Araştırma Makalesi/Original Article
Makale Dili "İngilizce"/Article Language "English"

Türk Hijyen ve Deneysel Biyoloji Dergisi

Nested-PCR assay for molecular identification of cutaneous leishmaniasis species using kinetoplast DNA gene in Mirjaveh and Reg-e Malek provinces (IRAN)

Mircave ve Reg-e Malek (İran) kentlerinde kutanöz leishmaniasis türlerinin Nested-PCR ile kinetoplast DNA geni kullanılarak yapılan moleküler tanımlanması

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ABSTRACT

Objective: As a global health challenge, especially in Iran, cutaneous leishmaniasis is one of the major health and medical problems in Sistan and Baluchestan Province and plays a significant role in the inhibition of socioeconomic growth of this deprived province. The aim of our study was molecular identification of cutaneous leishmaniasis species using kinetoplast DNA (kDNA) gene in in a small part (Mirjaveh and Reg-e Malek) of Sistan and Baluchestan region of Iran.

Methods: This descriptive cross-sectional study was carried out on patients suspected of cutaneous leishmaniasis with epidemiologics information. Samples of the skin lesions and ulcers suspected of cutaneous leishmaniasis were collected from patients with active ulcers, and the collected samples were diagnosed by of parasitologic (direct smears of the lesions and staining them using Giemsa Staining Method) and molecular methods. The specific Polymerase Chain Reaction (PCR) method was applied to the kinetoplast DNA's isolated

ÖZET

Amaç: Özellikle İran'da küresel bir sağlık sorunu olarak kütanöz leishmaniasis, Sistan ve Belucistan eyaletindeki başlıca sağlık sorunlarından biridir ve bu yoksun eyaletin sosyoekonomik büyümesinin önündeki en büyük engellerden biridir. Çalışmamızın amacı, İran'ın Sistan ve Belucistan bölgesinin küçük bir bölümünde (Mircave ve Reg-e Malek), kinetoplast DNA (kDNA) geni kullanılarak kütanöz leishmaniasis türlerinin moleküler tanımlanmasıdır.

Yöntem: Bu tanımlayıcı kesitsel çalışma, kutanöz leishmaniasisden şüphelenilen hastalarda yapılmıştır. Aktif ülseri olan hastaların kütanöz leishmaniasis şüphesi olanlarından deri lezyonları ve ülser örnekleri toplandı ve toplanan örneklere parazitolojik (direkt yayma ve Giemsa Boyama Metodu) ve moleküler yöntemlerle tanı konuldu. *Leishmania* cinsini ve türlerini belirlemek için doğrudan yaymalardan izole edilen kinetoplast DNA'larına spesifik Polimeraz Zincir Reaksiyonu (PCR) yöntemi uygulandı. Son

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Geliş Tarihi / Received : 04.02.2021
Kabul Tarihi / Accepted : 05.10.2021

DOI ID : 10.5505/TurkHijyen.2022.06887

Mirahmadi H, Gorgani F, Metanat M, Etemadi S, Tabatabaei SM, Momeni MK. Nested-PCR Assay for molecular identification of cutaneous leishmaniasis species using kinetoplast DNA gene in Mirjaveh and Reg-e Malek provinces (IRAN). Turk Hij Den Biyol Derg, 2022; 79(4): 690 - 701

from the direct smears to identify the genus and species of *Leishmania*. Finally, the resulting information was analyzed in SPSS software.

Results: The microscopic test results of 43 (57.9%) patients of the 76 patients suspected of cutaneous leishmaniasis were positive. In a PCR assay, the *Leishmania* kDNA fragment was identified in 50 patients (65.8%). Besides, 44 samples were diagnosed with *Leishmania major* and 6 samples were diagnosed with *Leishmania tropica* using species-specific primers. The agreement between the results of the molecular method and the parasitology method in the samples in Mirjaveh and Reg-e Malek was (Kappa=0.8) compelling agreement. There was a significant difference between the results of molecular with the parasitologic results obtained in the samples of cutaneous leishmaniasis in the city of Mirjaveh and the Reg-e Malek (Pv≤0.05).

