Original Article

Determination of Species of Leishmania Using *HSP70* Gene in Patients Referred to Selected Health Centers of Iran

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Abstract

Background: Cutaneous leishmaniasis is caused by Leishmania major and Leishmania tropaica. Iran is considered as one of the world's largest leishmaniosis centers. In this study, the restriction fragment length polymorphism (RFLP)-PCR method was used to determine the species of pathogenic parasites.

Materials and Methods: In this cross-sectional study, 120 patients with suspected cutaneous ulcer referring to the selected medical centers of Shahid Beheshti University of Medical Sciences were selected. Their demographic characteristics were recorded. After diagnosis of parasite by microscopy, positive samples were cultured in NNN medium. After DNA extraction of cultured parasite, PCR was performed for amplification of the *HSP70* gene. The PCR products were digested with *HaeIII* restriction enzyme; the obtained patterns were compared with the same genes in the GenBank database.

Results: A 1422 base pair fragment was detected in PCR of *HSP70* gene in 30 positive samples. After digestion, 16 (53.3%) of the samples, had an enzymatic digestive pattern compatible to *L. major* and 14 (46.7%) others had the *L.* tropica profile. More information concerning demographic aspects were obtained after analyses them with the infected samples.

Conclusion: Regarding the history of patient trips to their hometown, the transfer does not appear to have occurred in areas covered by the university's medical centers, but the presence of these patients in these areas requires careful monitoring them. Additionally, control of population and mosquito species is needed. The results of this study sustain that the *HSP70* gene still has sufficient ability to differentiate between two different species of *L. major* and *L. tropica*.

Keywords: Leishmaniasis, PCR_RFLP, *HSP70* gene, Medical centers

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Introduction

The world health organization (WHO) has prioritized 6 infectious diseases in tropical and subtropical regions, one of which is Leishmaniosis. The disease has been reported in 88 countries in Asia, Europe, Africa and America. It is one of the major health and economic problems in many countries of the world, including in Iran. Currently, 350 to 400 million people are at risk for the disease worldwide and about 2 million people are infected with new cases every year[1].

This disease is seen in both cutaneous and visceral forms in Iran. Cutaneous form is found in both rural and urban in referred country. The control and isolation of

the parasites are of great importance with regard to reservoirs and vectors. More than 23-30 species of *Leishmania* have been identified that cause infection in humans and many mammals[1-4]. About 20,000 new cases of the disease are reported each year in the country. That is certainly less than the actual statistics[5].

The agent of cutaneous leishmaniasis in Iran is *L. major* and *L. tropica*. All species belong to the *Leishmania* genus and are morphologically similar, but isolation of them is not possible just by parasitological methods. These methods included direct observation of the parasite in patient-made smears, cultivation in NNN, and serology method. For management the leishmaniasis, it is necessary to identify the pathogenic species using complementary methods. Then, planed a specific strategy for control and treatment with the lowest costs and side effects[6].

Advanced methods such as DNA analysis is need to distinguish the species of *leishmania* in addition to clinical signs and knowledge of geographical dispersal, and immunological features.

New molecular techniques such as PCR, RFLP-PCR and Real Time -PCR and different molecular markers are used to identify and differentiate parasite species[8-9]. Some of the genomes used for this purpose included internal spacers for transcription or ITS, gp63, *HSP70* gene, β-tubulin, kDNA, mini-exon, G6pd, cytochrome b and *HSP70*[10-12].

One of the proteins used in research work on *Leishmania* parasites is heat shock proteins. HSPs are found in all living organisms, from bacteria to humans[13]. *HSP70* is one of the *HSP* proteins that have highly conserved sequences and play an important role in stimulating immune responses, including humoral immunity[14]. The genes of this protein are conserved and specific for *Leishmania*, and it has been mentioned as a suitable target for the differentiation of *Leishmania* species[15-16]. The aim of this study was to use PCR to detect *Leishmania* genus the agent of cutaneous leishmaniosis in old world and to use RFLP-PCR to isolate *Leishmania* species (*L. tropica* and *L. major*, which is common in Iran) in patients referred to selected health centers of Shahid Beheshti University of Medical Sciences using *HSP70* gene.

Methods

Study area and sample collection

The study population was patients with suspected cutaneous leishmaniasis referred to selected health-diagnostic centers of Shahid Beheshti University of medical sciences (Shahid Jafari Center, Pakdasht and Pishva counties), from September 2017 to September 2018. These centers were selected based on statistics from previous years and the number of patients referred.

