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Comparison of PCR Assays for Diagnosis of Cutaneous Leishmaniasis

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Three PCR assays for diagnosing leishmaniasis were compared and validated against parasite cultures and microscopic evaluation of stained tissue smears using 92 specimens from suspected cases of cutaneous leishmaniasis (CL) in Israel and the West Bank. Samples from imported and locally acquired disease were examined. The kinetoplast DNA (kDNA) PCR showed the highest sensitivity (98.7%) of any assay, correctly diagnosing 77/78 of the confirmed positive samples, followed by the rRNA gene internal transcribed spacer 1 (ITS1) PCR (71/78 positive, 91.0% sensitivity) and then the spliced leader mini-exon PCR (42/78 positive, 53.8% sensitivity). Either parasite culture or microscopy alone detected 62.8% (49/78) or 74.4% (58/78) of the positive specimens, respectively, while culture and microscopy together improved overall sensitivity to 83.3% (65/78). Except for the kDNA PCR that had six false positives, all other assays were 100% specific. Further, restriction enzyme analysis of the ITS1 PCR product enabled identification of 74.6% of the positive samples, which included strains of *Leishmania major* (50.9%), *Leishmania tropica* (47.2%), and the *Leishmania braziliensis* complex (1.9%). This suggests that a PCR using kDNA should be used for the diagnosis of CL and that an ITS1 PCR can be reliably used for the diagnosis of CL when rapid species identification is needed.

Leishmaniasis is endemic in more than 88 countries and threatens more than 350 million people (9, 10). At least 21 species and subspecies of *Leishmania* have been recorded as being infective to humans, many of which cause extensive morbidity and, in some cases, mortality. Together, they are responsible for a wide spectrum of clinical symptoms (26, 27). Correct diagnosis and characterization of the particular parasite is important for evaluating prognosis and prescribing appropriate treatment (5, 18, 39). Until recently, diagnosis was based primarily on clinical symptoms, microscopic observation of parasites in stained tissue smears, and/or culture of promastigotes from tissue (18, 39). In those cases where promastigotes were cultured, additional serological, biochemical, biological, and other techniques had to be used to characterize the parasites (12, 30, 37, 41). Even today, microscopic identification and parasite cultivation are still primary diagnostic tools employed in many regions where leishmaniasis is endemic. When positive cultures are obtained, it may take long periods, possibly weeks, until sufficient parasites are available for species characterization by multilocus enzyme electrophoresis or other techniques.

Culture of promastigotes from infected tissues and/or direct identification of amastigotes in microscope smears has long been considered the standard for diagnosis. While these techniques are highly specific for diagnosing leishmaniasis, they are not sensitive. The different species of *Leishmania* are not equally easy to culture; contamination is a constant hazard, and variations in efficacy among different growth medium formulations or even batches may be encountered. Likewise, the percent success for microscopic identification of amastigotes in stained preparations varies depending on the number of parasites present and/or the experience of the person examining the slide (18). Unfortunately, today there is no single widely accepted standard procedure that can be used as a basis for evaluating new molecular diagnostic assays for leishmaniasis, though PCR methods using either genomic or kinetoplast DNA (kDNA) are now frequently cast in this role.

Many different PCR targets, including the coding and intergenic noncoding regions of the gp63 gene locus, splice leader mini-exon (SLME), and the SSU rRNA gene, have been used for the identification of parasites from cultures and for their direct detection in various animal, sand fly, and human tissues (6, 8, 20, 38, 40). Sensitivity is correlated with the copy number of the amplified region. The kDNA PCR is considered to be the most sensitive method for diagnosing leishmaniasis since there are ~10,000 minicircles per parasite. However, these reactions generally either amplify genus- and subgenus-specific conserved regions or require separate primer pairs for each species of *Leishmania* (1, 11). Diagnostic PCR assays using the internal transcribed spacer 1 (ITS1) region of the rRNA genes (40 to 200 copies) and the SLME (100 to 200 copies) have been shown to be sensitive methods for detecting cutaneous leishmaniasis (CL) (8, 20, 36). When either amplicon is digested with restriction enzymes, it is possible to identify almost all pathogenic *Leishmania* species by restriction fragment length polymorphism (RFLP), allowing direct, rapid characterization and identification of the infecting parasite.