Conclusion: According to the present research, cutaneous leishmaniasis prevalance caused by *Leishmania major* in Mirjaveh City in Sistan and Baluchestan Province in Iran was high. Our findings also confirmed the necessity of carrying out direct molecular tests (including direct smears) on the clinical samples of patients for negative results and species identification.

Key Words: *Leishmania major*, *Leishmania tropica*, PCR, kDNA, Sistan and Baluchestan, Iran

olarak elde edilen bilgiler SPSS yazılımında analiz edildi.

Bulgular: Kütanöz leishmaniasis şüphesi olan 76 hastanın 43 (%57.9)'ünün mikroskobik test sonuçları pozitifti. 50 hastada (%65,8) PCR ile *Leishmania* kDNA fragmanı belirlendi. Yanı sıra 44 örneğe *Leishmania major*, altı örneğe ise türe özgü primerler kullanılarak *Leishmania tropica* tanısı konuldu. Mirjaveh ve Reg-e Malek'teki örneklerde moleküler yöntem ile parazitoloji yönteminin sonuçları arasındaki uyum (Kappa=0,8) zorlayıcı bir uyumdu. Mirjaveh ve Reg-e Malek şehirlerindeki kutanöz leishmaniasis örneklerinde elde edilen parazitolojik sonuçlar ile moleküler sonuçları arasında anlamlı bir fark vardı (Pv≤0.05).

Sonuç: Yaptığımız araştırmaya göre, İran'da Sistan'ın Mirjaveh şehrinde ve Belucistan eyaletinde *Leishmania major*'un neden olduğu kütanöz leishmaniasis prevalansı yüksekti. Bulgularımız ayrıca negatif sonuçlar ve tür tanımlaması için hastaların klinik örneklerinde doğrudan moleküler testler (doğrudan yaymalar dahil) yapılmasının gerekliliğini doğruladı.

Anahtar Kelimeler: *Leishmania major*, *Leishmania tropica*, PCR, kDNA, Sistan ve Belucistan, İran

INTRODUCTION

As one of the most important zoonotic diseases in the tropical and subtropical regions, leishmaniasis is caused by various protozoan species of the *Leishmania*. This disease has various clinical manifestations in the form of cutaneous, visceral, and mucocutaneous leishmaniasis in humans and some animals, but the cutaneous and visceral types are prevalent in Iran (1,2). Leishmaniasis is also one of the most important and common endemic diseases in Iran and the second parasitic disease transmitted by arthropods after malaria. Approximately 20,000 cases of cutaneous leishmaniasis are reported in Iran every year (3).

The identification of the specifications of different species causing leishmaniasis is generally one of the most important principles in controlling and preventing leishmaniasis. Moreover, identifying the dominant strain in a region can serve pharmaceutical studies, preparation of vaccines, and preparation of the antigens required for diagnosis (4). Various

methods such as in vivo and in vitro culture, isoenzyme methods, serologic methods, immunohistochemical methods, and molecular methods are used in the differentiation of the species of *Leishmania* due to the importance of identifying of the species of *Leishmania* and its effect on the prevention, control, and treatment of leishmaniasis. The two genomic fragments of *Leishmania* parasite commonly used in molecular studies are the kinetoplast DNA (kDNA) and the internal transcribed spacer (ITS1) fragment of ribosomal DNA. The kDNA has thousands of minicircles, with each minicircle having a variable section and a conserve section. Therefore, the kDNA is a target for the diagnostic and species identification molecular studies (5,6). Depending on the specific characteristics of the genus, species, and host immune status, clinical forms may vary widely, but are generally seen in three clinical forms: cutaneous, muco cutaneous, and visceral. *Leishmania major* is originally a rodent parasite but can cause zoonotic cutaneous leishmaniasis in humans and *Leishmania tropica* is the causative agent of Anthropontic Cutaneous Leishmania (ACL) and the primary host is infected humans, who are also a major source of infection (7).

PCR technology has been successfully employed in numerous studies. This method diagnoses species-specific infections without the need for culturing (8). *Leishmania* kinoplasts are disc-shaped on the mitochondrial membrane at the base of the flagellum and consist of large and small DNA loops (maxi-circle and mini-circle kDNA). Existence of duplication and great variety of mini-circle kDNA used for the analysis of genetic and is a target for the identification of *Leishmania* species (9). In this study, presence of the *Leishmania* parasite were determined through the microscopic observation of the Giemsa-stained slides and PCR assays. Afterwards, these methods are compared and the species of *Leishmania* in Mirjaveh City and the Reg-e Malek district of Sistan and Baluchestan Province (Iran) were identified through PCR assays.

