

Molecular Identification of *Leishmania* Parasites in Sand Flies (Diptera, Psychodidae) of an Endemic Foci in Poldokhtar, Iran

Abstract

Background: Cutaneous leishmaniasis is one of the most important public health problems in many developing countries. Sand flies, as vectors, transmit infectious forms of the parasite to the vertebrate hosts. Poldokhtar, South West of Iran, is one of the endemic foci of diseases with a little information about it. In this paper, we have tried to gather some useful information to control and to prevent this disease in this region. **Materials and Methods:** The present study was conducted to determine the vector(s), the parasite, and the species composition of sand flies in the Poldokhtar County during the months from July to September 2015. Sticky paper traps were used to collect sand flies from July to September. Species identification was done based on available diagnostic keys. Nested-polymerase chain reaction was performed to diagnosis the *Leishmania* infection of sand flies, and restriction fragment length polymorphism was used to identify the *Leishmania* species. **Results:** A total of 2000 specimens comprising 8 species of sand flies (6 *Phlebotomus* and 2 *Sergentomyia*) were identified. *Phlebotomus papatasi* was the dominant species outdoor and *Sergentomyia sintoni* was the dominant species indoor. Among the 163 specimens of female *P. papatasi*, just 10 of them (6.1%) were positive to *Leishmania major* parasites. **Conclusion:** This is the first report of *Leishmania* infection of *P. papatasi* to *L. major* in this region. The results revealed that the high density of *P. papatasi* in outdoor and their infection with *L. major* is attributed that this species can play a major role as a principle vector in this region.

Keywords: Cutaneous leishmaniasis, nested-polymerase chain reaction, Poldokhtar, restriction fragment length polymorphism, sand fly

Introduction

Leishmaniasis is one of the six important infectious diseases in tropical and subtropical regions and also is a serious public health complication in the world. This disease is endemic in 88 countries, especially in the developing ones. More than 350 million people are considered at risk of contracting the disease and about 2 million of new cases occur each year.^[1]

Leishmaniasis is caused by an obligate intracellular parasite of the genus *Leishmania* and is transmitted to the vertebrate host mainly through the bite of female sand flies. The disease clinically has four main forms: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis, diffuse CL, and visceral leishmaniasis (VL).^[2]

Sand flies (family Psychodidae, subfamily Phlebotominae) are vectors of the disease. More than 800 sand flies species are known

worldwide that about 70 of them have medical importance. Vectors of this disease are belonging to the genus *Phlebotomus* in the old world and *Lutzomyia* in the new world.^[3]

Iran is known as one of the high-risk endemic countries of the disease, with >20,000 annual cases. *Leishmania major*, *Leishmania tropica*, and *Leishmania infantum* are the causative agents of zoonotic CL (ZCL), anthroponotic CL, and zoonotic VL, respectively, in Iran.^[4,5]

Despite efforts to prevent and to control leishmaniasis, this disease has been expanded and new foci of disease are reported every year, and at the moment, leishmaniasis is endemic in 17 out of 31 provinces. One of the newest reported hotspot foci is in the west of Iran, Poldokhtar. This region is in South of Lorestan Province and has common borders with Ilam and Khuzestan provinces.^[6]

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Increasing the number of people, infected with leishmaniasis, over the past 10 years in this region and its neighboring provinces as Khuzestan and Ilam (active foci of CL) could be indicators of an emerging endemic focus in this area.^[11]

One of the first practical approaches for investigating and controlling the disease is the evaluation of disease's vectors and their characteristics in an area. Since we did not find any reliable research on the vectors of leishmaniasis in this region up to now, designing a study to identify the vectors and their characteristics seems essential.

Materials and Methods

Study area

The North and East of Poldokhtar district is bordered to Khorramabad city, the North and North-West is bordered to Kuhdasht city, and the South is bordered to Khuzestan and Ilam provinces [Figure 1]. This city covers an area of 3615 km² and situated between 47°42' E and 33°9' N longitude, at altitude of 660 m above the sea level. The climate of this county is warm and semi-dry, with maximum and minimum recorded temperatures of 50°C and -2°C, respectively. Annual rainfall has been recorded as 450 mm³.^[6]

This region has been divided into 2 parts as Mamulan and Markazi and consists of 24 villages. We select 10 of villages randomly with 3 locations, triangularly, in each village for our investigations.

