Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi:

First molecular identification of *Leishmania* species in a new endemic area of cutaneous leishmaniasis in Lorestan, Iran

Farnaz Kheirandish[1*], Ali Chegeni Sharafi[2], Bahram Kazemi[3,4,5], Mojgan Bandehpour[3], Mohamad javad Tarahi[6], Ali Khamesipour[7]

[1]*Razi Herbal Medicines Research Center, Department of Parasitology and Mycology, Lorestan University of Medical Sciences, Khorramabad, Iran*
[2]*Department of Communicable Disease Control and Prevention, Deputy of Health, Lorestan University of Medical Sciences, Khorramabad, Iran*
[3]*Cellular and Molecular Biology Research Center, Shahid Beheshti University, M.C., Tehran, Iran*
[4]*Department of Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
[5]*Department of Biotechnology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran Iran*
[6]*Department of Epidemiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*
[7]*Center for Research and Training in Skin Disease and Leprosy, Tehran University of Medical Sciences, Tehran, Iran*

ARTICLE INFO

Article history:
Received 10 April 2013
Received in revised form 15 May 2013
Accepted 15 June 2013
Available online 20 September 2013

Keywords:
Cutaneous leishmaniasis
Leishmania tropica
Leishmania major
PCR
Lorestan
Iran

ABSTRACT

Objective: To identify *Leishmania* using PCR. **Methods:** This study was conducted from April 2009 to March 2011 in order to identify *Leishmania* species in a new endemic area of CL in Lorestan, Iran. Samples were taken from 62 patients that referred to the health centers in different cities of Lorestan province, the presence of *Leishmania* was confirmed using direct smear and then grown in NNN media and mass cultured in RPMI 1 640 medium supplemented with 10% heat-inactivated fetal bovine serum. DNA was extracted from cultured promastigotes and used in ITS-PCR. **Results:** 45(72.6%) samples out of 62 showed a band in the range of 485 bp and 17 (27.4%) with a band in the range of 626 bp which were similar to standard strains of *Leishmania tropica* (*L. tropica*) and *Leishmania major* (*L. major*), respectively. 50 (65.80%) of samples were collected from people with no history of travel in at least a year prior to the onset which shows that indigenous source of infection. **Conclusions:** Since the vector and reservoir of the two species are different, so precise and extensive control and prevention methods should be designed and carried out.

1. Introduction

Leishmaniasis is endemic in 88 countries with incidence rate of 1.5-2 million; the most common form of leishmaniasis is cutaneous leishmaniasis (CL) with 1.5 million new cases per year[1,2]. 90% of cutaneous leishmaniasis are reported from Iran, Afghanistan, Algeria, Iraq, Saudi Arabia, and Syria in the Old World; and Bolivia, Brazil, Colombia, and Peru in the New World[3].

In Iran, zoonotic CL (ZCL) and anthroponotic CL (ACL) are caused by *Leishmania major* (*L. major*) and *Leishmania tropica* (*L. tropica*), respectively. In recent years new foci of CL are reported from different parts of Iran[4,5]. Geographically prevalence of the disease is due to different factors which the most important ones are suitable climate conditions and related vectors and reservoirs[6]. In most of the cases, the lesion(s) heal after a few months to one year, but in some cases the lesion might last long[7]. In order to achieve effective control of CL, identification of *Leishmania* species is important[8]. The *Leishmania* species are the same in terms of morphology and the microscopically identification of *Leishmania* using direct smear and culture is impossible, also epidemiological and clinical findings are not sufficient to achieve this purpose[9-11]. Previously

*Corresponding author: Farnaz Kheirandish, Razi Herbal Medicines Research Center, Department of Parasitology and Mycology, Lorestan University of Medical Sciences, Khorramabad, Iran.
Tel/Fax: +98 661 6200133
E-mail: kheirandish81@yahoo.com

identification of isolates mostly was depend upon traditional methods such as epidemiological characteristics of the disease, etc which are not definite and perfect[11].

Differentiations among species require molecular or biochemical techniques such as PCR and isoenzymes analysis[11]. Biochemical methods are lengthy, complicated and expensive procedure, so nowadays molecular methods are more useful[12-15].

PCR as one of the molecular method has been broadly used for the detection and identification of *Leishmania* species in CL, visceral leishmaniasis and other forms of the disease[11,16].

