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Comparison of Molecular, Microscopic, and Culture Methods for Diagnosis of Cutaneous Leishmaniasis

Sima Rasti,[1] Baharak Ghorbanzadeh,[1] Farnaz Kheirandish,[2]* Seyed Gholamabbas Mousavi,[3] Ahmad Pirozmand,[4] Hossein Hooshyar,[1] and Bathol Abani[1]

[1]Department of Parasitology and Mycology, School of Medicine, Kashan University of Medical Sciences, Kashan, Iran

[2]Department of Parasitology and Mycology, School of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran

[3]Department of Statistic, School of Medicine, Kashan University of Medical Sciences, Kashan, Iran

[4]Department of Microbiology, School of Medicine, Kashan University of Medical Sciences, Kashan, Iran

Cutaneous leishmaniasis (CL) is endemic in the northwest of Isfahan province, Iran. Increase in the incidence of the disease in Kashan has made it necessary to find out the best method for diagnosis and molecular characterization of *Leishmania* species. In the present study, 130 patients suspected to cutaneous leishmaniosis referred to health care centers of Kashan were examined. Serosity of lesion was collected for smear preparation and cultured in Novy-Nicolle-McNeal medium. DNA was extracted from serosity, and *Leishmania* species was determined by polymerase chain reaction (PCR) and nested PCR using kinetoplast DNA (kDNA) specific primers. The diagnostic criteria of CL were based on the observation of amastigotes in the smear, promastigotes in culture, presence of expected bands in PCR, or nested PCR. Of 130 specimens, 87 (66.9%), 72 (56.2%), 98 (75.4 %), 96 (73.8%), and 99 (76.2%) were positive for microscopic culture, PCR, nested PCR, and combined PCR and microscopy (proposed method), respectively. Sensitivity, specificity, positive and negative predictive values of PCR were 99%, 100%, 100%, 96.9%, respectively, for microscopy 87.9%, 100%, 100%, 72.1%, for culture 72.7%, 100%, 100%, 53.4 %, and for nested PCR 97%, 100%, 100%, 91.2%, respectively. Based on the results of the study, kDNA-PCR was the most sensitive method for diagnosis of CL. J. Clin. Lab. Anal. 00:1–6, 2016.

Key words: diagnosis; cutaneous leishmaniasis; PCR; Kashan; Iran

INTRODUCTION

Cutaneous leishmaniasis (CL) is one of the most common dermal diseases and health problems in Mediterranean regions including Iran. According to the world health organization (WHO), more than 90% of CL occurs in Afghanistan, Pakistan, Algeria, Iran, Saudi Arabia, Brazil, Columbia, and Syria (1). In the last two decades, the disease has increased (2). CL is endemic in many parts of Iran, and is reported from at least 17 provinces of the country (3).

The major problem associated with CL treatment is incorrect diagnosis of CL in the scarce number of parasites in zoonotic CL (ZCL) and the presence of false-negative results in microscopic diagnosis (2, 4). Therefore, there is a need to use a sensitive method for influential diagnosis of parasite species for efficient treatment and control of the disease (5).

Nowadays, diagnosis is based on the microscopic observation of stained slides and finding amastigote in the

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*Correspondence to: Dr. Farnaz Kheirandish, Associate Professor, Department of Parasitology and Mycology, School of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran. E-mail: Kheirandish81@yahoo.com

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serosity of wounds. Although this method is cheaper and more accessible, it does not have sufficient sensitivity in terms of etiological diagnosis. Culture method has some limitations including the need of an incubator, long period of culture time, and probable microbial and fungal contamination. On the other hand, these two methods cannot be used for identifying parasite species (6).

Specificity of these two tests is high; however, their sensitivity is low. Furthermore, different sensitivities have been reported for them because of the number of parasites in sample, disease duration, type of culturing medium, and skill of lab experts (2).

Recently, PCR method with high quality and sensitivity has been widely used for diagnosing CL (7–10). PCR is suitable when there are atypical lesions of CL and few numbers of parasites, or when microscopic method is negative (11).

