Frequency of Cutaneous Leishmaniasis and Species Identification in Suspected Individuals from Golestan Province, Northern Iran in 2014

Farideh HEZARI [1], *Maryam NIYYATI [1,2], *Seyyed Javad SEYYED TABAEI [2], Mehdi MOHEBALI [3], Vahideh MOIN VAZIRI [2], Hamed BEHNIAFAR [2], Eznolah AZARGASHB [4], Niloofar TAGHIPOUR [2]

1. Infectious Diseases and Tropical Medicine Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
2. Dept. of Medical Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
3. Dept. of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
4. Dept. of Family Medicine, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Corresponding Authors: Emails: maryamniyati@yahoo.com, seyyed_tabaei@yahoo.co.in

Abstract

Background: Leishmaniasis is a zoonotic disease caused by species of protozoa of the genus *Leishmania*. In recent years, incidence of cutaneous leishmaniasis has increasing trend in Golestan Province, North of Iran. The aim of the present study was to identify the frequency of cutaneous leishmaniasis using PCR-RFLP in patients referred to Kalaleh Health Center, during 2013-14.

Methods: This descriptive cross-sectional study was conducted on 70 individuals with suspected cutaneous leishmaniasis that referred to health center of Kalaleh County, Golestan Province, Northern Iran, from Sep 2013 to Nov 2014. Samples of cutaneous lesions were examined microscopically. DNA was extracted from all of the positive smears and PCR was done on ITS-1 gene. RFLP was performed using HaeIII enzyme for species identification.

Results: Totally, 38 out of the 70 (54.3%) suspected individuals including 22 males (57.9%) were found positive by microscopic examination. All of microscopically positive samples were confirmed to be positive for *Leishmania* DNA (approximately 340 bp bands were detected). RFLP revealed 140 bp and 200 bp bands (approximate size), indicative of *L. major*.

Conclusion: The detected species of studied region was *L. major*. Cutaneous leishmaniasis has high prevalence in Kalaleh County, thus more studies on leishmaniasis in the animal reservoirs, comparison of homology of animal and human isolates and a survey regarding natural infection of vectors in this region is highly recommended.

Keywords: Leishmania major, ITS-1, Cutaneous leishmaniasis, Iran

Introduction

Leishmaniasis is one of the most important tropical diseases worldwide, that appears in three main forms including cutaneous, visceral (kala-azar) and mucocutaneous. This disease occurs in more than 98 countries across Asia, Europe, Africa, America and Australia (1, 2). Cutaneous leishmaniasis (CL) is considered as one of the major health problems in many countries around the world including Iran. Approximately 1-1.5 million new cases of CL are reported worldwide annually, and about 90% of cases occur in eight countries: Brazil, Afghanistan, Peru, Iran, Saudi Arabia, Syria, Sudan and Algeria (2, 3).

CL is one of the most important parasitic diseases in Iran, and is the most important arthropod-borne disease. The major causative agent of wet type is *L. major*, and rodents are major reservoir of its causative agent. This disease is endemic in rural regions of 17 out of the 31 provinces of Iran (4). In Iran Golestan Province and Lotf-Abad in northeast, Abar Dezh, Varamin, Isfahan and Yazd in the center, Fars and Sistan-o-Baluchestan provinces in south and southeast, and Ilam and Khuzestan provinces in southwest of the country are reported as endemic foci of the disease (1). *L. tropical* is causative agent of dry type of CL and primarily human and in the next level dogs are its reservoir. (1). Although, microscopic observation of parasites on direct stained direct smears from wounds is the simplest method to detect *Leishmania*, but low sensitivity for the detection of parasites in low-grade parasitemia is the problem with this method (3, 5). To date, species identification of *Leishmania* spp. is based on molecular approaches such as PCR methods. PCR methods have advantages such as need to small amounts of DNA, it is not affected by confounding conditions of environmental and host, examination of numerous samples in a short time and its high sensitivity (6, 7).

Kalaleh County is located in the east and it is the fifth populous county of the province. Meanwhile, the County is located near endemic counties (such as Gonbad-e Kavus, Marave Tappe, Minoo Dasht) and other provinces (such as North Khorasan and Semnan) for CL, so increasing trend in incidence of CL, is probable. A research carried out a study on vectors and reservoirs of leishmaniasis in the Kalaleh County during 2006-7, in this study *Phlebotomus papatasi* was found predominant species of sandflies (8). A study conducted on identification of *Leishmania* spp. in border villages of Gonbad-e-Qabus County using PCR method on ITS1 gene in 2007, in this study *L. major* was reported as the predominant species (9). Moreover, *Ph. papatasi* was reported as the predominant sandflies in the Marave Tappe, in 2011-2012 (10).

Due to endemicity of the CL in neighboring regions of the Kalaleh County, complex and vari-

ous features of the cutaneous lesions and different treatment protocols for different species, exact identifications of the causative agent is essential, on the other hand there is no published study for this aim.

The objectives of this study were a better understanding of the demographic situation of the disease and molecular identification of the parasite species on stained slides of the referred patients to health center of the studied region, during 2013-14.