MATERIAL and METHOD

Study area

Sistan and Baluchestan Province is located in the southeast of Iran. It is located at latitude 29° 1´9.4˝ and longitude 61°27´2.2˝with an elevation of 858 m of sea level. Mirjaveh is one of the cities of Sistan and Baluchestan Province of Iran that consists of Reg-e Malek and central districts. Due to the proximity of this city to the 32nd parallel, which overlaps with the global desert belt, this city is one of the most arid parts of Iran with an arid, hot, Low rain fall and desert climate.

Figure 1. Location of sampling zone (Statistical Center of IRAN, www.amar.org.ir and 8)

Sampling

This cross-sectional study was conducted February 2017 until September 2019 on the patients referred to the health centers of Mirjaveh City and Reg-e Malek in Sistan and Baluchestan Province Iran. A total of 76 patients diagnosed with cutaneous leishmaniasis were subjected to epidemiologics information (personal information, gender, ulcer type, ulcer location, and the number of ulcers) in this study. Diagnosis was carried out by collecting direct smears of the lesions and staining them using Giemsa Staining Method. This end, a small incision was made around the ulcer and a thin smear was isolated from the serous using microscopic slides. In the microscopic analysis, leishman bodies (amastigotes) were searched inside or outside of the macrophages. Total samples (in addition to microscopic experiments) with a specific PCR method were used to identify the Cutaneous leishmaniasis and *Leishmania* species.

Giemsa Staining Method

On a clean dry microscopic glass slide, make a direct smear of the lesions and leave to air dry. Dip the smear into pure methanol for fixation of the smear, leave to air dry for 30 seconds. In the following, flood the slide with 5% Giemsa stain solution for 20-30 minutes.

DNA extraction

The surface of each dry smear was carved using a separate scalpel and was transferred to a microtub under sterilized conditions. Afterwards, 200 µl of a lysis buffer and Proteinase K solution were added to each sample and each sample was incubated for 15 min 56°C.

The binding buffer was added thereafter. After centrifuging the samples at a high speed, adding isopropanol, and pipetting, the tube contents were transferred to Spin column DynaBio Blood/Tissue Genomic DNA Extraction Kit (Iran). Next, DNA extraction was carried out in accordance with the manufacturer's instructions.

Detection and identification of *Leishmania* species

The fragment kDNA was amplified in a two-step. sequences of the used primers are demonstrated in table 1.

The first PCR recruited CSB2XF and CSB2XR primers and the second PCR used primers 13ZF and LIR (11,12).

The first PCR reaction mixture contained 250 µM deoxynucleoside triphosphate (dNTP), 1.5 µM MgCl2, 1 U Taq polymerase,1 µM CSB2XF primer (forward), 1 µM CSB2XR primer (reverse) and 5 µL DNA in a final volume of 25 µL.

The reaction mixtures were incubated in a thermocycler (Flexcycler2, Germany) as follows: initial denaturation at 95°C for 5 minutes followed by 30 cycles of 94°C for 30 seconds, 59°C for 55 seconds and 72°C for 60 seconds and final extension at 72°C for 5 minutes. The PCR products were electrophoresed in 1.5% agarose gel.

Table 1. Primers used for defining the species of *leishmania* in Nested-PCR and their sequences

PCR step	Target	Primer name	Sequence
First step	kDNA	CSB2XF	5`CGAGTAGCAGAAACTCCCGTTA3`
		CSB2XR	5`ATTTTTCGCGATTTTCGCAGAACG3`
Second step	kDNA	13ZF	5`ACTGGGGTTGGTAAAATAG3`
		LIR	5`TCGCAGAACGCCCCT3`

The first-PCR product (2 µL of a 1:5 dilution in ddH2O) was used as template for the second PCR, in a total volume of 25 µL and in the same status the first PCR but using 13ZF and LIR as the primers in 30 cycles.