Microscopic examination is the routine method for diagnosing CL, thus the sensitivity of the test varies from 42% to 70% depending on the skill of the technicians (6,12–14), The sensitivity and specificity of PCR with variable region of kinetoplast DNA (kDNA) is 98.7–100% having 10,000 copies per cell. It is an ideal target that offers accurate discrimination between species. However, the sensitivity of PCR-RFLP using ITS1 primer is 82–91% having 40–200 copies per cell, whereas the specificity is 100% (9,14,15). The increasing trend of CL in Kashan, Iran, and contradictory reports regarding the sensitivity and specificity of PCR diagnostic methods and the need for accurate diagnosis of disease and *Leishmania* species in low parasite cases (11,14,16) for treatment regimen of patients and disease control programs (17) were considered. The present study was designed to determine the sensitivity and specificity, positive and negative predictive value of PCR, nested PCR, microscopic and culture methods for the diagnosis of CL.

MATERIALS AND METHODS

Study Area

Kashan is located in the northwest of Isfahan province, Iran. This is an ancient city with major tourist attractions in the central part of Iran.

Sample Collection

This diagnostic value study was performed on 130 patients suspected of cutaneous leishmaniosis. These patients were referred to health care centers of Kashan (Zidi and Shahid Beheshti Hospital Laboratory) from August 2012 to September 2013.

The demographic information and number, form, and location of wounds were recorded in questioner's forms. Serosity of ulcer was collected and examined by microscopy, culture, PCR, and nested PCR methods. The diagnostic criteria of CL were based on the observation of amastigotes within the smear, promastigotes in the culture, presence of expected bands in PCR, or nested PCR. Disease was confirmed when the results were positive for at least one of the four methods (smear, culture, PCR, or nested PCR).

Microscopic Examination

After disinfecting the ulcer by 70% alcohol, disposable lancet was used for collecting serosity from border of the ulcers and spread on two slides. After fixing and staining by Giemsa, it was examined for amastigote presence.

Parasite Culture

Additionally, the ulcer serosity inoculated into Novy-McNeal-Nicolle medium supplemented with RPMI-1640 cell culture medium was enriched with 10% fetal bovine serum. Then, culture mediums were incubated at 24°C up to 2 weeks and checked microscopically.

DNA Isolation

Serosity was directly collected and transferred to 1.5 microtubes containing 0.5 ml of sterilized normal saline and stored at −20°C for DNA extraction.

DNA Extraction

DNA of samples were extracted by Kit (Bioneer, Korea) according to the manufacturer's instruction and then stored at −20°C.

Nested PCR

The nested PCR was performed on all 130 extracted DNA samples. The external primers were CSB2X (Forward: 5′-CGAGTAGCAGAAACTCCCG TTCA-3′) and CSB1X (Reverse: 5′-ATTTTTCGCGATT TTCGCAGAACG-3′) for the first round, and internal primers 13Z (Forward: 5′-ACTGGGGG TTGGTGTAAAATAG-3′) and LiR (Reverse: 5′-TCGCAGAACGCCCCT-3′**)** for the second round as described previously to amplify the variable region of the minicircle kDNA of *Leishmania* (17).

First-round reaction was carried out in 20 μl reaction mixture containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol CSB2X, 10 pmol CSB1X, 1 U Taq DNA polymerase (Bioneer), 100 pg DNA (2 μl), and 1× PCR buffer. The PCR conditions consisted of one initial denaturing cycle at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 40 s, and finally one cycle extension at 72°C for 5 min. Second-round reaction was