Materials and Methods

In this descriptive cross-sectional study, samples were collected from 70-suspected individuals to CL that referred to laboratory of Kalaleh Health Center, Kalaleh County, Golestan Province, Northen Iran, from Sep 2013 to Nov 2014.

Informed consents were obtained from each patient. Demographic information of each patient including age, sex, locality, the onset of disease, number of lesions and location of lesions were recorded.

After disinfection of sampling sites, serous were collected from the margin of the lesions by sterile vaccine style. Thin smears were stained and slides were microscopically examined for the presence of Leishman bodies (inside or out of macrophages).

Molecular method was used for identification of species. Total genomic DNA was extracted from positive samples by phenol-chloroform method (11). ITS-1 fragment is useful for the identification of *Leishmania* species. This fragment was amplified by using LITSR (5' CTG-GATCATTTTCCGATG 3') as forward and L5.8S (5TGATACCACTTATCGCACTT3) as reverse primer. These primers amplify an approximately 340 bp fragments detectable on agarose gel.

In this study standard strains, *L. tropica* (standard) MHOM/IR/02/MHOM/R and *L. major* (standard) MHOM/IR/75/ER, obtained from School of Public Health, Tehran University of Medical Sciences were used as positive controls. PCR was

prepared using amplicon (Taq DNA Polymerase Master Mix) as ready-made mixture. PCR was performed in a 15 µL mixture containing 7.5 µL Taq Master Mix, 5 ng DNA, 10 pmol of each primer and 5.5 µL distilled water. All of the PCRs were carried out under following conditions: an initial denaturation step at 95 °C for 5 min, 30 repetitions at 94 °C for 30 sec, an annealing step at 47 °C for 30 sec, extension step at 72 °C for 1 min, and final extension 72 °C for 7 min. PCR products were examined using 1.5% gel agarose, stained with ethidium bromide solution, and were seen under UV light.

RFLP examination: RFLP was carried out on positive PCR products to determine the parasite species. HaeIII endonulease was used for digestion of PCR products. The cutting site of the enzyme is GG CC. This restriction enzyme distinguishes different species of *Leishmania* by different cutting patterns. The enzyme can cut PCR products to 200 and 140 bp fragments, and 200, 60 and 40 bp fragments in *L. major* and *L. tropica*, respectively. For this purpose 30 µL reactions were prepared containing 10 µL PCR product (equivalent 1 µg), 3 µL enzyme-specific buffer, 17 µL distilled water and 1 µL fast-acting HaeIII. After a very short centrifugation, reactions were incubated at 37 °C for 15 min to cut ITS-1 gene by the enzyme function. To confirm PCR-RFLP results, beside 100 bp DNA ladder (product of Sina Gene Company, Iran, cat no: s17031) products were separated using 2.5% gel agarose. Finally, obtained data were analyzed using SPSS software (version 11) and chi-squared test.

Results

Thirty-eight (54.3%) specimens out of the 70 specimens were found positive for Leishman body by microscopic examination of thin smears. Among patients, 22 cases (57.9%) were male (Table 1). DNAs were extracted only from positive specimens.

There was no significant difference between the two sexes. The youngest patient was 3 yr old and the oldest patient was 60-yr-old (Table 1). The most lesions were observed on hand, foot and face, respectively, but there was no statically significant difference in involvement of different organs (Table 1). The highest frequency was in the age group of 30-to 50-yr-old and the lowest frequency was in the age group of 1-9 yr old. 37% of patients were from urban regions (Kalaleh city) and 67% of patients were from rural regions (Table 1).

Table 1: Frequency of CL cases based on sex, age group and location of lesion in Kalaleh County, during 2013-14

Variables		Frequency	Percentage (%)
Sex	Male	22	57.9
	Female	16	42.1
Age group (yr)	<10	6	15.8
	10-15	6	15.8
	15-30	4	10.5
	30-50	19	50.0
	50<	3	07.9
Location of lesion	Hand	20	52.6
	Foot	8	21.0
	Face	4	10.5
	Hand and foot	4	10.5
	Hand and face	1	02.6
	Hand, foot and face	1	02.6