Sample collection

Sand flies were collected biweekly using sticky papers, placed indoors (living room, bedroom, bathroom, and toilet), and outdoor (cracks in the walls, rodent burrows, barn animals, and bird holes) during the months from July to September 2015. The traps were installed before sunset and the sand fly specimens were collected in the next morning at dawn.

Trapped sand flies were picked out from sticky papers using needles; they were put into the acetone as the degreasing solution for seconds and then were transferred into the vials, filled with 70% ethanol. All collected specimens

were transferred to the laboratory of the Department of Parasitology, Medicine School of Isfahan University of Medical Sciences, for the next investigations.

For species identification in the laboratory, head and abdominal genitalia segments of each sand fly were removed gently, mounted in Puri's medium, and identified after 24 h using a reliable diagnostic key.^[7]

DNA extraction

DNA was extracted and purified using a conventional phenol-chloroform protocol with a slight modification. Briefly, the ethanol-fixed whole bodies of individual sand flies were homogenized and transferred to 1.5 ml microtubes, containing 300 µl of lysis buffer and 300 µl glass bids, then were vortexed, and incubated at 95°C for 20 min. The 300 µl of phenol-chloroform (1:1) was added, then were vortexed, and centrifuged, and chloroform extraction was performed again. A 2.5 equal volume of absolute alcohol and 1/10 volume of sodium acetate (pH 5.2) were added to the supernatant and centrifuged for 10 min at 10,000 rpm, and the precipitant was washed with 70% ethanol and was centrifuged for 5 min at 5000 rpm. The pellet was air dried and suspended in 50 µl of distilled water (Tris-EDTA buffer) and stored at -20°C until use.^[8]

Identification of *Leishmania* species from sand flies by polymerase chain reaction and nested-polymerase chain reaction method

Polymerase chain reaction (PCR) amplification to detect the *Leishmania* species DNA was carried out in a reaction mixture. The first-stage PCR mixture contained 0.5 µl of each forward Leish out F (5'-AAA CTC CTC TCT GGT GCT TGC-3'), with the reverse primer Leish out R (5'-AAA CAA AGG TTG TCG GGG G-3'), external primers, 2 µl template DNA, 10 µl premix, and 7 µl H₂O in a volume of 20 µl. After an initial denaturation at 95°C for 5 min, the procedure was followed and carried out with 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 45 s and extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min with the BIO RAD System T100 Thermal Cycler.

For identifying the *Leishmania* genus, the first round reactions were performed in duplication with 1 and 1/100 µl volumes of DNA template; the second-round (nested) PCR was performed in a final volume of 20 µl containing µl of a 1:100 dilution in distilled water of the first-round PCR product as template, 0.5 µM of each forward (Leish in F as 5'-AAT TCA ACT TCG CGT TGG CC-3') and reverse (Leish in R as 5'-CCT CTC TTT TTT CTC TGT GC-3') internal primers, 10 µl of Premix, and 8 µl sterile distilled water to a final volume of 20 µl.

The reaction was started with an initial denaturation at 95°C for 2 min, followed by 25 cycles of denaturation at

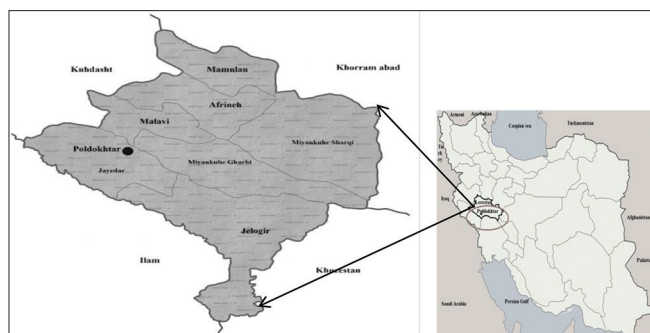


Figure 1: Map of study area

95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The final extension was carried out at 72°C for 10 min. Reference strains *L. major* (MRHO/IR/75/ER) were used as positive controls, and distilled water was used as negative controls. The PCR product of the negative control of the first-round PCR was used as the negative control in the second round, and also, the PCR product of the positive control of the first-round PCR was used as positive control in the second round.