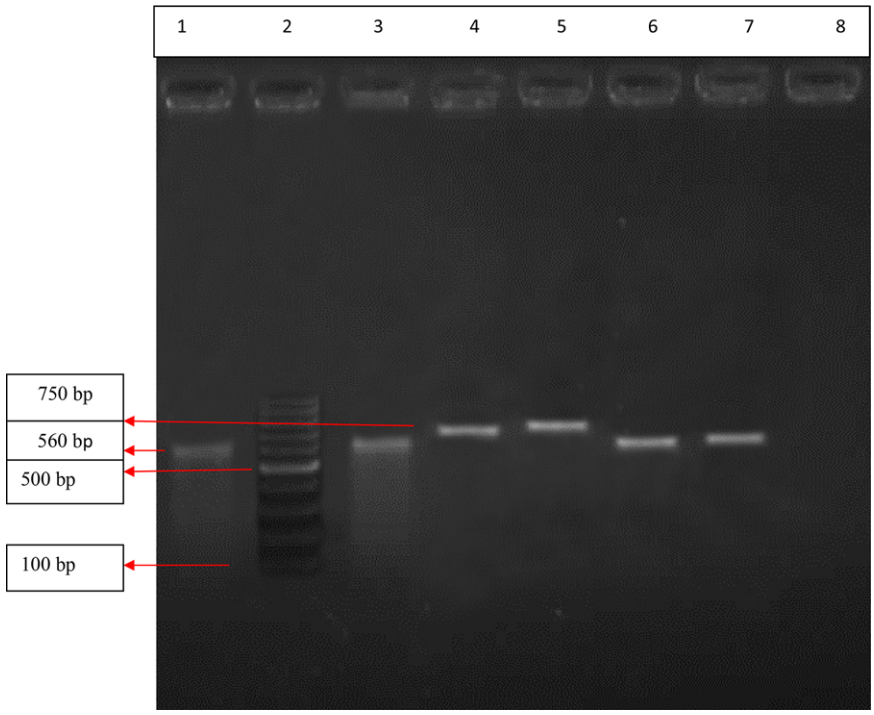

Fig. 1. Gel electrophoresis of nested PCR products of *Leishmania* isolates using CSB2X, CSB1X, 13Z, LiR primers. Lanes 1, 3, 7: *L. major* isolates; Lane 2: 100 bp DNA ladder marker; Lane 4: *L. tropica* isolate; Lane 5: reference strain of *L. tropica* (MHOM/IR/89/AR2); Lane 6: reference strain of *L. major* (MHOM/IR/54/LV39); Lane 8: negative control.

performed in 20 μl reaction mixture containing production of first-round reaction diluted 1:2 as template and followed as described in the previous step. PCR products were analyzed using 1.5% agarose gel electrophoresis. The sizes of the PCR products were 560 and 750 bp for *Leishmania major* and *Leishmania tropica*, respectively (Fig. 1).

Reference strains, *L. tropica* (MHOM/IR/89/AR2) and *L. major* (MHOM/IR/54/LV39), were used as positive controls. Samples from lesion with known skin conditions other than CL served as negative control.

PCR Method

The PCR was performed on all 130 DNA samples. Species-specific primers, LINR4 (Forward: 5′- GGG GTT GGT GTA AAA TAGGG-3′) and LIN17 (Reverse: 5′- TTT GAA re CGG GAT TTC TG-3′) were used as described by Aransay et al., to amplify the variable region of the minicircle kDNA of *Leishmania* (18).

PCR mixtures contained 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol LINR4, 10 pmol LIN17, 1 U Taq DNA polymerase, 100 pg DNA (2 μl), and 1× PCR buffer. The reaction mixtures were incubated at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 52°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 5 min.

PCR products were analyzed using 1.5% agarose gel electrophoresis. The sizes of the PCR products were 650 and 760 bp for *L. major* and *L. tropica*, respectively. Positive and negative controls, similar to nested PCR, were used.

Statistical Analysis

Data were analyzed by SPSS version 16 (SPSS, Inc., Chicago, IL) using chi-square. Efficiency of four methods was determined by calculating sensitivity, specificity, positive and negative predictive values, and kappa. This study was approved by the ethical committee of Kashan University of Medical Sciences, Iran.

RESULTS

One hundred thirty patients with suspected CL were investigated by four diagnostic techniques: microscopic culture, PCR, and nested PCR. All the examined people were identified as CL patients with a positive result in at least one of the four performed techniques.