The band size was 570 and 750 bp for *L. major* and *L. tropica*, respectively. Positive control (Accession Number: *L. tropica* (HM234012), *L. major* (KF701191) (13,14).

Statistical analysis

After recording of data, all information and test results, using SPSS version 22 software with descriptive statistics and chi-square test with confidence interval (CI 95%) was analyzed.

Pv ≤0.05 was considered statistically significant. The Cohen's kappa coefficient (κ) was used to obtain agreement between the tests.

This study was approved by the Zahedan University of Medical Sciences Ethics Committee (Date:22.02.2017 and No: IR. ZUMS.REC.1396.119).

RESULTS

Of the 76 suspected cutaneous leishmaniasis samples in the course of a year, leishman bodies were identified inside or outside of the macrophages of 43 samples (56.6%). (Fig 2 and Table 5).

Moreover, of the smear samples collected from Mirjaveh City with PCR assays, 27 samples were diagnosed with *Leishmania major* and 4 were diagnosed with *Leishmania tropica*. Of the 19 samples collected from Reg-e Malek, 17 and 2 samples had *Leishmania major* and *Leishmania tropica*, respectively. It should also be noted that 7 samples could not be diagnosed parasitological, but 7 samples diagnosed molecularly. Figure 3 shows the electrophoresis gel product PCR *Leishmania* isolates with positive and negative control samples using the Nested-PCR method.

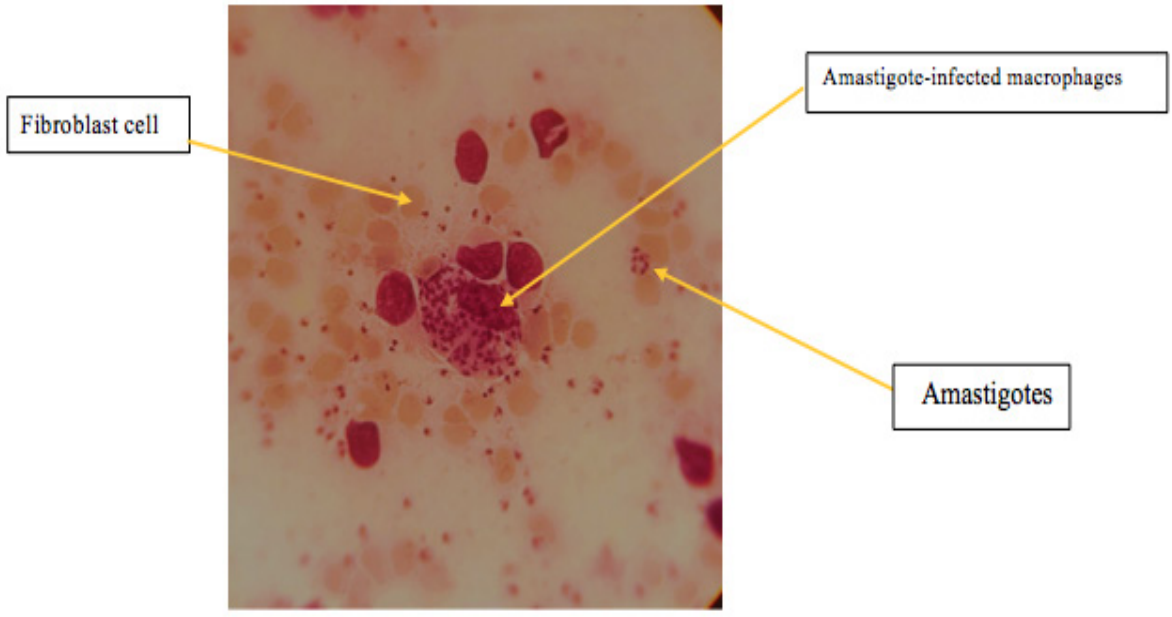

Figure 2. Leishman body (Amastigotes) of cutaneous leishmaniasis cases observed in prepared samples

