EDTA (Avicenne Groupe, France)/ 10 mM NaCl (Sigma, Japan) (NET 10), 1% SDS (Sigma, Japan) and 100 ug/ml of proteinase K (Invitrogen, Brazil) [7] followed by two phenol (Scharlau, Spain) extractions and ethanol (Sharlau, Spain) precipitation. The precipitate was dissolved in 20 μl of distilled water.

Polymerase chain reaction (PCR)

The primers used for the detection and identification of *Leishmania* parasite are located in the small subunit of the ribosomal gene as described by van Eys *et al.* [7]: 5'-GGTTCCTTTCCTGATTTACG -3', 5'-GGCCGGTAAAGGCCGAATAG- 3'. The reaction mixture consisted of 1x DNA polymerase buffer (Promega, USA), 100 μM dNTP (Promega, USA) and 150-180 pM of each primer (Bioprobe, France), 2 μl sample DNA, 0.5 U Taq polymerase (Promega, USA) in

a final volume of 50 µl. Each reaction was overlaid with 50 µl of mineral oil. Thirty-two cycles were performed in a thermocycler (Perkin Elmer Cetus). Each cycle consisted of 94°C denaturation (75 s), 60°C annealing (75 s), 72°C extension (2 m) [7]. In all assays, positive controls containing *Leishmania infantum* (MHOM/TN/80/IPT1), *Leishmania tropica* (MHOM/SU/74/K27) and *Leishmania major* (MHOM/SU/73/5ASKH) DNA and a negative control without DNA were included. Ten µl of the reaction mixture were visualised by 2% agarose gel electrophoresis (Scharlau, Spain).

The amplified product was digested with 10 units of RsaI (Promega, USA). Samples were run on a 2% agarose gel with reference strains *Leishmania infantum* (MHOM/TN/80/IPT1), *Leishmania tropica* (MHOM/SU/74/K27) and *Leishmania major* (MHOM/SU/73/5ASKH).

Diagnostic criteria for determining cutaneous leishmaniasis (CL)

Specimens were considered confirmed positives when cultures or stained tissue smears were positive for parasites. The sensitivity of the PCR test was assessed with samples from the patients with confirmed cutaneous leishmaniasis, whereas the specificity was calculated on the basis of the results for patients without leishmaniasis living in leishmaniasis-free regions.

Results

Detection of Leishmania parasites

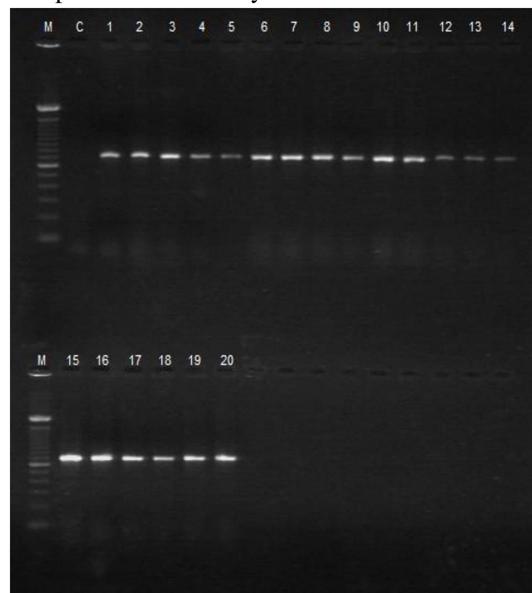
Specimens from 26 suspected cutaneous leishmaniasis patients in Morocco were examined by three diagnostic techniques.

One third of each biopsy was examined microscopically on the same day for the presence of amastigote. The second third was used for *in vitro* culture, and the last third was stored at -80°C for PCR processing on a later day.

Five samples from individuals with other skin diseases clinically similar to CL were also included.

For the PCR, we used the SSU rRNA gene present as 160 copies in the nuclear DNA and yielding a PCR product 650 bp in length. The PCR results were generally available within 24 hours.

Figure 1. Agarose gel electrophoresis of PCR amplification of extracted SSU rRNA gene DNA from patients infected by *Leishmania.*



M: DNA Marker (Invitrogen, Brazil). C: negative control with no template in the reaction. Lanes 1, 2 and 3 reference strains of *L. major* (MHOM/SU/73/5ASKH), *L. tropica* (MHOM/SU/74/K27) and *L. infantum* (MHOM/TN/80/IPT1). Lanes 4-20: patients living in endemic areas of leishmaniasis and infected by *Leishmania* : TN2, TN4, TN5, SM3, SM4, SM5a, SM5b, SM7a, SM8, NJ1, Y1, Y4, Y7, Y9, Y10, MR1and KH1

PCR amplification of Leishmania DNA from patients with confirmed cutaneous leishmaniasis

The results obtained from each assay were compared (Table 1). As defined by the consensus standards, both parasite cultures and microscopic examination of smears were highly specific for the diagnosis of CL, and when analyzed together, they correctly identified 13/26 of the suspected specimens. However, eight of the positives specimens were detected by one method but not the other, showing that for greater efficacy, they should be used together. The sensitivity of the both assays was 69%.