The frequency of suspected CL patients based on age groups is shown in Table 1. Of 130 specimens, 99 (76.2%) samples were positive and 31 (23.8%) samples were negative by the consensus criteria (Table 2). By PCR method, 71.4% and 26.6% of isolates were identified as *L. tropica* and *L. major*, respectively, and 2% were mixed. Sensitivity, specificity, and positive and negative predictive values of

TABLE 1. Frequency of Suspected Patients to CL Based on Age Groups

Age	Number	Percentage
<20	29	22.3
20–39	46	35.3
40–59	33	25.4
60–79	14	10.8
≥80	8	6.2
Total	130	100

TABLE 2. Frequency of Positive Cutaneous Leishmaniasis Based on Diagnostic Techniques

	Number (%)	
Assay	Positive	Negative
Microscopic	87 (66.9%)	43 (33.1%)
Culture	72 (56.2%)	58 (43.8%)
kDNA PCR	98 (75.4%)	32 (24.6%)
Nested PCR	96 (73.8%)	34 (26.2%)
Microscopic + PCR	99 (76.2%)	31 (23.8%)

TABLE 3. Efficiency of Four Diagnostic Methods for Cutaneous Leishmaniasis

Assay	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Microscopic	87.9	100	100	72.1
Culture	72.7	100	100	53.4
kDNA PCR	99	100	100	96.9
kDNA-nested PCR	97	100	100	91.2

PPV: positive predictive value; NPV: negative predictive value.

microscopic culture, PCR, and nested PCR are shown in Table 3. PCR and microscopic showed more correlation ($P = 0.001$), $K = 0.759$.

DISCUSSION

Applying a highly sensitive method to diagnose the disease and then using effective treatment of the patient definitely help to prevent the spread of the disease and decrease the source of the disease. Evaluation and comparison of diagnostic methods are very important in order to determine the best diagnostic method for treating CL effectively. Furthermore, by determining the parasite species, exact and efficient planning can be used for the disease control.

In the present study, the positive rates from 130 specimens were 87 (66.9%), 72 (56.2%), 98 (75.4%), and 96 (73.8%) for microscopic cultivation, kDNA-PCR, and nested PCR methods, respectively. Of 98 positive cases of PCR, 70 (71.4%), 26 (26.6%), and 2 (2%) *L. tropica*, *L. major*, and mixed cases, respectively, were diagnosed. Furthermore, the identified species were similar in PCR and nested PCR methods. Only two cases were positive in PCR, but negative in nested PCR method.

In the present study, using two diagnostic methods, PCR and microscopic 99 (76.2%) were positive for CL. It was reported as the proposed method. Hayat et al. also reported higher sensitivity of kDNA-PCR compared with other methods (19). In another study, of 51 suspected cases of CL, kDNA-PCR and microscopic methods found 29 (59.6%) and only 3 (5.9%) positive cases, respectively (11).

In the present study, the infection percentage by microscopic method was almost the same as the results found by Pourmohammadi and Kumar (14, 20). However, positive cases of two other studies using microscopic method were reported as 46.7% and 38.4%, which were less than the present study (21, 22). According to the results in Zahedan and Ahvaz cities, using PCR and mini-exon PCR methods, the infection percentage of 55.5% and 70.3%, respectively, was reported (21, 22).

Based on the findings of the present research using kDNA-PCR method, the infection percentage was higher than Zahedan study and similar to the Ahvaz research (21, 22). Pourmohamadi (20) and Kumar (14), using kDNA-PCR, reported the positive rate of 93.6% and 87.5%, respectively, which were higher than the results of the present study. This difference may be attributed to more sample size or higher parasite density in the samples.

Bensoussan et al. reported the sensitivity of microscopic culture, and kDNA-PCR methods as 83.3%, 83.3%, and 98.7%, respectively. Sensitivity of microscopic and PCR methods was almost similar to the present research, but sensitivity of cultivation was higher than the present research (23). Lemrani et al. reported the sensitivity of microscopic cultivation, and kDNA-PCR methods as 69.2%, 69.2%, and 84.6% and its specificity was 100% (24). The sensitivity of the three methods was less than the sensitivity of the present study, but specificity was similar.