CL is considered as one of the health problems in some provinces of Iran, including Lorestan.

According to the reports presented from 2006 to 2008, 191(63.66%), 32 (23.36%) and 19 (33.33%) cases of CL out of 300, 137 and 57 cases, respectively, were due to local transmission[17,18]. In 2006 an epidemic of the disease occurred in Lorestan. Since Lorestan is one of the areas of endemic for CL but the species of *Leishmania* so far was unknown, identification of species, in order to select an appropriate control measure against reservoirs, vectors and also to treat the patient appropriately is necessary. So the aim of present study was to identify the parasite species using PCR.

2. Materials and methods

2.1. Study area

This descriptive study was conducted from May 2009 to March 2011 in Lorestan province which is located between valleys of Zagros Mountain at the west of Iran covers an area of 28.392 km^2. The population of the province is approximately 1.6 million. Lorestan province is a mountainous region. Climatically, the province is divided into three parts: the mountainous regions that experience cold winter and moderate summer. In the central region, the spring season begins from mid-February and lasts till about mid May. The southern area is under the influence of the warm air currents of Khuzestan province which has a hot summer and relatively moderate winter.

2.2. Sample collection

A clinical history was completed to record the necessary information such as name, age, sex, sites of ulcer on the body, address, data and place of acquiring the disease, previous travel history and location of the study. Diagnosis was done by direct smear and/or culture.

2.3. DNA extraction

Samples were taken of 62 patients that referred to the Health centers in different cities of Lorestan province such as Poldokhtar, Kouhdasht, Nourabad, Doroud and Broujerd which the existence of parasite confirmed using direct dermal lesion smear and then grown in NNN and mass cultured in RPMI 1640 cell culture medium enriched with 10% fetal bovine serum.

Genomic DNA was extracted from cultured promastigotes using DNeasy Tissue Kit (Qiagen, Courtaboeuf, France) in accordance with the manufacturer's protocol. The extracted DNA was stored at −20 ℃ until use.

2.4. PCR reaction

ITS primers were used according to the previous study[12] with minor modifications. The PCR conditions consisted of one initial denaturing cycle at 94 ℃ for 5 min, followed by 30 cycles of 94 ℃ for 30 s, 56 ℃ for 30 s , 72 ℃ for 40 s and finally 1 cycle of 72 ℃ for 5 min. At the end, PCR products were analyzed using 1.5% agarose gel electrophoresis.

Standard strains of *L. major* (MRHO/IR/75/ER) and *L. tropica* (MHOM/IR/01/yaza) were used as positive controls. Expected PCR products of *L. major* and *L. tropica* were 626 bp and 485 bp, respectively.

The study was approved by the Medical Research Ethics Committee of Lorestan University of Medical Sciences.

2.5. DNA sequencing

PCR products were sequenced by dideoxy chain termination method. NCBI site was used to draw the phylogeny trees of *L. major* and *L. tropica*.

3. Results

Out of 62 patients, 26 (41.94%) were females and 36 (58.06%) were males. Patients' age was between 1 to 70 years (Table 1). The number of lesion was one lesion in 42 (67.7%) of the patients, two lesions in 12 (19.4%), three lesions in 5 (8.1%), four lesions in 1 (1.6%)and six lesions in 2 (3.2%) of the patients. Most of the patients were from Poldokhtar city with a total of 43 (69.4%) (Table 2).

Table 1

Distribution of cutaneouse leishmaniasis cases by age, Lorestan, Iran, April 2009 to March 2011.

Number of case (%)	Age group (year)
6 (9.68)	1–10
10 (16.13)	11–20
16 (25.81)	21–30
14 (22.58)	31–40
8 (12.90)	41–50
4 (6.45)	51–60
4 (6.45)	61–70
62 (100)	Total

Table 2

Characterization of *Leishmania* species isolated from human cutaneous lesions using PCR in Lorestan, Iran, April 2009 to March 2011.