In this experimental study, samples were collected from the individuals clinically suspected to CL and referred to mentioned medical centers. A questioner was completed to record the essential information such as demographic information, sites of ulcer on the body and travel history. After collecting a complete clinical history, finally 120 samples were taken for isolation of *Leishmania* species.

Sample preparation and microscopic examination

Skin suspected lesions were cleaned with 70% ethanol, and then we scratched the marginal side of the lesion and using its sera for direct smear preparation. After air-drying, slides were fixed with absolute methanol, stained with Giemsa 10%. The slides were examined under optical microscopy (magnification ×1000) for the existence of amastigote forms of the *leishmania.*

Cultivation the parasite

The positive sample sera were transferred to the NNN culture medium[17]. After parasite replication in NNN medium we sub cultured it in a tube of liquid medium, RPMI 1640 (Gibco, Germany) supplemented with 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco) for further replication. The promastigote were harvested after washing twice by PBS buffer and the pellet were frozen in -20° for DNA extraction.

DNA extraction and PCR

Genomic DNA was extracted from isolated parasites using the phenol, chloroform and stored at -20°C for PCR. A primer pair designed by Montalvo et al., 2004 was used to amplify the target DNA[7].

HSP70 sen:5'-GACGGTGCCTGCCTACTTCAA-3'

HSP70 ant:5'-CCGCCCATGCTCTGGTACATC-3'

The primers were synthesized by the Bioneer Corporation (Daejeon, South Korea) via Pishtazteb company Tehran Iran.

Considering the sequence reference and primer specificity in the Blast site of NCBI, expected a fragment of about 1400 bp for *L. major* and *L. tropica*. The PCR reaction was prepared in a volume of 20 µl containing 1 µg of genomic DNA, 1 U of Taq DNA polymerase (GeNet Bio, Korea), 10 µl Master Mix RED (Ampliqon) and 10 picomol of each primer and 8 µl double distilled water. Samples were amplified in (Techne thermocycler.UK) using the following cycling parameters:((1) initial denaturation at 94° C for 5 min; (2) 30 cycles of 94° C for 30 s, 59° C for 40s and 72° C for 30s ; and (3) a final extension step of 5 min at 72° C. Analysis on a 1.5% agarose gel (Invitrogen, Carlsbad, California, USA)which was ethidium bromide stained and used to verify the amplified product length of 1422 bp.

Validation of primers was done by using standard stains of *L. major* (MRHO/IR/75/ER10) and *L. tropica* (MHOM/IR/02/Mash10) prepared from the Tehran University of medical sciences.

RFLP analysis of *HSP70*

Testing the sequences of GenBank reference with HaeIII enzyme for restriction site informed us fragments from digestion with HaeIII would be expected to make different patterns as follows: L. major (351, 307, 246, 152, and 99 bp), L. tropica (354, 338, 246, 150 and 99 bp), Digestion was performed in a total of 30 ml 1× optimal buffer, using 1U HaeIII restriction enzyme. Reactions were incubated for 2 hours at 37˚C and completely analyzed by electrophoresis in a 3% small fragment agarose gel. The gels were subsequently ethidium bromide stained and subjected to electrophoresis along with the 100 bp DNA Ladder (MBI Fermentas) as a reference DNA size marker. After enzymatic digestion and comparison with standard urban and rural *leishmania* species, a sample of each, were sent for sequencing.

Results

In this study, 120 patients referred to the laboratories of Shahid Jafari, Pishva and Pakdasht Health Centers. Each patient had 1-3 lesion and we totally sampled from 190 lesions of all referred patients. While 48 (40%) samples were positive for *Leishmania* parasites and 72 (60%) isolates were negative. All positive samples were cultured in NNN medium but 18 isolates were contaminated by fungi and bacteria and promastigotes did not observe, although we used antibiotics, penicillin G (100 IU/ml) and streptomycin (100 µg/ml). Finally, we yielded 30 pure positive isolates and amplification of the HSP70 gene resulted in production of about 1,422 bp fragment in all 30 *Leishmania* isolates (fig-No:1).

HaeIII restriction enzyme was used to differentiate between two standard *L. tropica* strains (MHOM/IR/02/Mash10) and *L. major* strains (MRHO/IR/75/ER) that were identical to the digestions made on isolated samples (fig-No:2).