In the present study, sensitivity, specificity, and positive and negative predictive values of the proposed method (PCR and microscopic) compared to PCR were 99%, 100%, 100%, and 96.9%, respectively. These values were also 87.9%, 100%, 100%, and 72.1%, respectively, compared to microscopic method. Moreover, compared to culture method, these values were 72.7%, 100%, 100%, and 53.4%, respectively. As far as for nested PCR, these values were 97%, 100%, 100%, and 91.2%, respectively.

Based on the research in Zahedan, Iran, sensitivity, specificity, and positive and negative predicted values of PCR were 76%, 73%, 80%, and 68%, respectively (21), Furthermore, in Shiraz, Safai et al. used kDNA-PCR for

examining 32 patients. They reported the sensitivity and specificity of 92% and 100%, respectively (25). Results of these two studies (21, 25) were less than the present research. It might be due to the difference in the type of consumed primer.

Moreover, Tohidi and Barghae observed that sensitivity, specificity, and positive and negative predicted values of PCR were 76.8%, 100%, 100%, and 56.7%, respectively (26), which was less than the present research. Coefficient of agreement for PCR and nested PCR in diagnosing CL was 95.9%. In two cases, PCR was positive, while nested PCR was negative. In nested PCR, more materials and time were supposed to be used.

Moreover, coefficient of agreement for cultivation and microscopic methods was 72.9% and for cultivation and PCR methods was 54.4%. Tohidi et al. reported that coefficient of agreement for cultural and microscopic methods was estimated 82%, which was higher than the present research (27). According to the current results, kDNA-PCR is suitable for the diagnosis and determination of parasite species, especially in the cases with fewer parasites. Finally, the best method for diagnosing CL is the combination of kDNA-PCR and microscopic methods.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