Infected cities	Number of cases (%)		Total
	L. major	*L. tropica*	
Broujerd	1 (5.9)	2 (4.5)	3 (4.8)
Poldokhtar	6 (35.3)	37 (82.2)	43 (69.4)
Nourabad	9 (52.9)	1 (2.2)	10 (16.1)
Doroud	0 (0)	1 (2.2)	1 (1.6)
Kouhdasht	1 (5.9)	4 (8.9)	5 (8.1)
Total	17 (100)	45 (100)	62 (100)

After ITS-PCR, according to the pattern of electrophoresis, out of 62 samples, 45 (72.6%) had an amplification produced fragment of 485 bp and 17 (27.4%) a 626 bp band that were similar to *L. tropica* and *L. major* reference strains, respectively (Figure 1).

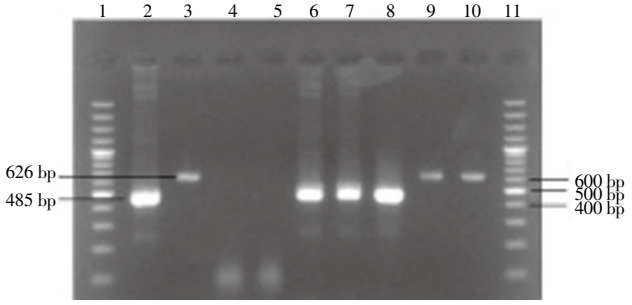

Figure 1. Agarose gel electrophoresis of PCR amplification for identification of *Leishmania* species.

Lane 1, 11: 100 bp DNA ladder marker; Lane 2: Reference strain of *L. tropica*; Lane 3: Reference strain of *L. major*; Lane 4,5: Negative controls; Lane 6–8: *L. tropica* isolates; Lane 9,10: *L. major* isolates.

Among the patients, 12 (19.35%) had history of travel to other provinces and 50 (80.65%) of the patients had no history of travel in the past year. From 50 patients resident of Lorestan, 41 (82%) infected with *L. tropica* and 9 (18%) with *L. major*.

Nucleotide sequence data reported here have been submitted to the GenBank database with accession numbers, *L. tropica* JX104546 and *L. major* JX025361. The phylogeny trees of *L. major* and *L. tropica* are shown in Figure 2 and Figure 3.

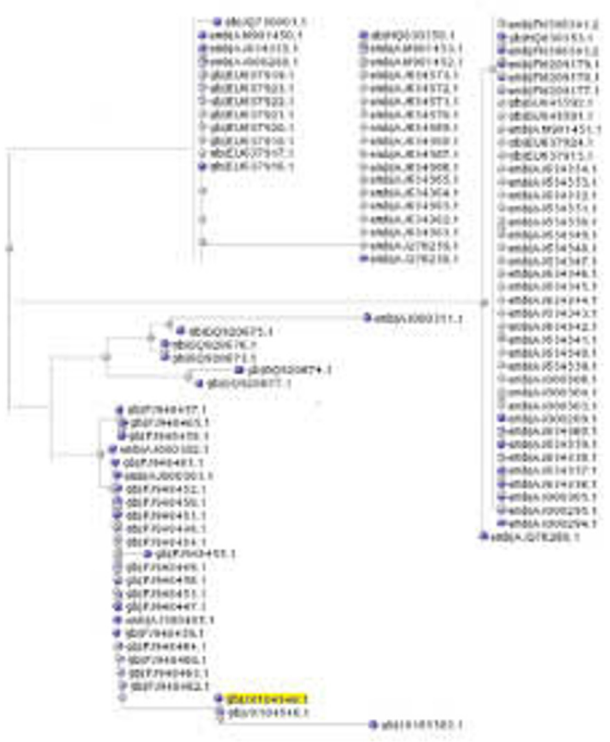

Figure 2. The phylogeny tree of *L. tropica* (Accession number JX104546)

Figure 3. The phylogeny tree of *L. major* (Accession number JX025361).

4. Discussion

CL is a major public health problem in 17 of 30 provinces in Iran[4]. The current study was performed using PCR for the first time in the Lorestan province to determine of

Leishmania species.

Since there are differences between the strategies of prevention and control against ACL and ZCL, to select an appropriate control measure the identification of the species is crucial[13]. The aim of this study was to identify the *Leishmania* species isolated from CL patients. The results of this research could the useful data for more accurate prevention programs.

Before the advent of new techniques, there were problems with traditional identification methods such as clinical signs, epidemiological characteristics[11].