Out of the 13 patients with proven cutaneous leishmaniasis (positive by culture and/or microscopy), 11 samples were also positive by PCR (Table 1). Our results clearly show that PCR had the highest sensitivity of any individual assay, correctly diagnosing 84,6% of patients with confirmed cutaneous leishmaniasis and missing 2 specimens. These two false negatives could be due to PCR inhibition (Table 2).

Biopsies from patients with skin diseases other than cutaneous leishmaniasis all gave

Table 1. Results of parasitologic methods and PCR assay for diagnosis of cutaneous leishmaniasis in twenty six patients with clinically suspected leishmaniasis.

Sample code	Smear	Biopsies culture	Diagnosis by currents methods	PCR
TN1	neg	neg	none	neg
TN 2	neg	POS	+ CL	POS
TN3	neg	neg	none	neg
TN4	neg	neg	none	POS
TN5	POS	POS	+ CL	POS
TN6	neg	neg	none	neg
SM1	neg	neg	none	neg
SM2	neg	neg	none	neg
SM3	neg	neg	none	POS
SM4	neg	neg	none	POS
SM5a	neg	neg	none	POS
SM5b	POS	neg	+ CL	POS
SM6	neg	neg	none	neg
SM7a	neg	neg	none	POS
SM7b	neg	neg	none	neg
SM8	POS	neg	+ CL	POS
NJ1	POS	POS	+ CL	POS
Y1	POS	POS	+ CL	POS
Y4	neg	neg	none	POS
Y7	POS	POS	+ CL	POS
Y9	POS	POS	+ CL	POS
Y10	neg	POS	+ CL	POS
Y12	neg	POS	+ CL	neg
Y13	neg	POS	+ CL	neg
MR1	POS	neg	+ CL	POS
KH 1	POS	neg	+ CL	POS

neg: negative; POS: positive, +CL: confirmed cutaneous leishmaniasis (smear and /or culture positive), none: non-confirmed cutaneous leishmaniasis

Table 2. Comparison of the abilities of various techniques: smear, culture and PCR to detect *Leishmania* parasite in ulcer biopsies from 13 patients with confirmed cutaneous leishmaniasis (smear stained and /or culture positive).

Methods compared	Positive of total	%
PCR	11 of 13	84,6%
Smear	9 of 13	69,2%
Biopsy culture	9 of 13	69,2%

Table 3: Clinical sensitivity and specificity of the PCR method for diagnosis of leishmaniasis.

Disease statute	No PCR positive	No PCR negative	Total
Confirmed leishmaniasis*	11	2	13
Other cutaneous disease**	0	5	5
Sensitivity	11 of 13 = 84,61%		
Specificity	5 of 5 = 100 %		

*: Leishmaniasis confirmed by currently recommended diagnostic methods (smear microscopy and/or culture); patients with non-leishmanial ethiology confirmed are excluded.
**: Patients with other diseases than leishmaniasis

negative results. The PCR used in this study had a specificity of 100% (Table 3).

PCR amplification of Leishmania DNA from suspected but unproved case of CL

Among 13 patients with suspected but unproved cutaneous leishmaniasis, PCR detected *Leishmania* DNA from 6 patients who were negative for smear and culture, thus showing the failure of these two methods in diagnosing cutaneous leishmaniasis, even when they were associated.

The nine negative PCR samples were purified by two- four- and 10-fold dilution, to eliminate possible PCR inhibition, but they remained negative.

Restriction enzyme analysis of PCR product

RsaI digestion of *L. infantum* gave three fragments, 325, 250 and 85 base pairs . RsaI digestion of *L. tropica* and *L. major* results in two fragments of 400 and 200 base pairs. We obtained one strain of *L. infantum* (NJ 1) and 16 of *L. major* or *L. tropica.*

Discussion

The diagnosis of CL is often difficult. Although the traditional diagnostic methods such as *in vitro* culture, smear, and direct examination are easily employed, they require the presence of relatively high numbers of viable or morphologically intact micro-organisms. The values reported in this study for diagnosis of CL by microscopy (69, 23%) or parasite culture (69, 23%) are comparable to those reported by others groups [10,12,13]. Sensitivity of microscopic techniques, *i.e.*, histopathology and tissue smears, touch preparations and exudates, has been reported to range from 17% to 83% for CL [14-18] depending on clinical presentation, parasite species, technical expertise, and other factors. Likewise, sensitivity of culturing parasites has been reported to vary from 27% to 85% [14,16,17]. In addition, it can take several days to weeks until parasites are observed, depending on the species and number of parasites seeded at the time of the biopsy, and cultures may be contaminated, in some cases reaching 30% of the samples.

