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Original paper

A comparison of the efficiency of molecular and conventional methods in the diagnosis of cutaneous leishmaniosis

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ABSTRACT. Leishmaniosis is one of the most important vector borne diseases. Among different forms of the disease, cutaneous leishmaniosis (CL) is the most common. Determining the method of definitive diagnosis for the disease has been the aim of various studies. Therefore this study afforded an opportunity to investigate this subject. To diagnose CL in 150 suspected patients referred to Mehran and Dehloran health centers during June 2018 to November 2019, two polymerase chain reaction (PCR) methods were performed and compared with the in vitro culture and microscopic evaluation of stained slides. The smears were stained with Giemsa for microscopy and cultured in Novy-Nicolle-McNeal (NNN) blood agar for promastigote growth. For semi-nested PCR and PCR-RFLP, the tissue and serosity from the lesions were used for DNA extraction. The semi-nested PCR technique using minicircle kDNA gene showed the highest positivity rates among all diagnostic assays with 114/150 (76%) of the samples and was used as reference standard, followed by the PCR-RFLP test using ITS1 gene with 112/150, (74.7%) positivity rates, microscopy with 101/150 (67.3%) and then culture 72/150 (48%). microscopy and culture methods together improved overall positivity rates to 68.7% (103/150). The all positive samples using molecular technique were identified as *Leishmania major*. The highest sensitivity (98.3%), specificity (100%), accuracy (98.8%), negative predictive value (94.7%) and κ coefficient (0.96=almost perfect) was observed by comparing PCR-RFLP and semi-nested PCR. kDNA-semi-nested PCR and ITS1-PCR-RFLP presented an interesting alternative to conventional methods for the identification of CL and improved its diagnostic value significantly in suspected patients with negative direct smears.

Keywords: *Leishmania*, cutaneous leishmaniosis, Nested PCR, RFLP, diagnosis

Introduction

Leishmaniosis is an endemic disease in more than 88 countries of Asia, Africa, the Americas, and the Mediterranean region [1]. At least twenty species of *Leishmania* parasites are infectious to

humans and can cause a wide range of symptoms [2]. Cutaneous leishmaniosis (CL), which is the most common form, is one the major health problems in some countries such as Iran. CL is endemic in 17 out of 31 provinces in Iran and more than 20,000 new cases are reported annually in this country [3,4]. Using diagnosis tests with high sensitivity and specificity will be an effective step towards timely treatment and control of the disease, especially in humans [5]. It seems that in areas where CL is endemic, isolation of the parasite from the lesions and identifying it in stained smears using direct microscopy as well as culture are still basic methods of diagnosis. Although these methods are highly specific, they are not sensitive; therefore, differentiating *Leishmania* species with these methods is not possible. In recent years, PCR-based molecular methods with high sensitivity and specificity have been used to identify *Leishmania* in various forms of the disease [6,7]. In these studies, genomic or kDNA have been used. kDNA PCR is considered the most sensitive method for the detection of *Leishmania* [8,9]. Furthermore, PCR using the ITS1 gene has also been identified as a sensitive method [10,11]. In the present study, we compared the sensitivity and specificity of two PCR techniques (minicircle kDNA and ITS1) against microscopic and culture methods for the diagnosis of *Leishmania* in CL lesions to gain further insight on the disease.

Materials and Methods

Samples were obtained from 150 patients with clinically suspected CL lesions who referred to Mehran and Dehloran health centers, to the border between Iran and Iraq during June 2018 to November 2019. Serosity and materials obtained from the lesions were prepared for the following three diagnostic methods: microscopic direct smear, culture, and two molecular techniques for each case.

From the border of the lesions, two smears were prepared on slides and fixed with methanol (Sigma Chemistry, Tehran, Iran). After Giemsa staining, light microscopy was used to confirm the existence of *Leishmania* amastigotes.

Some of the cells and serosity from the lesions were collected in eppendorf tubes containing complete RPMI 1640 medium (Gibco, Frankfurt, Germany) as transfer medium and cultured in blood agar based, NNN medium. The culture media were maintained at 24°C for 4 weeks and the positive cultures were passaged in complete RPMI 1640 medium plus 100U/ml penicillin and 100µg/ml streptomycin.