CL is reported in several provinces of Iran and identification of the species in some of the foci was done using PCR method. For example, in rural area in Kerman province, it was determined that the causative agent of CL was *L. major* using RAPD-PCR[19].

In another study on the cutaneous ulcers in Mashhad, using ITS-PCR it has been determined that *L. tropica* was the dominant species[12]. In another study in Mashhad using PCR-RFLP, it was proven the dominance of *L. tropica*[20]. Also a study on the patients with scar in rural areas in Shiraz using Nested PCR has shown that the dominant species was *L. major*[21]. In order to identify *Leishmania* species using Nested-PCR, a study on the samples collected from 100 patients with CL was performed in Shush city in Khuzestan province and shown that the predominant species was *L. major*[22]. In other countries, the molecular techniques were used to identify *Leishmania* species in the patients, reservoir and vectors. For example, in Morocco detection of *Leishmania* parasites in the skin biopsies was performed using PCR and showed that the method is highly sensitive (84.6%)[23]. In Venezuela, *Leishmania* species was identified in the sand flies using Multiplex-PCR[24]. In Brazil, detection of *Leishmania* parasite in biopsies from 109 patients with CL using PCR was shown that patients were infected with *Leishmania* braziliensis[25].

In current study, 62 samples of cutaneous lesion were assessed by PCR in order to identify *Leishmania* species in Lorestan province. Among them, 50 samples were for residence of the province with no history of travel in at least a year prior to the onset and both *L. tropica* and *L. major* were identified from the collected samples and *L. tropica* was prodominant. Since the vectors of these two species are different and ACL is the main reservoir, identification of species is important for control and treatment. Due to drug resistance in 10 to 15 percent of CL within one province of Iran, therefore an appropriate treatment of all the patients in case of ACL is necessary[26]. In the other hand there are numerous ancient sites including the historic castle Falak_al_aflak, 1 700 years old, which attracts tourists to the area of Lorestan province so special attention should be made to clearly define which parts of the province is endemic.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

Hereby the authors appreciate the Deputy of Research and Technology and co-researchers of Lorestan University of Medical Sciences. Also all the patients who participated in the study are sincerely appreciated. This study was funded by a grant from Lorestan University of Medical Sciences (11/19/2008, No. 1121).

References

[1] Desjeux P. Leishmaniasis: Current situation and new perspectives. *Comp Immunol Microbiol Infect Dis* 2004; **27**: 305-318.

[2] WHO. Leishmaniasis: the global trend[Online]. Available from: http://www.who.into /neglected_disease/integrated-media_Leishmaniasis/en/index.html. [Accessed on 2009].

[3] Herwaldt BL, Magill AJ. Leishmaniasis, Cutaneous, Infectious diseases related to travel, Centers for Disease Control and Prevention, Traveler's health. Yellow book, 2012; Chapter 3.

[4] Zoonoses Control Office (2010) CDC, Ministry of Health and Medical Education, Iran.

[5] arahmand M, Nahrevanian H, Atashi Shirazi H, Naeimi S, Farzanehnejad Z. An overview of a diagnostic and epidemiologic reappraisal of cutaneous leishmaniasis in Iran. *Infect Dis* 2011; **15**: 17-21.

[6] Sharma U, Singh S. Insect vectors of *Leishmania*: distribution, physiology and their control. *J Vector Borne Dis* 2008; **45**: 255-272.

[7] WHO. Report of *WHO expert Committee on the Control of Leishmaniasis. WHO Technical Report Series 949*. Geneva: WHO; 2010.

[8] Mahdy MAK, Al-Mekhlafi HM, Al-Mekhlafi AM, Lim YAL, Bin Shuaib NOM, Azazy AA, et al. Molecular characterization of *Leishmania* species isolated from cutaneous leishmaniasis in Yemen. *PLoS ONE* 2010; **5**: e12879.

[9] Shalaby I, Gherbawy Y, Jamjoom M, Banaja AE. Genotypic characterization of cutaneous leishmaniasis at al Baha and Al Qasim Provinces (Saudi Arabia). *Vector Borne Zoonotic Dis* 2011; **11**: 807-813.