PCR amplification was followed by restriction fragment length polymorphism (RFLP) technique using MNL1 enzymes for final species identification of the parasite.

Restriction analysis was performed in a total volume of 15 µl solution including 10 µl nested-PCR products, 0.3 µl MNL1 enzyme, 1 µl enzyme reaction buffer, and 3.7 µl ddH₂O, at 37°C for 90 min. Restriction fragments were analyzed in 1.5% agarose gel using in Tris/Borate/EDTA buffer (0.09 mM Tris, 0.09 mM boric acid, and 20 mM ethylenediaminetetraacetic acid, pH 8.3).^[9] Figures 2 and 3 show Nested PCR and RFLP products on the agarose gel respectively.

Results

In this study, in total, 2000 sand flies were collected (966 females, 48.3% and 1034 males, 51.7%) and eight species of sand flies (6 of genus *Phlebotomus* and 2 of genus *Sergentomyia*) were identified. These eight species were included *Phlebotomus papatasi* (27.65%), *Phlebotomus alexandri* (23.9%), *Phlebotomus sergenti* (5.65%), *Pelodytes caucasicus ssp.* (0.1%), *Phlebotomus salehi* (0.05%), and *Phlebotomus kazeruni* (0.05%) and two *Sergentomyia* species, i.e., *Sergentomyia sintoni* (41.7%) and *Sergentomyia tiberiadis* (0.9%) in the studied area [Table 1].

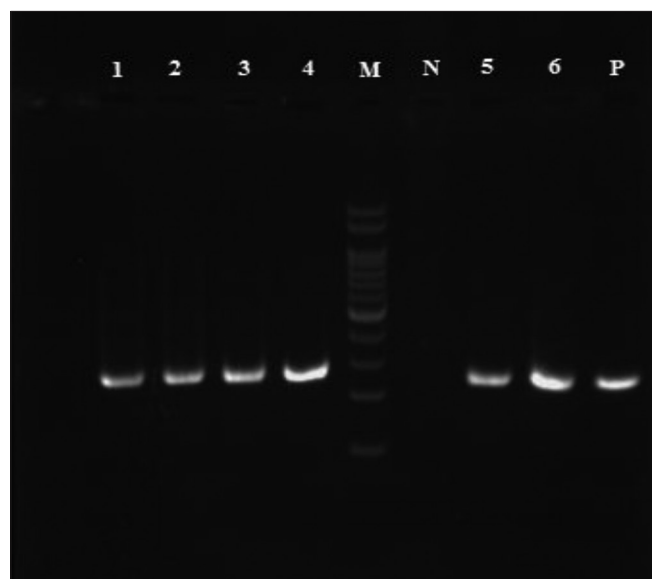


Figure 2: Agarose gel electrophoresis of nested-polymerase chain reaction products. M: 100 bp ladder, N: negative control (distilled water), P: Positive control of *L. major*

P. papatasi was the dominant outdoor and *S. sintoni* was the dominant indoor species. The sex ratio of collected specimens also has been shown in Table 2.

Table 1: Species of collected sand flies form Poldokhtar, Lorestan, Iran, July-September 2015

Month species	Total	September	August	July
<i>P. papatasi</i>	209	90	257	556
<i>P. papatasi</i>	199	38	241	478
<i>P. sergenti</i>	47	10	56	113
<i>P. caucasicus ssp.</i>	2	0	0	2
<i>P. salehi</i>	0	0	1	1
<i>P. kazeruni</i>	0	0	1	1
<i>S. sintoni</i>	172	654	5	831
<i>S. tiberiadis</i>	7	5	6	18
Total	636	797	567	2000

P. papatasi: *Phlebotomus papatasi*, *P. sergenti*: *Phlebotomus sergenti*, *P. caucasicus*: *Phlebotomus caucasicus*, *P. salehi*: *Phlebotomus salehi*, *P. kazeruni*: *Phlebotomus kazeruni*, *S. sintoni*: *Sergentomyia sintoni*, *S. tiberiadis*: *Sergentomyia tiberiadis*