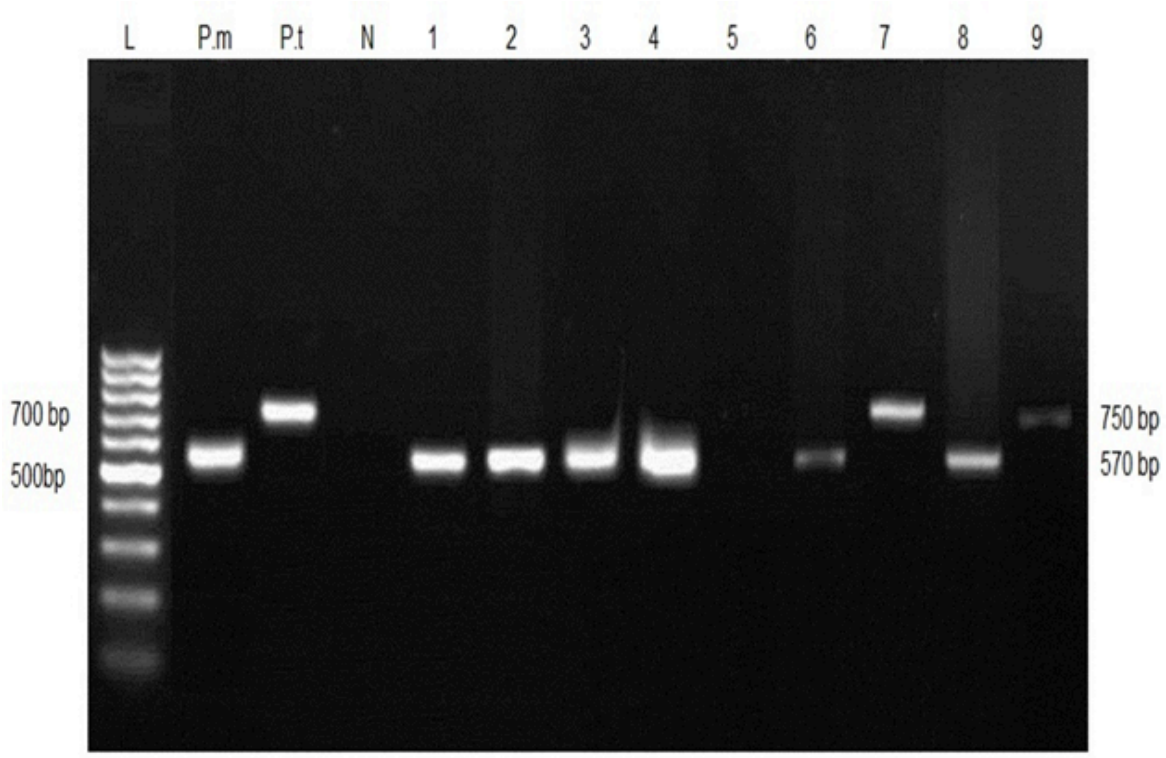

Figure 3. Gel electrophoresis of PCR products of *leishmania* isolates using Nested-PCR technique. 100 bp Size Marker (L), Positive Control *L. major* (Pm), Positive Control *L. tropica* (Pt). Negative Control (N), Patients with *L. major* (1, 2, 3, 4, 6, 8), Patients with *L. tropica* (7,9)

In this study, 39 samples were collected from men (33 (66%) *Leishmania major* and 6 (12%) *Leishmania tropica*, while 11 samples were collected from women (11 (22%) *Leishmania major*. *Leishmania tropica* was not present in the female samples. The frequencies of the *Leishmania* species are classified in Table 2 and Table 3 by location and the number of lesions in the suspected samples. Due to the fact that from each person suspected of cutaneous leishmaniasis, 3 to 4 samples were prepared from different location of lesions and all dry smears were used for parasitological testing and DNA extraction, this method automatically increased the sensitivity of the tests.

The agreement between the results of the molecular method and the parasitology method in the samples in Mirjaveh and Reg-e Malek was (Kappa=0.8) compelling agreement. There was a significant difference between the results of molecular with the parasitologic results obtained in the samples of cutaneous leishmaniasis in the city of Mirjaveh and the Reg-e Malek Pv ≤ 0.05.(Table 4)

No significant difference was found between the percentage's species of cutaneous leishmaniasis in Mirjaveh and Reg-e Malek. Also, there was no significant difference in the distribution of species of cutaneous leishmaniasis in the two regions in males and females Pv ≥ 0.05. (Table 5).