After enzymatic digestion of patients samples, *L. tropica* with 246bp, 307bp and 338bp bands and *L. major* parasites with bands 130, 246 and 350 were identified (Fig. 3). In spite of the enzymatic digestion observed, all samples were divided into two groups of *L. tropica* and *L. major* similar to standard ones. For certainty and definitive diagnosis, one sample of each

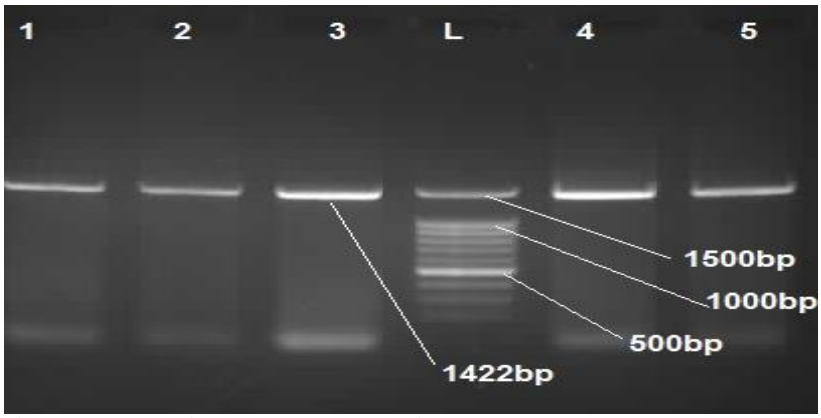

Figure 1. Amplification of the *HSP70* gene of L.tropica and L.major from isolated samples on 1% agarose gel.
No 1-5: isolated samples from patients
L: 100bp size marker (Fermentas).

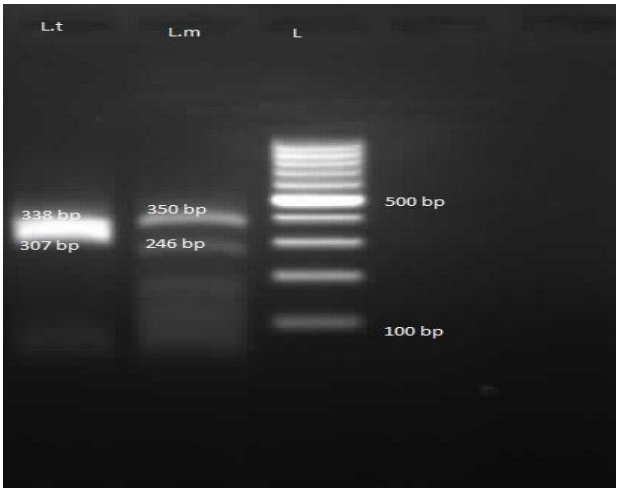

Figure 2. Digestion pattern of HaeIII enzyme on *HSP70* gene of standard samples *of L. major and L.tropica.* L.t : standard samples of *L. tropica* . L.m: standard samples of *L. major*. L: 100bp size marker (Fermentas).

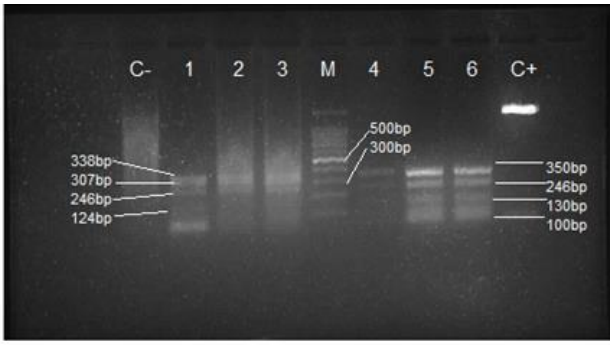

Figure 3. The digestion patterns of (HaeIII) enzyme on *Hsp70* gene of isolated samples from patients on 2.5% agarose gel. C-: negative control, C+: undigested positive control, M: 100bp size marker,1-3 *L. tropica* ,4-6 *L.major*.

shear pattern was sent to Bioneer Company Korea for sequencing (by ABI sequencer) through Iran Takapozist Company. The results re confirmed the type of *L. tropica* that corresponded to a clade of *L. tropica* registered in gene bank under accession numbers LC328563 and the *L. major* corresponded to a

Table 1: Frequency of *L. major* and *L.tropica* isolates collected from health centers.