Some of the obtained samples containing tissue and serosity from the lesions were stored in sterile normal saline at -20°C for molecular study. DNA was extracted using the Genomic DNA Isolation kit (GeNet Bio, South Korea) according to the manufacturer's instructions.

ITS1 region was employed to detect and identify the *Leishmania* species. A total of 150 extracted DNA were examined using the following primers: LITSR (5'-CTGGATCATTTTCCGATG-3') and L5.8S (5'-TGATACCACTTATCGCACTT-3'). The pre-prepared Master Mix (Roche, Germany) was used to amplify DNA. Amplification was performed in a Perkin Elmer (GeneAmp®PCR) Thermocycler 9700 (0.2 ml block) at 94°C for 5 min followed by 35 cycles, each consisting of 30s at 94°C, 30s at 48°C and 60s at 72°C. After the last cycle, the extension was continued for a further 10 min then held at 4°C. For RFLP analysis, the PCR product including the amplified ITS1 were digested with 2µl of *HaeIII* at 37°C for 4 hours without prior purification using conditions recommended by the supplier (Fermentas Life Sciences, Germany). PCR productions and restriction fragments were analyzed using 1.5% agarose gel electrophoresis, respectively. Standard parasite of *L. major* (MRHO/ IR/75/ER), which was prepared from Skin Diseases and Leishmaniasis Research Center of Isfahan, and distilled water were used as positive and negative control, respectively.

The set of primers LINR4 (forward) (59-GGG GTT GGT GTA AAA TAG GG-39), LIN17 (reverse) (59-TTT GAA CGG GAT TTC TG-39), and LIN19 (reverse) (59-CAG AAC GCC CCT ACC CG-39) were used in a semi-nested PCR technique to amplify the conserved area of the minicircle kDNA [12] along with reference strains, *L. major* (MRHO/IR/75/ER). The thermocycler used was set to 5 min at 94°C followed by 17 cycles, each consisting of 30s at 94°C, 30s at 52°C, and 30s at 72°C, and then a final extension was continued for a further 10 min.

All statistical analyses were performed using SPSS software (SPSS 24.0, Chicago, IL, USA). Efficiency of four methods was determined by calculating sensitivity, specificity, positive and negative predictive values, and kappa coefficient.

Ethical approval. The current study was approved by the Ethics Committee of Isfahan

Table 1. Concordance (coefficient kappa) and accuracy analysis between semi nested PCR results and microscopic, culture, RFLP and smear+culture methods in the diagnosis of cutaneous leishmaniosis

Nested -PCR	Microscopic			Culture			RFLP			Microscopic+Culture		
	Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total
Positive	101	13	114	72	42	114	112	2	114	103	11	114
Negative	0	36	36	0	36	36	0	36	36	0	36	36
Total	101	49	150	72	78	150	112	38	150	103	47	150
	%(95% CI)			%(95% CI)			%(95% CI)			%(95% CI)		
Sensitivity	88.6 (81.46-93.21)			63.16 (54.01-71.45)			98.25 (93.83-99.52)			90.35 (83.54-94.53)		
Specificity	100 (90.36-100)			100 (90.36-100)			100 (90.36-100)			100 (90.36-100)		
PPV	100 (96.34-100)			100 (94.93-100)			100 (96.68-100)			100 (96.4-100)		
NPV	73.47 (59.74-83.79)			46.15 (35.53-57-14)			94.74 (82.71-98.54)			76.6 (62.78-86.4)		
Kappa index	0.78 (0.63-0.94)			0.45 (0.31-0.58)			0.96 (0.80-1.12)			0.81 (0.66-0.97)		
Accuracy	91.33 (85.74-94.87)			72 (64.33-78-57)			98.67 (95.27-99.63)			92.67 (87.35–95.86)		