Table 2. The frequency of *Leishmania* species according to the location of lesions in the cutaneous leishmaniasis cases

Leishmania species	Place of lesion				
	Hand & Foot n (%)	Face n (%)	Nose n (%)	Hand, Foot, Face n (%)	Samples from other parts of body n (%)
L. major	12 (24)	10 (20)	2 (4)	2 (4)	18 (36)
L. tropica	3 (6)	0 (0)	0 (0)	3 (6)	0 (0)
Total	15 (30)	10 (20)	2 (4)	5 (10)	18 (36)

*n: number

Table 3. The frequency of *Leishmania* species according to the number of lesions in the cutaneous leishmaniasis cases

Leishmania species	Number of lesions						
	1 n (%)	2 n (%)	3 n (%)	4 n (%)	5 n (%)	6 n (%)	7 n (%)
L. major	15 (30)	12 (24)	6 (12)	4 (8)	4 (8)	0 (0)	3 (6)
L. tropica	3 (6)	2 (4)	1 (2)	0 (0)	0 (0)	0 (0)	0 (0)
Total	18 (36)	14 (28)	7 (14)	4 (8)	4 (8)	0 (0)	3 (6)

*n: number

Table 4. The agreement rate (Kappa) of the molecular test results by PCR with parasitology method in the samples studied

Diagnostic techniques		PCR		Total	Kappa	*Pv*
		Positive n (%)	Negative n (%)			
Parasitology	Pos	43 (100)	0 (0)	43	0.808	0.001
	Neg	7 (21.2)	26 (78.8)	33		
Total		50 (65.8)	26 (34.2)	76		

*n: number

Table 5. Frequency of *Leishmania* species based on sex and area of study

Leishmania species	Gender		Place	
	Male n (%)	Female n (%)	Mirjaveh n (%)	Reg-e Malek n (%)
L. major	33 (84.6)	11 (100)	27 (85.2)	17 (89.5)
L. tropica	6 (15.4)	0 (0.0)	4 (14.8)	2 (10.5)
Pv	0.516		0.341	

*n: number

DISCUSSION

The kDNA of trypanosomatids comprises of catenated molecules minicircle and maxicircle (15). Each minicircle has one to four (150-200 bp) conserved regions which includes repeating regions for both strands, and a variable region usually is a single guide RNA (gRNA) gene (almost 150 bp long), and can be used for detection and exact difference between Leishmania species and between strains (16).

Cutaneous leishmaniasis is one of the chief health and medical problems in the deprived areas of Iran. Unfortunately, it has adversely affected the mental, physical, and material dimensions of the patients' lives. Hence, the timely diagnosis and treatment of this disease are vital (17).

The diagnosis of cutaneous leishmaniasis through parasitologic methods such as microscopic tests, direct observation of leishman bodies, and culturing the margins of ulcers have low sensitivity and only determine the parasite genus (18). Therefore, these methods are incapable of identifying the pathogen species. In addition, serologic tests fail to differentiate between the new infections and the old infections because the antibody produced for *leishmania* parasite interferes with the antibodies of trypanosomiasis, tuberculosis, and toxoplasmosis (19).

In recent decades, several molecular methods for the epidemiologic analysis of leishmaniasis have been proposed. PCR technology has been successfully employed in these studies. This technology diagnoses species-specific infections without culturing (18).

According to the findings from different studies, PCR method, which functions using the kDNA gene of *Leishmania* parasite, shows sensitivity in the diagnosis of diseases, especially chronic diseases. Moreover, the kDNA gene is preferred over the ITS1 gene for the diagnosis of *Leishmania* parasite and it can serve as an effective molecular marker for the diagnosis of different *Leishmania* species (20,21). Hence, the kDNA gene was used in the present study.

This study aimed for the identification of the parasite causing cutaneous leishmaniasis in Mirjaveh City and Reg-e Malek. It was concluded that the *Leishmania major* was the prevalent species of *Leishmania* and the number of men suffering from cutaneous leishmaniasis was higher than women. This finding could be attributed to the stronger presence of men in open environments, the fewer pieces of clothing worn by men, the stronger presence of men in the deserted areas, and higher exposure of men to mosquitos.