Health centers	Leishmania spp.		Leishmania spp		
			L. major	L. tropica	Total
	Shahid jafari	Count	4	10	14
		% within healthcenter name	28.6%	71.4%	100.0%
	Pishva	Count	9	1	10
		% within healthcenter name	90.0%	10.0%	100.0%
	Pakdasht	Count	3	3	6
		% within healthcenter name	50.0%	50.0%	100.0%
Total		Count	16	14	30
		% within hospital name	53.3%	46.7%	100.0%

Table 2: Frequency of L. major and L. tropica Specimens Depending on Patients' Travel to their hometown collected.

city/ health center name	leishmania	city/leishmania								Total
		Isfahan major	Isfahan tropica	Mashhad major	Mashhad tropica	Yazd major	Sabzevar major	Sabzevar Tropica	Shahrood major	
Shahid jafari	Count	3	8	0	1	0	0	1	1	14
	% within health center name	21.4%	57.1%	.0%	7.1%	.0%	.0%	7.1%	7.1%	100.0 %
Pishva	Count	0	0	4	0	1	4	1	0	10
	% within health center name	.0%	.0%	40.0%	.0%	10.0%	40.0%	10.0%	.0%	100.0 %
Pakdasht	Count	0	3	1	0	1	1	0	0	6
	% within health center name	.0%	50.0%	16.7%	0%	16.7%	16.7%	.0%	.0%	100.0 %
Total	Count	3	11	5	2	1	5	2	1	30
	% within health center name	10.0%	36.7%	16.7%	3.3%	6.7%	16.7%	6.7%	3.3%	100.0 %

clade of *L. major* registered in gene bank under accession numbers HF586344.

Totally 10 (71.4%) samples collected from Shahid Jafari Health Center. 6 (42.4%) were *L. tropica* and 4 (28.6%) samples *L. major*. Pishva positive samples 9 (90%) were *L. major* and 1 (10%) were *L. tropica* and both statistically were significant (pvalue<0.012), but in Pakdasht center we had 3 *L. tropica* and 3 *L. major* positive samples which they were equal and it was not significant (Table 1).

We used Pearson Chi-Square to analyze the recorded data of patients. All who participated in this study had a history of travel to their hometowns such as Isfahan, Mashhad, Yazd, Sabzevar and Shahroud. These cities are all endemic areas of leishmaniasis. The frequency of samples in the cities traveled and the type of parasite recorded indicated that most of the samples belong to the Shahid Jafari Center with 14 patients, 71.4% of *L. tropica* and 28.6% of *L. major*. The Pishva Center with 10 patients, 90% *L. major* and and 1 patient 10% *L. tropica*, and Pakdasht Center with 3 patients 50% *L. tropica* and 3 patients 50% *L. major*, are less in importance. The prevalence of the disease in individuals is significant due to the frequency of the type of parasites in these areas (P value <0.03) (Table 2).

According to the collected data, the proportion of infected persons in males was more than females and was the most frequent in the age group of 30-40 years, none of which was statistically significant (Table 3).

Table 3: Frequency of Leishmaniasis patients referred to Shahid Jafari, Pakdasht and Pishva health centers by Age and Sex in.

gender / age		gender		
		male	**female**	**Total**
<10	Count	1	1	2
	% within age	50.0%	50.0%	100.0%
10-20	Count	2	1	3
	% within age	66.7%	33.3%	100.0%
20-30	Count	3	0	3
	% within age	100.0%	.0%	100.0%
30-40	Count	7	7	14
	% within age	50.0%	50.0%	100.0%
40-50	Count	3	2	5
	% within age	60.0%	40.0%	100.0%
>50	Count	2	1	3
	% within age	66.7%	33.3%	100.0%
Total	Count	18	12	30
	% within age	60.0%	40.0%	100.0%

When looking at the bite location in different cities it can be seen the difference of bite location is similar in all cities and shows the tendency of the biters in all cities to the exposed location (Table 4).

Discussion

Leishmaniasis is endemic in Iran and the presence of this disease in this country has a long history. The Traditional medicine practitioners like Ibne Sina, were familiar with this disease and name it Salak (one year) because of its long duration. Complications of this disease are seen in two forms of wet and dry lesion due to the presence of two different species of *L. tropica* (Anthropometrics) and *L. major* (zoonotic). The disease has been reported in recent years in Evin[18], Yousef Abad, Vanak and Punk, which are north and northwest of Tehran[19]. The high number of referred patients with suspected lesions to cutaneous leishmaniasis at the health centers of Shahid Beheshti University of Medical Sciences in southeastern Tehran over the years had courage us to a detailed assessment of the infection and the possibility of transmission and species of parasites in this area.

