# Molecular evaluation of *Leishmania* species in negative ulcer smears from patients suspected of having cutaneous leishmaniasis referred to health center of Aq Qala city during 2019-2020

**Running title:** Molecular evaluation of *Leishmania* species in negative ulcer smears

**Authors:**

Fatemeh Livani[1], Faramarz Koohsar[2], Farideh Tohidi[2], Mitra Sharbatkhori[2], Roghiyeh Faridnia[2], Ayeneh Hajieh Pangh[2], Mehdi Khoshrou[1], Hamed Kalani[1,*]

**Affiliation:**

[1] Infectious Diseases Research Center, Golestan University of Medical Sciences, Gorgan, Iran

[2] Laboratory Sciences Research Center, Golestan University of Medical Sciences, Gorgan, Iran

[*]**Corresponding author:**

Hamed Kalani, Infectious Diseases Research Center, Golestan University of Medical Sciences, Gorgan, Iran; Email: hamed.kalani@yahoo.com

**Abstract**

**Background:** Cutaneous leishmaniasis (CL) is a prevalent infectious zoonotic disease between human and animals. Golestan province is one of the important centers of CL in Iran. Current method for detecting *Leishmania* parasite in patients is Giemsa-stained direct smear from skin ulcers; however, PCR is a strongly recommended method for epidemiological studies. The aim of this study is to investigate *Leishmania* species in negative ulcer smears from patients suspected of having CL referred to Aq Qala health center using multiplex nested PCR method.

**Methods:** This study was performed on 72 negative ulcer smears from patients suspected of having CL referred to Aq Qala health center, Golestan province, northeastern Iran, during August 2019 to April 2020 using multiplex nested PCR method to detect *Leishmania major* and *Leishmania tropica* species.

**Results:** Out of 72 samples, 4 (5.55%) samples were positive by multiplex nested PCR. Moreover, all positive samples are related to *Leishmania major* species.

**Conclusion:** Detecting of *Leishmania* species is strongly recommended in negative ulcer smears from patients suspected of having CL using multiplex nested PCR method.

**Keywords:** Cutaneous leishmaniasis, Negative ulcer smear, *Leishmania major*, *Leishmania tropica*, multiplex nested PCR

## Introduction

Cutaneous leishmaniasis (CL) is a prevalent infectious zoonotic disease between human and animals, which is caused by different species of the genus *Leishmania*. This disease is transmitted to a new host by an infected mosquito bite (1). According to the reports of the General Department of Care and Prevention of Diseases, every year about 20,000 cases of CL are reported in Iran. It is estimated that the actual number of cases is 6-10 times more than that of is reported (2). Golestan province is one of the important centers of CL in Iran which is mostly reported from rural populations and the margin of cities (3).

The prevention and control of CL varies depending on the species of the parasite. In endemic areas where the prevalence of CL is high, the correct and timely diagnosis of this disease is of particular importance, so the use of diagnostic methods with high sensitivity and specificity such as PCR is suggested (4). PCR technique is considered as a valuable method in epidemiological studies (5). In comparison, current methods for detecting of CL such as microscopic examination of Giemsa-stained smears may report as false negative especially in low parasitic burden (6). Misdiagnosed cases may lead to subsequent consequences for the patient and the delay in healing the lesions, and of course, the spread of the disease in the region (7).

Therefore, the aim of this study is to examine *Leishmania* species in negative ulcer smears from patients suspected of having CL referred to Aq Qala health center, Golestan province, northeastern Iran, using multiplex nested PCR method.

## Methods

This retrospective cross-sectional study was performed on 72 negative ulcer smears from patients suspected of having CL referred to the health center of Aq Qala city during August 2019 to April 2020. These smears had been prepared by Giemsa stain and examined under a light microscope at 400 and 1,000 magnifications. The final report of examiner was negative for detecting of *Leishmania* species.

DNA was extracted from the smears using Blood DNA isolation kit (DENA Zist Asia, Mashhad, Iran) according to manufacturer's instruction. The quantity and quality of the extracted DNA was analyzed using NanoDrop ND-1000 spectrophotometer (PeqLab, Erlangen, Germany).