As regards the number of lesions, 36%, 28%, and 36% of the patients had only one lesion, two lesions, and more than one lesion, respectively. As for the lesion location, 30%, 20%, and 10% of the patients had lesions on their feet/hands, face, and face/feet/hands, respectively. Besides, 40% of the patients had lesions on other parts of their bodies. Hence, it is concluded that the aforesaid organs are among the uncovered parts of the body that are subjected to bloodsucking vectors.

There was a significant difference between the molecular and parasitological findings in the diagnosis of cutaneous leishmaniasis in the present study. Considering the importance of detecting negative cases in the diagnosis of the disease, and detecting negative cases of illness can release of people from taking drugs and wasting time and money. Therefore, molecular methods based on functional sequence gene in diagnosis can be a great step in the treatment of disease.

In the present study, no significant differences were found between the results of cutaneous leishmaniasis and the two studied regions and sex. the study area and sex factor were not significantly associated with the infecting *Leishmania* species because samples size was inadequate. On the other hand, the frequency of *Leishmania tropica* was expected to be higher depending on the

region, but this study showed different results.

The findings from the studies by Omidian in Ahwaz (South West Iran) and Yaghubi Ershadi in Yazd) central of Iran), who carried out direct and PCR tests, suggest that these areas are the centers of zoonotic cutaneous leishmaniasis caused by *Leishmania major* (22, 23). According to the study by Naseri in Torbat Heydariyeh (Northeast of Iran), The most identified species (*Leishmania tropica*) were using molecular tests (24). Also, Saad Abadi conducted a study in Neishabour City to identify different species of *Leishmania* parasite using the RAPD-PCR technique and reported the presence of *Leishmania tropica* in all samples (25). The results of this study are not consistent with our study with the frequency of *Leishmania major*.

Moreover, in Yaman, Mahdi identified the leishmania species in patients suffering from cutaneous leishmaniasis using the nested PCR method and the ITS-1 gene. Their findings revealed the presence of *Leishmania tropica* in 16 samples (26).

Molecular differentiation in cutaneous leishmaniasis plays an important role in the treatment of patients because the treatment of these people does not depend only on direct examination and clinical signs, and the results of such studies help the process of treatment and control of the disease (27).

A comparative analysis with six different molecular targets using PCR for diagnosis of old world leishmaniasis showed that sensitivity of different gene targets from highest to lowest is in the order of kDNA-PCR > SSU rRNA-PCR > ITS2-PCR > ITS1-PCR > ME-PCR > HSP70-PCR (28). The results of this study emphasize the high sensitivity of the KDNA gene target and the kDNA genomic region is the most sensitive for routine diagnosis of leishmaniasis.

The kDNA-PCR method for detect American tegumentary leishmaniasis (ATL) in Brazil showed that 87.5% diagnosis with this method, while the other methods demonstrated the following percentages of positivity: 62.8% for the Montenegro skin test, 61.8% for parasitology techniques, and 19.3% for in vitro culture (29).

Therefore, the results of the present study do not comply with the findings from the three aforesaid studies with respect to the prevalence of the *Leishmania* species. The prevalence of different *Leishmania* species in different parts of the world is determined by numerous factors such as weather differences, ecological conditions, life style changes, trips, uncontrolled immigration, and the large population of the war refugees.

CONCLUSION

Considering the importance of identifying the parasite species for the epidemiologic analysis of the disease and formulation of control plans, molecular methods are highly sensitive to the parasitologic techniques, especially the microscopic method, in the diagnosis phase.also kDNA-PCR have high sensitivity for diagnosis of cutaneous leishmaniasis (*Leishmania tropica* and *Leishmania major*) because can be used for detection and exact difference between *Leishmania* species and between strains.

ACKNOWLEDGMENT

* This study was financially supported by Zahedan University of Medical Sciences for the financial support (Grant no: 8294) (Thesis code: T/792). This project is from Dr. Farzaneh Gorgani's (infectious specialist student) thesis.

ETHICS COMITTEE APPROVAL

* This study was approved by the Zahedan University of Medical Sciences Ethics Committee (Date:22.02.2017 and No: IR.ZUMS.REC.1396.119).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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