After enzymatic digestion we could distinguish both *L. major* and *L. tropica* in our samples, that was similar to Frega et al study which used *HSP70* gene for old World Leishmania including *L. major*, *L. Tropica* and *L. donovani* detection in Cuba 2010[15]. In the other study Cruz et al, isolated the sub species of Leishmania and Viania using *HSP70*, Gp63 and cytochrome b genes, but they had concluded that *HSP70* gene is the best gene and the Nested-PCR method is the best method to determine the parasite species in clinical specimens. In agreement of our study, especially in case where the parasite sample and the DNA concentration was low[20]. In China phylogenetic analysis of Leishmania was performed using cytochrome b and *HSP70* genes by Dongmei et al in 2016. They collected samples from humans, dogs, mice and mosquitoes, and all species were successfully differentiated by using *HSP70* gene, therefore they declared that *HSP70* gene was more

Table 4: Frequency of cutaneous leishmaniasis based on place of lesions, parasite type and travel location in.

Place of lesion / leishmania/city			Leishmania major /city						
			Isfahan	Mashhad	Sabzevar	Isfahan	Mashhad	Sabzevar	Total
	Face	Count	2	1	2	2	0	0	7
		% within Place of lesion	28.6%	14.3%	28.6%	28.6%	.0%	.0%	100.0%
	Arm	Count	2	2	0	0	0	1	5
		% within Place of lesion	40.0%	40.0%	.0%	.0%	.0%	20.0%	100.0%
	wrist	Count	2	2	0	1	1	0	6
		% within Place of lesion	33.3%	33.3%	.0%	16.7%	16.7%	.0%	100.0%
	body	Count	0	0	0	1	1	0	2
		% within Place of lesion	.0%	.0%	.0%	50.0%	50.0%	.0%	100.0%
	fore arm	Count	1	1	0	2	1	1	6
		% within Place of lesion	16.7%	16.7%	.0%	33.3%	16.7%	16.7%	100.0%
	foot	Count	0	1	1	2	1	2	7
		% within Place of lesion	.0%	14.3%	14.3%	28.6%	14.3%	28.6%	100.0%
Total		Count	7	7	3	8	4	4	33
		% within Place of lesion	21.2%	21.2%	9.1%	24.2%	12.1%	12.1%	100.0%

powerful than others[21]. The next study which confirmed the capability of *HSP70* gene in Colombia 2016 was the study of Montalvo et al which used this gene for separation the sub spices of the Leishmania donovani and Viania complexes[22]. Two other researchers, Mir Ahmadi et al and Nemati et al, used the *HSP70* gene to identify Leishmania parasites in Sistan and Baluchestan south east of Iran and Tehran provinces, indicated that the *HSP70* gene in Iran can be used to isolate species, which is in agreement with the present study[23-24]. After enzymatic digestion of 30 positive samples from culture media[16], *L. major* and *L. tropica*[14] were identified that, indicated the presence of both species in our patients, but Fattahi Bafghi and Arjmand stated that *L. major* as the dominant specie[25-26], in their study or Saadabadi stated that *L. tropica* was dominant species[27] in their study, and both these results were opposed to the present study.

A short glance at demographic data informed us that, the ratio infection of male to female contamination was higher but not statistically significant, and our result was in agreement with results of studies of Akhlagh and

Feiz hadda[28-29]. Most infections were in the age group of 30-40 years, and disagreement with Feiz haddad studies that reported infection rates between 21-30 years or Ahmadi and Mesgarian studies in Kashan and Gonbad Cavus cities which reported infection rates in ages 1-10 years old respectively [30,33].

It is necessary to point out that the largest patient population was in the Jafari health center rather than Pishva and Pakdasht centers, which indicates the percentage of immigrants in this area was more than others and needs more attention to the population of sand fly for prevention of transmission. Considering the high frequency of patients trips to Isfahan, Mashhad and Sabzevar (endemic area of leishmaniasis) respectively, in last 6 months, confirmed the presence of both cutaneous leishmania species, thus it can be stated that the above-mentioned cases were just imported and no transitions were happened in these areas. This study was not matching with Ahmadi study et al, in Kashan[30] and Abdullah Zadeh et al, in South Khorasan[23] and Khajeh Deloey et al, in Khorasan Razavi[24], because they have described their study areas as the hotspot for leishmaniasis transmission[15]. While evidence suggests that all positive cases in this study had a history of trip to endemic areas of leishmaniasis.

Conclusion

This study indicated that considered patients were infected to common types of cutaneous leishmaniasis (*L. major* and *L. tropica*). All of them had a trip history to endemic area (their hometowns) for the last six months. We successfully detected different types of cutaneous leishmaniasis by molecular method using *HSP70* gene and *Haelll* restriction enzyme. Due to the presence of phlebotomine sand flies in the covered area, and the presence of immigrant patients, there is a high risk of leishmaniasis epidemic in the area if they are not properly monitored and controlled.

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