PrimerPlex v2.6 software was used in order to design primers for multiplex nested PCR. Two pairs of primers were used for each studied gene. Accordingly, the product of first reaction was considered as the target of the second reaction. Therefore, two pairs of primers, one for the first reaction and the other for the second reaction, were considered for each of the examined parasites, *Leishmania major* and *Leishmania tropica*. Primers were designed for SSU rDNA gene of the mentioned parasites. Furthermore, DNA for fragments 3 and 4 of histone protein were used to evaluate the accuracy of the multiplex nested PCR reaction as an internal control. The specifications of the primers used are given in Tables 1 and 2.

Multiplex TEMPase 2x Master Mix (Ampliqon, Denmark) was used for this aim according to manufacturer's instruction. The reactions were performed in 50 µL final volume including 25 µL of the 2x Master Mix, 1 µL of each primer, target DNA, and PCR-grade $H_2O$ to a total reaction volume of 50 µl. Reaction 1 included 3 steps as follows: 1) denaturation at 94 °C for 2 min, 2) 35 cycles including denaturation at 94 °C for 15 s, annealing at 52.2 °C for 30 s, extension at 72 °C for 30 s, 3) final extension at 72 °C for 2 min. Reaction 2 was carried out in the same way as reaction 1, except that the annealing was carried out at 50.3 °C.

The PCR product was run on 1.5% agarose gel in TBE buffer. After electrophoresis, the gel was placed in the UV transilluminator and it was expected to visualize at least two bands related to two

internal control genes. If the sample is positive for each of the two parasites under investigation, the bands related to each of those two would be visible. Band lengths are given in Table 1 and 2. The number of positive cases of each species of *Leishmania major* and *Leishmania tropica* was reported as frequency and percentage.

**Results and Discussion**

Out of 72 negative smears from the skin ulcers of patient, 4 (5.55%) samples were positive by multiplex nested PCR. Furthermore, all positive samples were related to *Leishmania major* species (Figure. 1). Out of the four positive cases, three were female and one was male. The ages of these individuals were 18, 32, 34, and 58 years. All four resided in rural areas and had no prior history of cutaneous leishmaniasis. The lesions in each case were located on the ankle, with a single lesion present in each individual. None of the positive cases had any underlying diseases. The duration of the lesions at the time of diagnosis ranged from one to three weeks.

The current method of examining suspected cases of having CL includes preparation of a smear from the margin of ulcer and staining with the Giemsa method and microscopic examination. Depending on the expertise of the examiner, the parasite burden of the sample, and the quality of the staining, the results may be associated with false negative. Therefore, the microscopic examination method has low sensitivity as compared to a highly sensitive molecular method for diagnosis of CL such as PCR (8-9).

In a study conducted on 29 microscopically negative samples from patients suspected of having CL, 18 (62%) samples were positive by PCR method, which shows the superiority of the PCR method compared to the microscopic examination method (10). Moreover, the difference between the number of positive cases in negative samples between the present study (5.55%) and the latter study (62%) shows the significant impact of individual expertise in the microscopic diagnosis of *Leishmania*. In the present study, all the positive cases were *Leishmania major*, which was consistent with the results of Hezari et al. in Golestan province, who reported only *Leishmania major* species (11). One of the advantages of the PCR method over the microscopic method can be considered the possibility of determining the parasite species (10).

In another study in Gonbad-e Kavus, out of 65 negative samples in microscopic examination from patients suspected of having CL, 34 (53.3%) cases were found to be positive by PCR method. Positive samples were *Leishmania major* and it was similar with the findings of the present study (12).

The results of a study conducted on 62 microscopically negative smears for *Leishmania* showed that 35 (4.56%) specimens were positive for *Leishmania* parasite by PCR method, which was in line with the results of the present study (13). Mohaghegh el al. showed 1.11% of the samples that were reported as negative microscopically were positive by PCR, which was consistent with the results of the present study, but the type of parasite found in all positive cases was *Leishmania tropica*. The difference between the parasite species found in the current study and the latter one is related to the geographical area (14).

In another study, out of 30 negative samples microscopically, 13 (3.43%) samples were positive by PCR method (15). A comparison among the microscopic method and 3 different diagnostic methods based on PCR showed that the sensitivity of the microscopic method was 22% and the sensitivity of the 3 PCR methods was reported between 64 and 100% (16). In order to decide on the use of different methods for the diagnosis of CL, in addition to the sensitivity and specificity of the methods used, the costs incurred have always been taken into consideration, and the higher cost of the PCR method compared to the microscopic method is one of the considerable points.