PPV: Positive predictive value; NPV: Negative predictive value

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Results

A total of 150 patients with suspicious CL lesions were studied using four diagnostic methods of direct smear, culture, and two molecular techniques. In this study, cases of infection that were confirmed by at least one of the methods were included as positive samples. *Leishmania* promastigotes from 72 (48%) patients were isolated in NNN medium. In direct microscopy amastigote forms of *Leishmania* were observed in 101(67.3%) samples in slide smears staining with Giemsa. ITS1 PCR–RFLP analysis by *HaeIII* confirmed infection with *L. major* in 112 (74.7%) samples. Two fragments of 140 and 220 bp in length appeared on agarose gel after PCR product electrophoresis (Fig. 1). By amplification of a minicircle kDNA fragments with a length of 650 bp in semi-nested PCR, *L. major* was detected in 114 (76%) samples (Fig. 1) and was used as reference standard (Table 1). Among 150 patients with suspected CL, 117 patients were positive for infection using at least one of the methods used in the study, and no infection was detected in 33 samples (Table 2). The results were confirmed using four PCR products which were selected randomly and minicircle kDNA and ITS1 gene regions of *L. major* sequencing was performed. The highest sensitivity (98.3%), specificity (100%), accuracy (98.8%), negative predictive value (94.7%) and κ coefficient (0.96=almost perfect) was observed by comparing PCR-RFLP and semi-nested PCR. As well as the comparison of culture with reference standard technique revealed lowest sensitivity (63.2%), specificity (100%), accuracy (72%), negative predictive value (46.2%) and κ coefficient (0.45=moderate) in the CL diagnosis (Table 1).

Discussion

L. major and *L. tropica* are major etiological agents of CL in the Middle East [13]. Effective approaches with high sensitivity and specificity that were cost and time effective are needed to combat leishmaniosis. The study area, the western border of

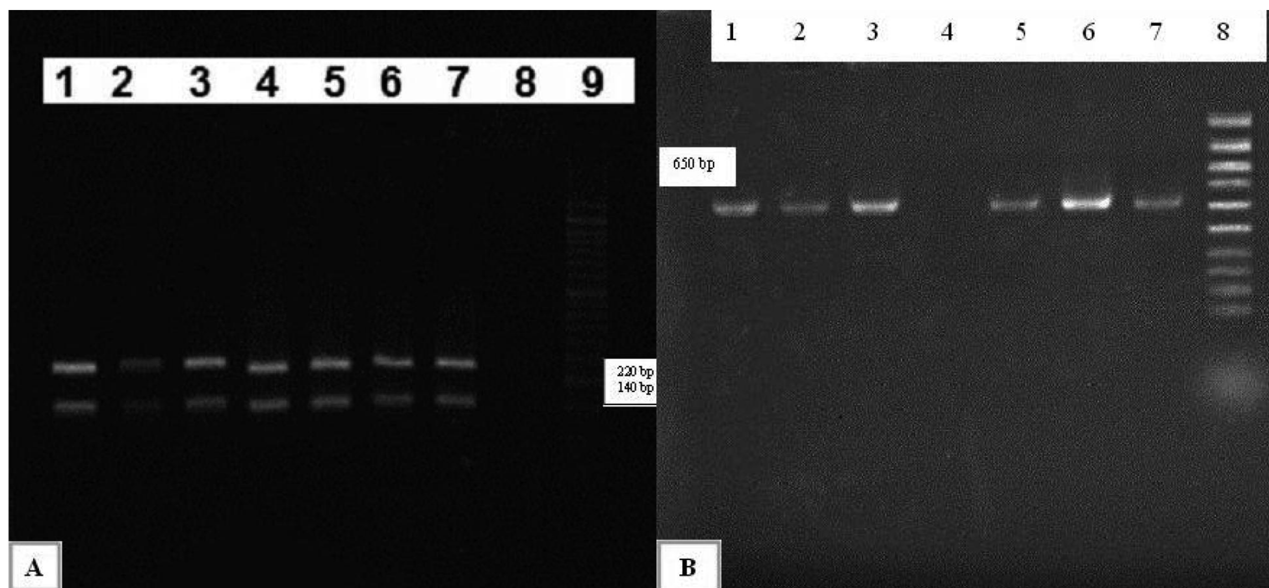

Figure 1. **A.** RFLP patterns of *Leishmania* species after restriction enzyme digest with *Hae* III**.** Lanes: 1, *L. major* (positive control: 140 and 220bp); 2–7, *L. major* isolates obtained from the lesions; 8, Negative control; 9, DNA size marker (100 bp). **B.** Agarose gel electrophoresis of *Leishmania* isolates from the human lesions in semi nested PCR using the primers LINR4/LIN17and LIN19: Lanes 1,2,3,6 and 7, *L. major* isolates isolates obtained from the lesions; 4, Negative control; 5 *L. major* (positive control 650 bp); 8, DNA size marker (100 bp).