Several studies have compared the PCR diagnosis with conventional techniques. Except for a few cases, PCR-based assays were found to be significantly more sensitive than the classical parasitologic methods of diagnosis. In their study, Akkafa *et al.* [10] demonstrated that PCR had a sensitivity of 96% in diagnosis of cutaneous leishmaniasis. In contrast, direct microscopy smears had a sensitivity of 67%. Ramirez *et al.* [19] reported that conventional diagnostic methods for cutaneous leishmaniasis, such as culture and histopathologic examination of biopsies, were less sensitive, 67.5% and 64.3% respectively, than PCR (90.4%). Our study confirms these findings, showing that PCR was superior to traditional methods for the diagnosis of CL (84,6%), identifying additional patients that had been missed by either microscopic examination or culture.

Two patients whose PCR false negatives (Y12 and Y13 [Table 1]) were positive for cutaneous leishmaniasis by culture, suggesting that additional factors, including sampling site, play a role in determining the outcome of any diagnostic assay for CL. Parasite loads and, correspondingly, diagnostic sensitivities for both PCR and conventional diagnostic assays have been shown to vary spatially within a lesion for CL [6,19].

The sequences from the small subunit ribosomal gene as a template for PCR amplification has an obvious advantage because ribosomal genes are highly repeated (about 160 copies) in *Leishmania* genome [7]. This target has been found to be highly efficient for the diagnosis of leishmaniasis from human clinical material [7,20]. Using ribosomal primers in a PCR Assay, van Eys *et al.* [7] reported that DNA corresponding to less than 10 promastigotes was successfully amplified. In the present study, we used these PCR primers and demonstrated the successful application of this PCR for the detection of *Leishmania* DNA in skin biopsies of Moroccan patients.

Emphasis should be given to the fact that in the group of CL-suspected patients, PCR was positive in six of 13 patients (46%) who were negative for direct microscopy smear and culture, thus showing the failure of these 2 methods in diagnosing CL, even when they were associated. The high sensitivity and specificity of PCR, the case history (i.e., whether the individuals were exposed to risk of acquiring the disease), and the clinical examination of lesions confirmed that these samples are not false positives, but instead true positives that contain very few parasites.

Negative control samples included in every PCR showed no bands owing to contamination. Thus, PCR applied to biopsies was the most sensitive and specific diagnostic assay compared to conventional methods, proving to be a good tool for the differential diagnosis of cutaneous lesions of other etiologies. Accurate diagnosis and effective treatment of the disease in its early stage is important to avoid the development of long-lasting chronic disease and disfiguring scars. It is also of paramount importance for reduction of the human reservoir. Failure to promptly diagnose and treat all cases will result in continued dissemination of the parasite.

After restriction for amplified products of 17 biopsies, we obtained 1 profile of *L. Infantum*; this case came from an area in Taounate province, a focus of cutaneous leishmaniasis due to *L. infantum* [21].

The other 16 biopsies presented the profile of *L. major* and *L. tropica*. Among these 16 biopsies, 9 (TN2, TN4, TN5, SM3, SM4, SM5a, SM5b, SM7a, SM8) came from Tanant and Smimou, Northern Slope of the High Atlas, which is an endemic zone of cutaneous leishmaniasis to *L. tropica* [22]. TN2 and TN5, which were culture positive, were identified as *L. tropica* by enzyme electrophoresis analysis of 15 enzymes (data not shown). Five biopsies (Y1,Y4,Y7,Y9,Y10) came from southern Morocco, a focus of cutaneous leishmaniasis to *L. major* [23]. Isolates Y1,Y7,Y9,Y10,Y12 and Y13 were characterized by isoenzyme electrophoresis as *L. major* (data not shown). MR1 and KH1 were from Fes province, a new focus of cutaneous leishmaniasis due to *Leishmania tropica* [24].

PCR RFLP used in this study gave the same profile for both *L. tropica* and *L. major*, but if we take into account the geographical origin of the patient, we can say it is one or the other species. Indeed in Morocco, these two species have different geographical distributions: *L. major* is largely confined to the arid Saharan region, while *L. tropica* has the largest geographic distribution, having been reported in Azilal, Essaouira, Taza, Fes and central Morocco [25]. The ability to identify species is especially important to the prognosis of the disease and in deciding appropriate therapy.

In conclusion, our data confirm the value of PCR as an alternative laboratory method for diagnosing CL, particularly in those cases where conventional techniques failed to detect the disease. In term of clinical utility, PCR offers several advantages: it is highly sensitive and specific, and, most importantly, it is more rapid than the current conventional methods. However, major hurdles, such as cost and the need of laboratory facilities, must be overcome before this approach can be implemented in endemic areas.

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