[10]Gomes AH, Ferreira IM, Lima ML, Cunha EA, Garcia AS, Araujo FL, et al. PCR identification of *Leishmania* in diagnosis and control of canine leishmaniasis. *Vet Parasitol* 2007; **144**: 234-

241.

[11]Eroglu F, Koltas IS, Genc A. Identification of causative species in cutaneous leishmaniasis patients using PCR-RFLP. *J Bacteriol Parasitol* 2011; **2**:113.

[12]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**: 1159-1162.

[13]Alimoradi S, Hajjaran H, Mohebali M, Mansouri F. Molecular identification of *Leishmania* species isolated human cutaneous leishmaniasis by RAPD-PCR. *Iran J Public Health* 2009; **38**: 44-50.

[14]Mahmoodi MR, Mohajery M, Tavakkol Afshari J, Shakeri MT, Yazdan panah MJ, Berenji F, et al. Molecular identification of *Leishmania* species causing cutaneous leishmaniasis in Mashhad, Iran. *Jundishapur J Microbiol* 2010; **3**: 195-200

[15]Martínez LP, Rebollo JA, Luna AL, Cochero S, Bejarano EE. Molecular identification of the parasites causing cutaneous leishmaniasis on the Caribbean coast of Colombia. *Parasitol Res* 2010; **106**: 647-652.

[16]Tavares RG, Staggemeier R, Borges ALP, Rodrigues, Castelan LA, Vasconcelos J, et al. Molecular techniques for the study and diagnosis of parasite infection. *J Venom Anim Toxins incl Trop Dis* 2011; **17**: 239-248.

[17]Chegeni Sharafi A, Amani H, Kayedi MH, Yarahahmadi A, Saki M, Mehrdad M, et al. Epidemiological survey of cutaneous leishmaniasis in Lorestan province(Iran) and introduction of disease transmission in new local areas. *JIUMS* 2010; **19**: 54-60.

[18]Communicable disease management office, CDC, Ministry of Health and Medical Education, Iran; 2008.

[19]Akhavan AA , Yaghoobi-Ershadi MR, Hasibi F, Jafari R, Abdoli H, Arandian MH, et al. Emergence of cutaneous leishmaniasis due to *Leishmania major* in a new focus of southern Iran. *Iranian J Arthropod-Borne Dis* 2007; **1**: 1-8.

[20]Vaeznia H, Dalimi A, Sadraei J, Pirstani M. Determination of *Leishmania* species causing cutaneous leishmaniasis in Mashhad by PCR-RFLP method. *Archives of Razi Institute* 2009; **64**: 39-44.

[21]Razmjou S, Hejazya H, Motazedianb MH, Baghaeia M, Emamyc M, Kalantaryb M. A new focus of zoonotic cutaneous leishmaniasis in Shiraz, Iran. *Trop Med Hyg* 2009; **103**: 727-730.

[22]Maraghi S, Samarbaf Zadeh A, Sarlak AA, Ghasemian, Vazirianzadeh B. Identification of cutaneous leishmaniasis agents by nested polymerase chain reaction (Nested-PCR) in Shush city, Khuzestan province, Iran. *Iranian J Parasitol* 2007; **2**: 13-15.

[23]Lemrani M, Hamdi S, Laamrani A, Hassar M. PCR detection of *Leishmania* in skin biopsies. *J Infect Developing Countries* 2009; **3**: 115-122.

[24]Jorquera A, González R, Marchán-Marcano E, Ovied M, Matos M. Multiplex-PCR for detection of natural *Leishmania* infection in *Lutzomyia* spp. captured in an endemic region for cutaneous leishmaniasis in state of Sucre, Venezuela. *Mem Inst Oswaldo Cruz, Rio de Janeiro* 2005; **100**: 43-46.

[25]Gomes AHS, Armelin IM, Menon SZ, Pereira-Chioccola VL. *Leishmania* (V.) *braziliensis:* detection by PCR in biopsies from patients with cutaneous leishmaniasis. *Exp Parasitol* 2008; **119**: 319-324.

[26]Hadighi R, Boucher P, Khamesipour A, Meamar AR, Roy G, Ouellette M, et al. Glucantime-resistant *Leishmania tropica* isolated from Iranian patients with cutaneous leishmaniasis are sensitive to alternative anti*leishmania* drugs. *Parasitol Res* 2007; **101**: 1319-1322.