## Conclusion

The results of this study showed the superiority of the multiplex nested PCR method over the microscopic method. In cases suspected of having CL, multiplex nested PCR can be used to avoid inappropriate treatment for patients where the samples are reported negative microscopically. Using the multiplex nested PCR method to determine the type of parasite that causes CL can provide researchers with more accurate information about the prevalence of the disease in epidemiological studies, which helps in making preventive decisions.


## Declarations

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## Ethics approvals

The Ethics Committee of Golestan University of Medical Sciences (ethical code: IR.GOUMS.REC.1400.166). This article has not been published in other journals, nor will it be, and is not under review in any journal.

## Conflict of interest

The authors declare that they have no conflict of interest.

## References


1. Torres-Guerrero E, Quintanilla-Cedillo MR, Ruiz-Esmenjaud J, Arenas R. *Leishmaniasis: a review*. F1000Res. 2017; 1(1):5-21.
2. Akhtari J, Faridnia R, Kalani H, Bastani R, Fakhar M, Rezvan H, et al. *Potent in vitro antileishmanial activity of a nanoformulation of cisplatin with carbon nanotubes against Leishmania major*. J Glob Antimicrob Resist. 2019; 2(4):16:11-6.
3. Weigle KA, Labrada LA, Lozano C, Santrich C, Barker DC. *PCR-based diagnosis of acute and chronic cutaneous leishmaniasis caused by Leishmania (Viannia).* J Clin Microbiol. 2002; 40(2):601-606.
4. Aviles H, Belli A, Armijos R, Monroy FP, Harris E. *PCR detection and identification of Leishmania parasites in clinical specimens in Ecuador: a comparison with classical diagnostic methods*. J Parasitol 1999; 85(2):181-187.
5. Mahboudi F, Abolhassani M, Tehrani SR, Azimi M, Asmar M. *Differentiation of old and new world leishmania species at complex and species levels by PCR.* Scand J Infect Dis. 2002; 34(10):756-758.
6. Pourmohammadi B, Motazedian MH , Hatam GR, Kalantari M, Habibi P, Sarkari B. *Comparison of Three Methods for Diagnosis of Cutaneous Leishmaniasis.* Iran J Parasitol. 2010; 5(4): 1–8.
7. Thakur Sh, Joshi J, KaurS. *Leishmaniasis diagnosis: an update on the use of parasitological, immunological and molecular methods*. J Parasit Dis. 2020; 44(2): 253–272.
8. Rodrigues E, De Brito M, Mendonca M. Werkhauser RP, Coutinho EM, Souza WV, et al. *Evaluation of PCR for diagnosis of Ameri-can cutaneous leishmaniasis in an area of endemicity in Northeastern Brazil*. J Clin Microbiol. 2002; 40:3572-3576.
9. Bensoussan E, Nasereddin A, Jonas F, Schnur LF, Jaffe CL. *Comparison of PCR assays for diagnosis of cutaneous leishmaniasis.* J Clin Microbiol. 2006; 44:1435-1439.
10. Aghamolaei S, Behniafar H, Behravan M, Hajjaran H, Vaziri VM. *Probability of false-negative results in microscopical detection of cutaneous leishmaniasis: more accurate screening by kDNA-PCR during epidemiological survey*. J Parasit Dis.2020; 44(4):781-784.
11. Hezari F, Niyyati M, Tabaei SJ, Mohebali M, Vaziri VM, Behniafar H, Azargashb E, Taghipour N. *Frequency of cutaneous leishmaniasis and species identification in suspected individuals from Golestan province, Northern Iran in 2014*. Iran J Public Health. 2016; 45(10):1348.

12. Pakheh, Abdul Sattar, Fakhar, Sharif, Danesh, Vahid, Ahmadi, Ziba. *Epidemiological study of cutaneous leishmaniasis with Leishmania tropica in a new center of Khorasan Razavi province*. J Maz Univ Med Sci. 2014; 23 (103): 46-52.
13. Fakhar M, Mikaeili F, Hatam GR, Habibi P, Karamian M, Motazedian M, Banimostafavi E. *A molecular epidemiology survey of cutaneous leishmaniasis in patient referring to parasitology lab at shiraz school of medicine and the importance of PCR assay*. Pars J Med Sci. 2014; 8(2):311.
14. Mohaghegh MA, Fata A, Salehi GH, Berenji F. *Molecular identification of Leishmania species using samples obtained from negative stained smears.* Iran J Parasitol. 2013; 8(2):337.
15. Karamian M, Faroghi Bojd MS, Hemmati M, Saadatjoo A, Barati DA. *Molecular identification of cutaneous leishmaniasis agents in Birjand, Iran.* J Birjand Univ Med Sci. 2013; 20(2):183-190.
16. Merdekios B, Pareyn M, Tadesse D, Getu S, Admassu B, Girma N, Leirs H, van Griensven J. *Detection of Cutaneous Leishmaniasis Foci in South Ethiopia*. Am J Trop Med Hyg. 2021; 51 (2): 79-86.