While a number of studies have examined the sensitivity and specificity of PCR assays against conventional diagnostic techniques for CL, only a few studies have evaluated these parameters among different PCR assays. In this study, we compared the sensitivities and specificities of three PCR assays (kDNA, SLME, and ITS1) used for parasite identification with leishmanial culture and microscopic detection in order to validate these PCR techniques for the molecular diagnosis of CL.

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MATERIALS AND METHODS

Sampling. Samples were taken from 92 patients referred to the Dermatology Department of the Hadassah Hospital, Jerusalem, Israel, with suspected CL. Most patients were infected in either Israel or the West Bank region ($n = 83$). The remaining cases were infected during travel either in South America ($n = 4$) or elsewhere in the Middle East ($n = 3$). The location in which two of the patients acquired their infections was not known. Ages varied from 2 to 75 years. Males comprised 63% and females 37% of the population.

Lesions and the adjacent normal-looking skin around them were cleaned and sterilized with disinfectant. Sterile saline (0.1 to 0.2 ml) was drawn into a syringe (1-ml, 25-gauge needle), and the needle was inserted into the nodule or ulcer's margin and rotated gently several times. A small amount of saline was expressed into the tissue, the needle was rotated, and some tissue aspirate and freed tissue were withdrawn. The syringe was removed from the lesion, and some of its contents were expressed into tubes containing semisolid normal rabbit blood agar medium (33). Tissue for making stained smears was taken using a disposable scalpel blade (no. 11). A small incision was made in the cleaned margin of nodules and 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 smeared on a clean glass microscope slide. After smears had dried completely, they were fixed with 100% methanol, allowed to dry again, and stained with Wright's Giemsa for microscopic examination. Sterile Whatman 3 MM filter papers were touched onto the lesion at the site of the cuts, allowed to air dry thoroughly, and individually wrapped in aluminum foil. Samples on filter papers were analyzed by PCR blindly. Filter papers were stored with silica gel at 4°C until DNA extraction, which was routinely carried out 2 to 14 days after sampling. Eleven samples stored on filter papers before the onset of this study were extracted 23 to 33 months after sampling. Cultures were incubated at 26°C and examined for parasite growth by phase microscopy at 320× magnification every 3 to 4 days until promastigotes were seen or up to 1 month before being discarded as negative. The stained tissue smears were examined for amastigotes by light microscopy using a Zeiss microscope at 400× magnification. The Helsinki Committee for Human Research of the Hadassah Hospital, Ein Kerem, Jerusalem, Israel, approved this study.

DNA extraction and PCR analysis. Each specimen was cut from the filter paper with a disposable sterile scalpel and incubated in 250 μl cell lysis buffer as previously described (36). DNA was extracted from the lysates with phenol-chloroform, and the pellets were air dried. After being dissolved in 50 μl Tris-EDTA buffer (10 mM Tris and 1 mM EDTA, pH 8.0), the DNA was kept at 4°C until analyzed by PCR. Clean filter paper was used as a negative control for DNA extraction. Each specimen was analyzed using three different pairs of PCR primers. The sensitivity of each PCR was optimized on pure *Leishmania* DNA prior to use for diagnosis. The SLME PCR was carried out as described by Marfurt et al. (20), using the forward and reverse primers Fme (5′-TAT TGG TAT GCG AAA CTT CCG-3′) and Rme (5′-ACA GAA ACT GAT ACT TAT ATA GCG-3′), respectively, except that 75 mM KCl and 2.5 U *Taq* polymerase were added in order to optimize the reaction. The ITS1 PCR using the primers LITSR (5′-CTG GAT CAT TTT CCG ATG-3′) and L5.8S (5′-TGA TAC CAC TTA TCG CAC TT-3′) was carried out as described by Schonian et al. (36), except that 300 nM primers, 1.5 mM $MgCl_2$, and 2.5% dimethyl sulfoxide were used. The kDNA PCR using the primers 13A (5′-GTG GGG GAG GGG CGT TCT-3′) and 13B (5′-ATT TTC CAC CAA CCC CCA GTT-3′) was carried out essentially as described by Reale et al. (29), except that the reaction was carried out in 50 μl, using 135 μM deoxynucleoside triphosphates. Leishmanial DNA (20 ng) isolated from reference strains (see below) was used as a positive control. Reaction buffers without leishmanial DNA were also included as negative controls in each PCR analysis. All PCRs were carried out in a 50-μl volume, using the optimal annealing temperatures, concentrations of primers, deoxynucleoside triphosphates, Mg ions, *Taq* polymerase, and additives as necessary. Inhibition was monitored when all PCRs were negative by adding a control plasmid (leishmanial protein kinase A gene flanked by the 5′ and 3′ ITS1 and SLME PCR primers; 100 ng/reaction; product size, 784 bp) to patient DNA extracted from the filter papers and by carrying out separate PCRs. Inhibition control reactions, using the conditions described above for each primer pair with plasmid or patient DNA alone, were carried out in parallel (not shown).