Table 2: The sex ratio of collected sand flies

Species	Male	Female	Sex ratio
<i>P. papatasi</i>	390	162	2.4
<i>P. alexandri</i>	352	124	2.9
<i>P. sergenti</i>	113	-	-
<i>P. caucasicus ssp.</i>	2	2	1
<i>P. kazeruni</i>	1	1	1
<i>P. salehi</i>	1	-	-
<i>S. sintoni</i>	170	664	0.26
<i>S. tiberiadis</i>	3	15	0.19
Total	1032	968	1.06

P. papatasi: *Phlebotomus papatasi*, *P. sergenti*: *Phlebotomus sergenti*, *P. caucasicus*: *Phlebotomus caucasicus*, *P. salehi*: *Phlebotomus salehi*, *P. kazeruni*: *Phlebotomus kazeruni*, *S. sintoni*: *Sergentomyia sintoni*, *S. tiberiadis*: *Sergentomyia tiberiadis*

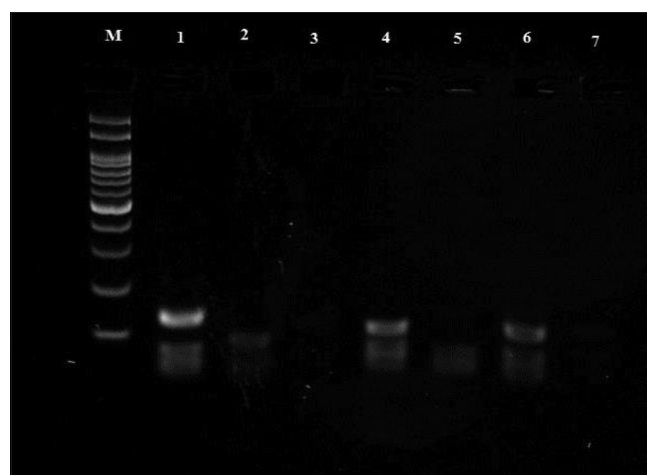


Figure 3: Polymerase chain reaction- restriction fragment length polymorphism analysis of ITS2 region for identification the *Leishmania* species using MNL1 M marker, 1-5: Infected *Phlebotomus papatasi* to *Leishmania major*, 6-7: Infected *Phlebotomus alexandri* to *Leishmania major*

All female sand flies were checked for their abdominal status as gravid, semi-gravid, blood-fed, and nonfed. This examination revealed that >90.68% of them were empty or nonfed [Table 3].

Seasonal abundance of sand flies in studied area and peaks of every species during the time period of investigations also have been shown below as Figure 4.

Two hundred and ninety specimens from indoor and outdoor including 163 *P. papatasi*, 124 *P. alexandri*, 2 *P. caucasicus*, and 1 *P. kazeruni* were examined for *Leishmania* infection using Nested-PCR. Ten *P. papatasi* and 8 *P. alexandri* were positive for *Leishmania*.

These 18 samples were examined again using PCR-RFLP to detect the species of *Leishmania* Parasites. All of these females were infected just with the *L. major* species.

Discussion

Determining of leishmaniasis vectors species, their characteristics, and their infection to *Leishmania* parasites are useful and important data for planning a suitable disease control program in an endemic area. Hence, the main goals of this study were identifying the sand fly vectors species and their *Leishmania* parasite infection with

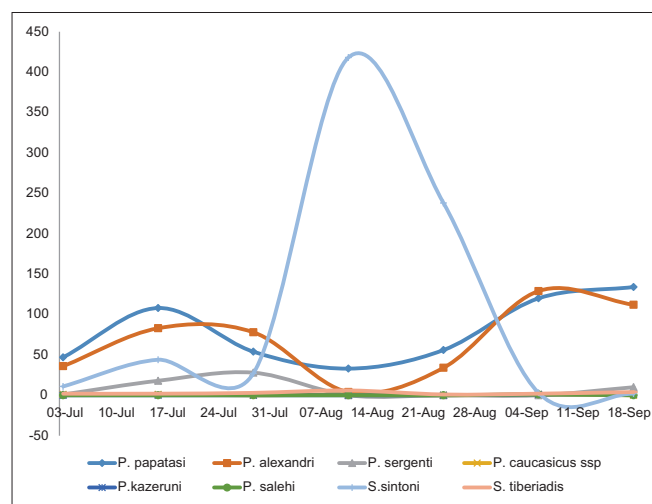


Figure 4: Seasonal abundance of sand flies' species

the same species that is responsible for recent cases of CL in Poldokhtar region as the studied area.