**Table 1.** Design primers for the first step PCR reaction

| Sequence | Forward primer (**5'→3'**) | Reverse primer (**5'→3'**) | Product length |
|---|---|---|---|
| H1F4 | CTAATTTCAGTTATGTGGCAG | CCGAGTTCCAAACACGGCA | 195 |
| H1F3 | CAGTTGCGCCTGCCTTCTTCG | GGAAGAGAGATTTGCAAATG | 514 |
| ITS1 Leishmania major | TGGCCAACGCGAAGTTGAAT | CTAACGTGTCGCGATGGA | 581 |
| ITS1 Leishmania tropica | ACGTTATGTGAGCCGTTAT | TATCCGCCCGAAAGTTCACC | 342 |

**Table 2.** Design primers for the second step PCR reaction

| Sequence | Forward primer (**5'→3'**) | Reverse primer (**5'→3'**) | Product length |
|---|---|---|---|
| H1F4 | GGTTACAAGCCTACTGGTT | CCTTCGGAAATAATGTCAGT | 106 |
| H1F3 | TAAGGAAAACATGAAAGT | AGACAATAAGTAATCTCA | 362 |
| Leishmania *major* ITS1 | GAGAATCATTCAATTACC | TTAATAATCCTAATCACAG | 465 |
| Leishmania *tropica* ITS1 | AAAGTTCACCGATATTTCTT | CGCCGTATATTTGTATAAAC | 278 |

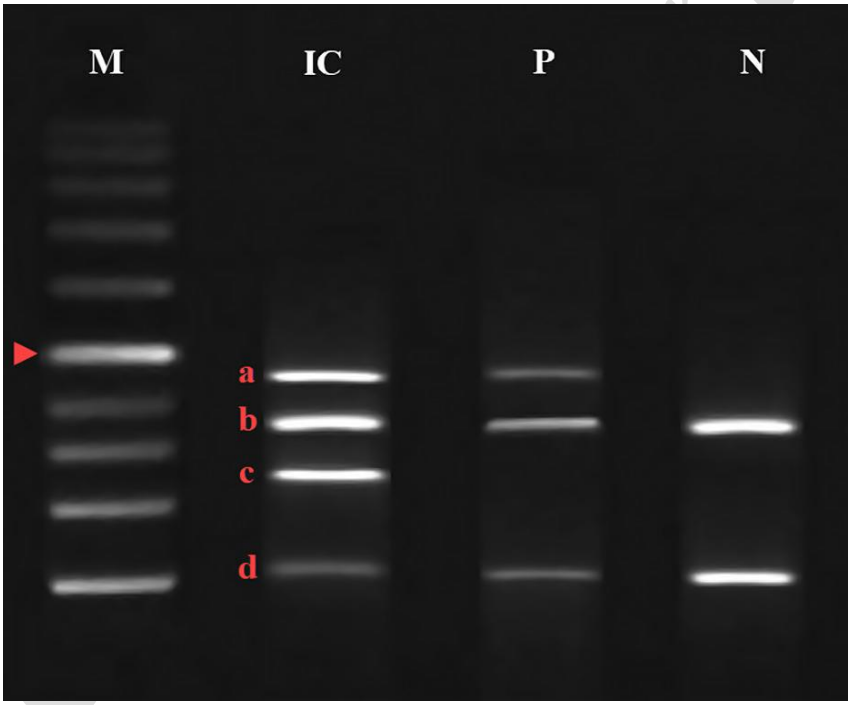


**Figure 1.** Bands obtained from multiplex nested PCR negative slides obtained from samples suspected of cutaneous leishmaniasis. M = standard DNA marker (1000 bp); IC = internal control to determine the correct operation of multiplex PCR; P = positive sample; N = negative sample; a = 465 bp band related to *Leishmania major*; b = 362 bp band related to fragment 3 of histone protein 1; c = 278 bp band related to *Leishmania tropica*; d = 106 bp band corresponding to fragment 4 of histone protein 1. Arrowhead = 500 bp band