Amplicons were analyzed on 1.5% agarose gels by electrophoresis at 100 V in 1× Tris-acetate-EDTA buffer (0.04 M Tris-acetate and 1 mM EDTA, pH 8.0) and visualized by UV light after being stained with ethidium bromide (0.3 μg/ml). A PCR was considered positive when a band of correct size (kDNA, ~120 bp; ITS1, ~300 to 350 bp; and SLME, ~220 to 443 bp) was observed. Product sizes of the ITS1 and SLME PCRs vary with *Leishmania* species (20, 36).

Leishmanial species DNA markers were prepared from promastigotes of *Leishmania major* (MHOM/PS/1998/ISL389), *Leishmania tropica* (ISER/IL/1998/LRC-L747), *Leishmania braziliensis* (MHOM/BO/2000/LRC-L785), *Leishmania donovani* (MHOM/IN/1980/DD8), and *Leishmania infantum* (MCAN/IL/1997/LRC-L720). These strains were obtained from the WHO Reference Centre, Jerusalem, Israel, and their DNA was prepared as previously described (36).

TABLE 1. Sources of samples analyzed and species of *Leishmania* identified by PCR

Geographic location	No. of C-Pos samples[b]	No. of C-Neg samples[c]	*Leishmania* species[d]
Israel	37	8	*L. major, L. tropica*
West Bank	34	4	*L. major, L. tropica*
South America	4	0	*L. braziliensis* complex
Other[a]	2	1	*L. major*
Unknown	1	1	*L. tropica*
All	78	14	*L. major, L. tropica, L. braziliensis* complex

[a] Other locations include Egypt, Jordan, and Afghanistan.
[b] Positive result by parasite culture, stained smear examination, or at least two PCR assays.
[c] Negative in all assays or positive in only one PCR assay.
[d] Species in positive samples, as identified by the ITS1 or SLME PCR and RFLP.

RFLP analysis of the ITS1 PCR amplicon. PCR products (8 to 20 μl) were digested with BsuRI (MBI Fermentas), a HaeIII prototype, according to the manufacturer's instructions, and the restriction fragments were analyzed by gel electrophoresis at 120 V in 1× Tris-acetate-EDTA buffer in 2.5% agarose gels (FMC BioProducts, Rockland, ME). The fragments were visualized by UV light and the sizes of the restriction products determined.

Serological characterization. Excreted factor (EF) serotyping was carried out using spent medium from cultures of the isolated parasite and comparing the reactions to standard reference EFs as previously described (34, 35).

Statistics. Specimens were considered confirmed positives (C-Pos) when cultures or stained tissue smears were positive for parasites or at least two PCR assays were positive for leishmanial DNA. When all five assays were negative or only one PCR was positive for parasite DNA, specimens were considered confirmed negatives (C-Neg). These values were used as the "consensus standards" against which each individual diagnostic assay was compared. Data were analyzed using the online statistics calculator at http://www.graphpad.com/quickcalcs/index.cfm. Sensitivity, specificity, positive predictive values (PPV), negative predictive values (NPV), and Cohen's kappa coefficient (κ) were determined. Cohen's kappa coefficient is a measure of the agreement between two tests beyond that expected by chance, where 0 is chance agreement and 1 is perfect agreement (16).