According of our data, all infected *P. papatasi* were collected in August and September 2015 that indicate the probable transmission time of parasite to human. Eight species of sand flies (6 of genus *Phlebotomus* and 2 of genus *Sergentomyia*) were identified. The most prevalent sand flies species were *S. sintoni*, *P. papatasi*, and *P. alexandri*, but just the second one has been known as the main vector of ZCL in different parts of Iran. Furthermore, *P. papatasi* was the dominant species outdoor (27.8%) and *S. sintoni* (41/55%) was the dominant one indoor; this result could be useful for determining the main control approach, such as outdoor insecticides spraying, using impregnated bed nets, public education, and trainings, in this region.

In according to other studies, *L. major* and *P. papatasi* reported as the main agent and vector of ZCL in study countries of the old world, such as: Iran, Egypt, Israel, Jordan, Saudi Arabia, Tunisia, Turkmenistan, Afghanistan, Algeria, Morocco and suspected vector in Azerbaijan, Iraq, Kazakhstan, Libya, Oman, Pakistan, Palestine, Sudan, Yemen, and Syria.^[10]

Furthermore, the predominance of CL due to *L. major* has already been confirmed mainly in Khuzestan, Kermanshah, and Ilam in previous studies too.^[11,12] These provinces are the closest ones to our study area, so the results could be overlapped together. There are some other studies on leishmaniasis in different neighborhood regions of Poldokhtar, but we have mentioned just the ones that are more similar to our investigation in goals, methods, and findings. All these investigation's findings are according to our data to a large extent. We have mentioned results of the closest ones geographically.

Jahanifard *et al.*, in Khuzestan, studied the biodiversity of sand flies, with emphasis on medically important species. She found that the main dominant species were *P. papatasi* and *S. sintoni*.^[11]

Vahabi also determined the vector(s), the parasite, and the species composition of sand flies in the Dehloran County (Ilam). *P. papatasi* has been dominant in outdoor and

Table 3: Abdominal status of female sand flies

Species	Abdominal status				
	n (%)	Un fed/empty (%)	Gravid (%)	Semi gravid (%)	Blood fed (%)
<i>P. papatasi</i>	163 (16.87)	100	4	5	54
<i>P. alexandri</i>	124 (12.83)	109	1	1	13
<i>P. kazeruni</i>	1 (0.10)	0	0	0	1
<i>P. caucasicus spp.</i>	2 (0.20)	2	0	0	0
<i>S. sintoni</i>	661 (68.42)	652	1	1	7
<i>S. tiberiadis</i>	15 (1.55)	13	1	0	1
Total	966 (100)	876 (90/68)	7 (0.72)	7 (0.72)	76 (7/86)

P. papatasi: *Phlebotomus papatasi*, *P. sergenti*: *Phlebotomus sergenti*, *P. caucasicus*: *Phlebotomus caucasicus*, *P. salehi*: *Phlebotomus salehi*, *P. kazeruni*: *Phlebotomus kazeruni*, *S. sintoni*: *Sergentomyia sintoni*, *S. tiberiadis*: *Sergentomyia tiberiadis*

indoor resting places and just 5 of them (1.8%) have been positive to *L. major* parasites. The results of his study have been indicated that *P. papatasi* is only species found infected by *L. major* and is the principal vector of the disease.^[12]

Beiranvand in a study, conducted in Lorestan Province, has been identified the parasite species causing CL by molecular methods in Poldokhtar district (Lorestan). She founded that most of the recognized cases of CL in Poldokhtar were due to *L. major*; also, she mentioned that the most prevalent cases of CL in Kermanshah are due to *L. major* too.^[13]

Repeatedly again and similar to these mentioned studies, we show that this new foci of CL have been established in Poldokhtar County with *L. major* as agent and *P. papatasi* as suspected vector.

PCR-based diagnostic techniques have shown high sensitivity in detecting and identifying *Leishmania* DNA in sand flies, so these techniques were used to diagnosis the parasite and its species in collected sand flies.^[10] Strictly, the results of molecular investigations revealed that the high density of *P. papatasi* and their infection with *L. major* is attributed that these two species can play major roles as principle vector and pathogen, responsible for ZCL, in this region.