the two countries, Iran and Iraq, has been recognized as an endemic area of leishmaniosis due to its appropriate climatic conditions [14]. This border, especially the cities of Mehran and Dehloran, is a very important area for religious tourism, which annually welcomes thousands of people from both of these and other neighboring countries such as Afghanistan, Pakistan, and India [15]. Understanding the biological properties of CL agents in this region, including the prevailing species and access to definitive diagnostic methods, can considerably reduce the complications and crises caused by the outbreak of the disease. In this study, for the first time in Ilam province, west Iran, we compared the traditional and molecular methods of CL detection. The only species identified was *L. major* and the positivity rates of culture, smear, PCR-RFLP and semi-nested PCR methods for diagnosis of disease were 61.5%, 86.3%, 95.7% and 97.4%, respectively. This study showed that molecular studies have more sensitivity than parasitological methods. In some studies, the sensitivity of culture ranged from 40 to 75%, and 74–92% for direct microscopy [16–19] which is consistent with our study. The culture method had the least sensitivity due to limiting factors such as microbial and fungal contamination of the lesions, which impedes the growth of *Leishmania* parasites in the culture medium and increases false negative results [20]. Other factors affecting culture can be species of parasite and the number of parasites isolated from the lesion [21,22]. The negative direct smears can be attributed to factors such as dependence on the skills of the staff, the form of the lesion, the parasite species, and the sampling error. In this study, to reduce sampling error, two slides were prepared from each lesion. Studies have shown that molecular methods are more sensitive than conventional parasitological methods [23–25]. The results of this study confirm these findings: 45 cases of false negative in culture and 16 cases of false negative in direct microscopy studies were identified as positive in PCR-based methods. Concurrent use of direct microscopy and culture methods in this study increased detection sensitivity (90.35%) comparing semi-nested PCR.

Identifying the species of *Leishmania* parasites is inevitably important in the prevention and treatment of the disease [4]. However, this process is not possible with conventional parasitological methods; therefore, the usefulness of molecular methods are apparent. Similar to the results of various studies in Iran and other countries [19,26], the minicircle kDNA method had a higher sensitivity in this study. This may be due to the high copy number of the kDNA fragment in the *Leishmania* genome (tens of thousands of copies) [14]. In this study, two samples proved to be positive only when semi-nested PCR

Table 2. Results of the different methods in the diagnosis of cutaneous leishmaniosis

Methods	Positive number (total: 150)
Culture(+) Smear(+) PCR-RFLP(+) SNP*(+)	70
Culture(+) Smear(-) PCR-RFLP(+) SNP(+)	2
Culture(-) Smear(+) PCR-RFLP(+) SNP(+)	31
Culture(-) Smear(-) PCR-RFLP(+) SNP(+)	10
Culture(-) Smear(+) PCR-RFLP(-) SNP(-)	1
Culture(-) Smear(+) PCR-RFLP(-) SNP(+)	1
Culture(-) Smear(-) PCR-RFLP(-) SNP(+)	2
Total	117

*Semi nested PCR

method was used (Table 2). To confirm the results, the DNA product of these two samples were sequenced and compared with the identified sequence in the GenBank and verified. In this method, only *L. major* yields a band of 650 bp, while other species are expected to create a band of 720 bp [12]. The PCR-RFLP method with a reported sensitivity and specificity of over 90% in different studies [16,19], including the present study (98.25%), as well as its ability to differentiate between *L. major* and *L. tropica* isolates can be a useful method for detection of the disease too. However, due to the limitation of PCR-RFLP in differentiating *L. donovani* complex species, sequencing is still needed to determine the parasite species. In the present study, all cases of infection were identified as *L. major* which seems to be the only cause of the disease in the region. This study showed that the development of molecular methods improves the quality of CL diagnosis and the combination of the conventional and molecular methods should be used for diagnosis. We suggest that the efficiency of other molecular techniques to be compared with nested PCR for diagnosis of cutaneous leishmaniosis. Also species identification with molecular techniques is a vital target in the epidemiology, control and therapy of the disease.

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