RESULTS

Specimens from 92 suspected CL patients infected in Israel, the West Bank, other Middle Eastern regions, and South America (Table 1) were examined by five diagnostic techniques. Out of the 92 specimens received for diagnosis, 78 samples, 84.8%, were C-Pos and 14 samples, 15.2%, were C-Neg by the consensus criteria (Table 2).

The results obtained with each assay were compared (Table 2). As defined by the consensus standards, both parasite cultures and microscopic examination of smears were highly specific (100%) for the diagnosis of CL, and when analyzed together, they correctly identified 65/78 (83.3%) of the C-Pos specimens. However, 23 of the C-Pos specimens were detected by one method and not the other, showing that for greater efficacy, they should be used together. The individual sensitivity of each assay was lower, 62.8 and 74.4% for cultures and smear examination, respectively, missing 29 (culture) and 20 (microscopy) of the C-Pos samples (false negatives). Bacterial or fungal contamination of cultures was not observed. The

TABLE 2. Analysis of five diagnostic assays for cutaneous leishmaniasis[a]

Assay	No. of samples		% of samples		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	C-Pos	C-Neg	C-Pos	C-Neg				
Culture	49	43	53.3	46.7	62.8	100	100	32.6
Smear	58	34	63.0	37.0	74.4	100	100	41.2
ITS1 PCR	71	21	77.2	22.8	91.0	100	100	66.7
kDNA PCR	83	9	90.2	9.8	98.7	57.1	92.8	88.9
SLME PCR	42	50	45.7	54.3	53.8	100	100	28.0
All	78	14	84.8	15.2				

[a] The sensitivity and specificity for each assay were compared to the total test results.

NPV of culturing (32.6%) or examining smears (41.2%) was low. The specificity and PPV of culturing or examining smears are of little relevance, since a positive result for either was always considered to be a true positive. The level of agreement, Cohen's kappa coefficient ($\kappa \pm$ standard error [SE]), between diagnosis by culture and smear, 0.491 ± 0.102, was only moderate; and the levels of agreement between culture or smear and the confirmed results (C-Pos and C-Neg) were 0.340 ± 0.078 (fair) and 0.469 ± 0.088 (moderate), respectively.

Out of the 65 specimens that were positive by culture and/or microscopy, all except for 1 sample were also confirmed by at least one of the PCR techniques used in this study. Using the kDNA PCR, 83/92 samples were positive (90.2%). The kDNA PCR had the highest sensitivity of any individual assay, correctly diagnosing 98.7% of the C-Pos and missing only 1/78 positive specimens. This one false negative was shown to be due to PCR inhibition. Inhibition was not observed for the other negative samples (data not shown). False positives, positive by kDNA PCR but negative by all the remaining assays, were observed for six samples. The PPV and NPV for this assay were 92.8% and 88.9%, respectively. The measure of agreement, 0.654 ± 0.101, indicates good agreement between the confirmed results and the kDNA PCR.

The ITS1 PCR correctly identified 71/78 of the C-Pos specimens, and the sensitivity of this assay was somewhat lower, 91.0%, than that of the kDNA PCR. No false positives were seen with the ITS1 PCR (specificity = 100%), but this assay did miss six positives that were detected by the kDNA PCR. The PPV and the NPV for the ITS1 assay were 100% and 66.7%, respectively. Agreement between the confirmed results and the ITS1 PCR was good: 0.755 ± 0.101.

Surprisingly, the SLME PCR gave the poorest results of any of the assays used. Only 42/92 samples (45.7%) were positive. While there were no false positives using this technique (specificity = 100%; PPV = 100%), the sensitivity was 53.8% (number of false negatives = 36) and the NPV was 28%. The agreement between the SLME PCR and the confirmed results was only fair: 0.262 ± 0.070.