Conclusion

The main objective of this study was to identify the sand fly vectors and the etiological agents, responsible for the recent cases of ZCL in Poldokhtar region, as new foci of the disease, for the first time.

The results of the current study indicated that the main vector of ZCL, same as the other western and southeastern parts of Iran, is *P. papatasi*. Furthermore, most of the CL cases in Poldokhtar have been happened due to *L. major*, so *L. major* is the most predominant parasite, responsible for ZCL.

All in all, further studies should be done in future on vectors, parasites, reservoirs, and also their relations and their roles for outbreaks of ZCL (and even other forms of human leishmaniasis) in Poldokhtar district.

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Conflicts of interest

There are no conflicts of interest.

References

1. Control of the leishmaniasis. Report of a WHO expert committee. World Health Organ Tech Rep Ser 1990; 793:1-58.
2. Desjeux P. Leishmaniasis: Current situation and new perspectives. Comp Immunol Microbiol Infect Dis 2004; 27:305-18.
3. Alten B, Ozbel Y, Ergunay K, Kasap OE, Cull B, Antoniou M, *et al.* Sampling strategies for phlebotomine sand flies (Diptera: Psychodidae) in Europe. Bull Entomol Res 2015; 105:664-78.
4. Yaghoobi-Ershadi M. Phlebotomine sand flies (Diptera: Psychodidae) in Iran and their role on *Leishmania* transmission. J Arthropod Borne Dis 2012; 6:1-7.
5. Abedi-Astaneh F, Akhavan AA, Shirzadi MR, Rassi Y, Yaghoobi-Ershadi MR, Hanafi-Bojd AA, *et al.* Species diversity of sand flies and ecological niche model of *Phlebotomus papatasi* in central Iran. Acta Trop 2015; 149:246-53.
6. Pol-e Dokhtar; 2017. Available from: <https://www.fa.wikipedia.org/wiki/>. [Last accessed on 2017 Aug 11].
7. Yaghoobi-Ershadi MR, Marvi-Moghadam N, Jafari R, Akhavan AA, Solimani H, Zahrai-Ramazani AR, *et al.* Some epidemiological aspects of cutaneous leishmaniasis in a new focus, central Iran. Dermatol Res Pract 2015; 2015:286408.
8. Akhavan AA, Mirhendi H, Khamesipour A, Alimohammadian MH, Rassi Y, Bates P, *et al.* *Leishmania* species: Detection and identification by nested PCR assay from skin samples of rodent reservoirs. Exp Parasitol 2010; 126:552-6.
9. Akhavan AA, Yaghoobi-Ershadi MR, Khamesipour A, Mirhendi H, Alimohammadian MH, Rassi Y, *et al.* Dynamics of *Leishmania* infection rates in *Rhombomys opimus* (Rodentia: Gerbillinae) population of an endemic focus of zoonotic cutaneous leishmaniasis in Iran. Bull Soc Pathol Exot 2010; 103:84-9.
10. Kassiri H, Nadda F, Mohebbali M, Javadian EA. Molecular characterization of *Leishmania* infection in sand flies from Sistan Va Baluchistan Province, Southeastern Iran. Jundishapur J Microbiol 2012; 2012:430-1.
11. Jahanifard E, Yaghoobi-Ershadi MR, Akhavan AA, Akbarzadeh K, Hanafi-Bojd AA, Rassi Y, *et al.* Diversity of sand flies (Diptera, Psychodidae) in southwest Iran with emphasis on synanthropy of *Phlebotomus papatasi* and *Phlebotomus alexandri*. Acta Trop 2014; 140:173-80.
12. Vahabi A, Rassi Y, Oshaghi MA, Sayyadi M, Rafizadeh S. Detection of *Leishmania* major DNA within wild caught *Phlebotomus papatasi* and species composition of sand flies in endemic focus of cutaneous leishmaniasis, in western Iran. J Parasit Dis 2016; 40:69-74.
13. Beiranvand E, Kalantari M, Rastgar HA, Amraee K. Molecular identification of *Leishmania* species isolated from human cutaneous leishmaniasis in Poledokhtar District, Lorestan Province, Iran. Jundishapur J Microbiol 2013; 6:1-4.