REFERENCES

1. WHO. Report of WHO expert Committee on the Control of Leishmaniasis. WHO Technical Report Series 949. Geneva: WHO; 2010.
2. Reithinger R, Dujardin J-C, Louzir H, Pirmez C, Alexander B, Brooker S. Cutaneous leishmaniasis. Lancet Infect Dis 2007;7(9):581–596.
3. Zoonoses Control Office. Ministry of Health and Medical Education, Iran; 2010.
4. Aronson N, Weller PF, Baron EL. Clinical manifestations and diagnosis of cutaneous leishmaniasis; 2014. Available at: https://www.uptodate.com.
5. Bulm J, Desjeux P, Schwartz E, Beck B, Htz C. Treatment of CL among travellers. J Antimicrob Chemother 2004;53:153–166.
6. Aviles H, Belli A, Armijos R. PCR detection and identification of *Leishmania* parasite in clinical specimens in Ecuador: A comparison with classical diagnostic methods. J Parasitol 1999;85(2):181–187.
7. Kheirandish F, Chegeni Sharafi A, Kazemi B, et al. Identification of *Leishmania* species using PCR assay on Giemsa-stained slides prepared from cutaneous leishmaniasis patients. Iranian J Parasitol 2013;8(3):382–388.
8. Kheirandish F, Chegeni Sharafi A, Kazemi B, Bandehpour M, Tarahi MJ, Khamesipour A. First molecular identification of *Leishmania* species in a new endemic area of cutaneous leishmaniasis in Lorestan, Iran. Asian Pacif J Trop Med 2013;6(9):713–717.
9. Al-Jawabreh A, Schoenian G, Hamarsheh O, Presber W. Clinical diagnosis of cutaneous leishmaniasis: A comparison study between standardized graded direct microscopy and ITS1-PCR of Giemsa-stained smears. Acta Trop 2006;99(1):55–61.
10. Hajjaran H, Vasigheh F, Mohebali M, Rezaei S, Mamishi S, Charedar S. Direct diagnosis of *Leishmania* species on serosity materials punctured from cutaneous leishmaniasis patients using PCR-RFLP. J Clin Lab Anal 2011;25(1):20–24.
11. Karamian M, Motazedian MH, Fakhar M, Pakshir K, Jowkar F, Rezanezhad H. Atypical presentation of old-world cutaneous leishmaniasis, diagnosis and species identification by PCR. J Eur Acad Dermatol Venereol 2008;22(8):958–962.
12. Goto H, Lindoso JA. Current diagnosis and treatment of cutaneous and mucocutaneous leishmaniasis. Expert Rev Anti Infect Ther 2010;8(4):419–433.
13. Faramand M, Nahrevanian H, Atashi Shirazi H, Naeimi S, Farzanehnejad Z. An overview of a diagnostic and epidemiologic reappraisal of cutaneous leishmaniasis in Iran. Braz J Infect Dis 2011;15(1):17–21.
14. Kumar R, Bumb RA, Ansari NA, Mehta RD, Salotra P. Cutaneous leishmaniasis caused by *Leishmania tropica* in Bikaner, India: Parasite identification and characterization using molecular and immunologic tools. Am J Trop Med Hyg 2007;76(5):896–901.
15. Bensoussan E, Nasereddin A, Jonas F, Schnur LF, Jaffe CL. Comparison of PCR assays for diagnosis of cutaneous leishmaniasis. J Clin Microbiol 2006;44(4):1435–1439.
16. Shahbazi F, Shahabi S, Kazemi B, Mohebali M, Abadi AR, Zare Z. Evaluation of PCR assay in diagnosis and identification of cutaneous leishmaniasis: A comparison with the parasitological methods. Parasitol Res 2008;103(5):1159–1162.
17. Novyes HA, Reyburn H, Bailey JW, Smith DA. Nested-PCR-based schizodeme method for identifying *Leishmania* kinetoplast minicircle classes directly from clinical samples and its application to the study of the epidemiology of *Leishmania tropica* in Pakistan. J Clin Microbiol 1998;36(10):2877–2881.
18. Aransay A, Scoulica E, Tselentis Y. Detection and identification of *Leishmania* DNA within naturally infected sand flies by seminested PCR on minicircle kinetoplastic DNA. Appl Environ Microbiol 2000;66(5):1933–1938.
19. Hayat M, Ahmad I, Afaq U, Munir SH. Prevalence and molecular diagnosis of cutaneous leishmaniasis in local population of Dir district, Khyber Pakhtunkhwa, Pakistan. J Pharmaceut Sci Rev 2013;21(2):359–364.
20. Pourmohammadi B, Motazedian MH, Hatam GR. Comparison of three methods for diagnosis of cutaneous leishmaniasis Iranian. J Parasitol 2010;5(4):1–8.
21. Fazaeli A, Fuladi B, Hashemi Shahri M, Sharifi I. Evaluation of a direct PCR in comparison with routine microscopy and in vitro culture for diagnosis of cutaneous leishmaniasis. Iranian J Publ Health 2007;36(1 Suppl):1–2.
22. Khademvatan SH, Saki J, Maraghi S. Comparison of traditional methods and PCR for diagnosis of cutaneous leishmaniasis in south-west of Iran. Zahedan J Res Med Sci 2012;14(8):1–5.
23. Bensoussan E, Nasereddin A, Jonas F, Schnur LF, Jaffe CL. Comparison of PCR assays for diagnosis of cutaneous leishmaniasis. J Clin Microbiol 2006;44(4):1435–1439.

24. Lemrani M, Hamdi S, Laamrani A, Hassar M. PCR detection of leishmania in skin biopsies. J infec Dev Ctries 2007;3(2):45–46.
25. Safaei A, Motazedian MH. Polymerase chain reaction for diagnosis of cutaneous leishmaniasis in histologically positive, suspicious and negative skin biopsies. J Dermatol 2002;205(1):18–24.
26. Tohidi F, Barghae A. Cutaneous leishmaniasis parasite identification via PCR in the infected areas in Golestan province. Knowl Health J 2012;6(2):26–31.
27. Tohidi F, Qorbani M. The comparison of direct microscopic examination and culture results in diagnosis of cutaneous leishmaniasis. Med Lab J 2008;2(2):56–57.