TABLE 3. Effect of sample storage on efficiency of PCR assays for cutaneous leishmaniasis

Sample type[a]	% of samples							
	SLME PCR		ITS1 PCR		kDNA PCR		Total	
	C-Pos	C-Neg	C-Pos	C-Neg	C-Pos	C-Neg	C-Pos	C-Neg
Old	18.2	81.8	72.7	27.3	90.9	9.1	81.8	18.2
New	49.4	50.6	77.8	22.2	90.1	9.9	85.2	14.8

[a] Old, filter papers containing the patient samples were stored desiccated at 4°C for 2 to 3 years prior to DNA extraction and analysis by PCR. New, DNA was extracted and analyzed within 14 days of sample receipt.

Storage of tissue specimens on different supports was previously shown to affect PCR sensitivity (37). Since 11/92 samples were stored for 2 to 3 years prior to DNA extraction and PCR, the results seen with the diagnostic assays were analyzed separately for each group. Storage of the filters had little or no effect on either sensitivity or specificity and on the percentage of positive and negative samples observed when the ITS1 and kDNA PCRs were carried out. The values for each assay remained essentially unchanged whether the DNA was extracted and analyzed upon receipt or after being stored for 2 to 3 years (Table 3 and data not shown). However, when these same DNA samples were analyzed by the SLME PCR, a drastic drop in sensitivity was observed, from 58% for newly extracted samples to 22% for old samples. This was also reflected in the percentages of positive and negative samples (Table 3). DNA from the same extraction was used in parallel for all three PCR assays, suggesting that the target of the SLME PCR is more sensitive to storage on a solid support prior to extraction, perhaps degrading with time.

Finally, in the case of the ITS1 and SLME PCRs, it was possible to characterize species of *Leishmania* by digesting the amplicon with restriction enzymes, such as HaeIII, and by analyzing the fragment lengths by RFLP (data not shown). Except for the South American samples, this analysis was carried out only with the ITS1 PCR products. Parasite species could be determined for 53/71 ITS1 positives (74.6%) by RFLP and included strains belonging to the *L. braziliensis* complex ($n = 1$; 1.9%), *L. major* ($n = 27$; 50.9%), and *L. tropica* ($n = 25$; 47.2%). Thirty of the specimens where the species of *Leishmania* were identified by RFLP were also positive for promastigotes by culture. EF serotyping, using spent medium from these positive cultures, confirmed the species designation found by RFLP, and the two methods showed 100% correlation (data not shown). The patterns generated after restriction digestion of the PCR amplicons with HaeIII for the remaining ITS1 positives were too weak to see when the agarose gels were stained with ethidium bromide.

DISCUSSION

The use of PCRs has slowly become the preferred way for diagnosing leishmaniasis since conventional parasitological methods are not sufficiently sensitive. The values reported in this study for diagnosis of CL by microscopy (74.4%) or par-

asite culture (62.8%) fall into the high end of the spectrum. 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 (2, 3, 11, 13, 22, 23, 31), 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% (3, 13, 22, 25, 31). In addition, it can take 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 (31).

Several studies of South and Central American CL caused by parasites of the *L. braziliensis* and *Leishmania mexicana* complexes have compared the kDNA PCR diagnosis with conventional techniques. Except for a few cases, PCR-based assays were found to be significantly more sensitive than the classical parasitological methods of diagnosis (3, 11, 23, 31). These findings are supported by a few studies of Old World CL caused by *L. tropica* in Iran (32), *L. major* in Sudan (2), and *Leishmania aethiopica* in Ethiopia (17). Our study confirms these findings, showing that the kDNA PCR is superior to parasitological methods for the diagnosis of CL, identifying additional patients missed by either microscopic examination ($n = 20$) or culture ($n = 29$). However, the sensitivities of conventional diagnostic techniques (culture, 62.5%; microscopy, 74.4%) are improved if both methods (sensitivity = 83.3%) are used in combination. In the absence of PCR-based assays, both parasite culture and microscopic examination should be routinely employed for diagnosing CL.

PCR assays that amplify multicopy nuclear DNA targets, such as the SSU rRNA (40 to 200 copies/cell) and gp63 (7 to 22 copies/cell), have also been used for diagnosis, with good results (38, 40). However, most PCR-based assays using nuclear and kinetoplast DNA targets identify leishmanial parasites only to the generic and/or subgeneric level or, at best, discriminate between a few species in a geographically restricted area (1, 11). Recently, two PCR RFLP assays targeting intergenic regions in nuclear DNA, the SLME and ITS1 PCRs, have been shown to be useful in the diagnosis and identification of pathogenic species of *Leishmania*. While the sensitivity and specificity of PCR assays for detecting leishmanial parasites have frequently been compared to those of conventional parasitological techniques, rarely have these parameters been evaluated in parallel for several PCR-based assays (15, 19, 36, 40).

The kDNA PCR, as expected, was the most sensitive of the PCR-based assays examined. The copy number of the kDNA target is between 50- and 250-fold higher than those of the SLME and ITS1 target regions; the amplicon size is smaller, and titrations using parasite DNA have shown that the kDNA PCR using the 13A and 13B primers can detect DNA equivalent to <0.001 parasites/reaction (15), while the SLME and ITS1 PCRs detect DNA equivalent to 10 and 0.2 parasites/reaction, respectively (20, 36). Six of the samples from suspected CL patients were positive only by the kDNA PCR. Since these results were not confirmed by any additional assay, they were considered negative. However, due to the high sensitivity of the kDNA PCR for leishmanial parasites, it is possible 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. Methods that detect lower amounts of PCR product than those seen by staining with ethidium bromide can increase assay sensitivity even further. Indeed, four of these samples were later found to be positive using a reverse line blot assay targeting the ITS1 region, which is more sensitive than the ITS1 PCR (unpublished data).

The ITS1 PCR was also shown to be more sensitive (91.0%) than conventional techniques of microscopy and culture used alone or in combination. However, this PCR assay was slightly less sensitive than the kDNA PCR, missing the six C-Pos samples (7.7%) identified by the latter technique. While differences in the PCR assays' detection limits explain this discrepancy in part, six of seven of the ITS1 PCR false negatives (85.7%) were also positive by at least one conventional parasitological method, microscopy, and/or 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 South American CL (28). All three PCR assays were performed in parallel on DNA extracted from the same patient specimen spotted on filter paper, and material obtained for culture and microscopic evaluation was taken from similar but not identical sites within the lesion. Therefore, in lesions where only a few amastigotes were present, variations in parasite load between specimens used for conventional and molecular techniques might have resulted in the false negatives by the ITS1 PCR but confirmed positives using the kDNA PCR, where the assay has a higher sensitivity.

Surprisingly, the SLME PCR showed the lowest sensitivity (53.8%) of the three molecular diagnostic assays examined. This result was unexpected since an earlier study (19) showed the SLME PCR to be highly sensitive (89.7%), using fresh biopsy specimens and lesion aspirates from CL and visceral leishmaniasis patients. In this study, samples examined by PCR were obtained as lesion scrapings applied to sterile filter paper, dried, and stored for a few weeks before the DNA was extracted. In the case of a few samples, the filter papers were stored for approximately 2 years prior to processing. Collection of patient specimens on filter papers for PCR diagnosis is a convenient method for storage and transfer to central diagnostic facilities. DNA in specimens collected on filter papers have been shown to be stable (4, 7, 21, 24), although the sensitivity of PCR on fresh samples may be higher than that on samples collected on filter papers or may vary depending on the DNA extraction and purification technique (4, 14).

Finally, both the SLME and ITS1 PCRs have the added advantage that species belonging to the genus *Leishmania* can be easily characterized by RFLP analysis of the amplicon. Indeed, digestion of the amplification product successfully identified the species of *Leishmania* in 77.4% of the ITS1 PCR positives. Utilization of alternative DNA gel stains, more sensitive than ethidium bromide, or a nested PCR should further increase the number of samples in which the parasite's species can be determined. The ability to identify species is especially important in prognosis of the disease and in deciding appropriate therapy, especially in regions where more than one type of species and disease are seen by clinicians.

In summary, the kDNA PCR was the most sensitive diag-

nostic assay for CL and should be employed as the new standard for routine diagnosis when species identification is not required. However, when further parasite characterization is needed, the ITS1 PCR is both highly sensitive and specific and enables one to identify the *Leishmania* species present in the lesion in a high percentage of CL cases.

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