All of the 38 positive smears were found positive by PCR analysis, too, and an approximately 340 bp bands were detected under UV light after transferring PCR products of both of positive specimens and standard strains to 1.5% agarose gel (Fig. 1). After carrying out RFLP, 140 bp and 200 bp bands were detected under UV light (Fig. 2). These bands revealed that all of our isolates were belonged to *L. major*. Treatment of the 38 patients with cutaneous leishmaniasis was also followed up.
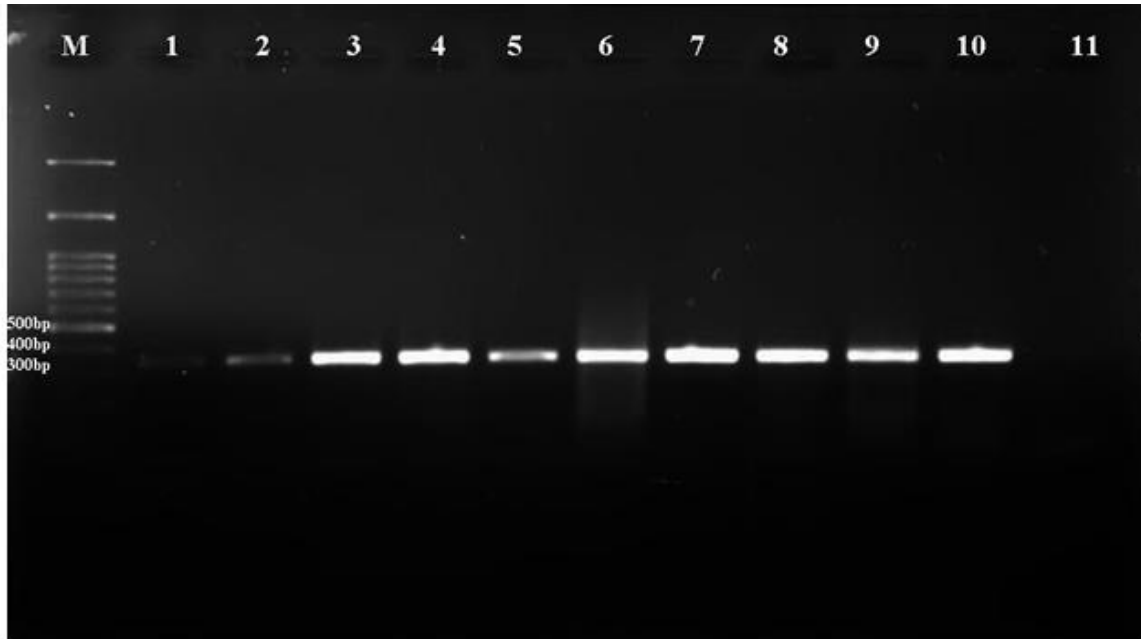
Fig. 1: Agarose (1.5%) gel showing the PCR products (340 bp) of amplified from standard strain and samples. Lane M is DNA size marker, lane 1 is standard strain of *L. major*, lanes 2-10 are positive samples and lane 11 is negative control
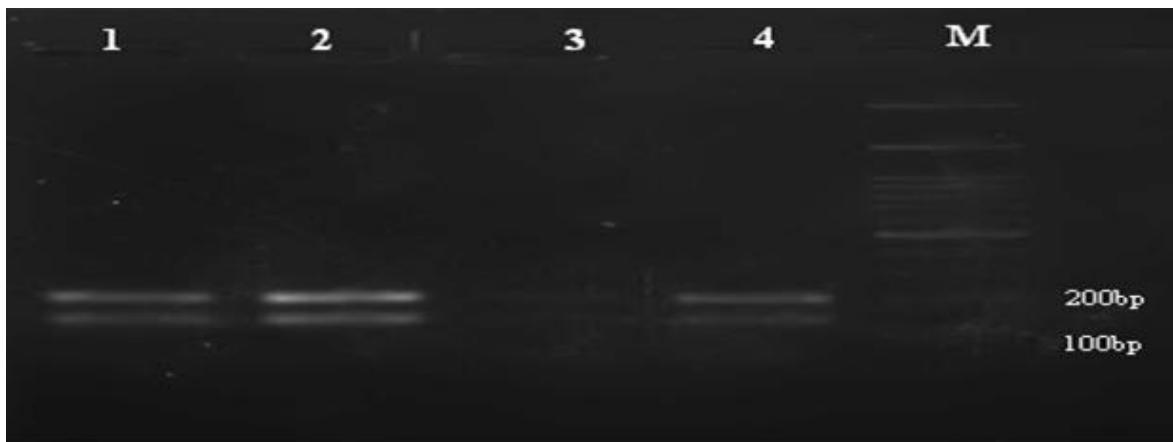
Fig. 2: Agarose (2.5%) gel showing the PCR-RFLP pattern of ITS-1 gene cut with HaeIII endonuclease (approximately 200 bp and 140 bp bands). Lane M indicating DNA size marker, lanes 1-4 indicating RFLP pattern of *L. major*

Treatment protocol in the studied region was five times and weekly oblique injection of 5 ml glucantim (Lab osterop company, Belgium) under the edge of lesion and at four points, among the aforementioned patients, 10 were not admitted for treatment due to commuting costs, and presence of lesion in the unexposed parts of body. Because of increased awareness of endemic people about establishment of immunity against CL while lacking treatment, if lesions locate in the unexposed parts of the body, majority of patients are not eager to complete treatment.

Discussion

The present study is the first study on frequency of CL in Kalaleh County, Northern Iran. In this study, 70 specimens were examined during 15 months, by both microscopic and molecular methods 38 specimens (54.3%) were found positive. PCR-RFLP results revealed that all of isolates belonged to *L. major*. The most affected parts of body were hand, foot, and face, respectively. Generally, lesions of CL are observed on exposed parts of the body to mosquito bites. The most affected parts of body are hands and feet in rural CL and face in urban CL (1).

In this study, most patients (47.4%) had only one lesion. About 2, 3 and 7 lesions were seen in 16 (42.1%), 3 and 1 cases, respectively. These findings (number of lesions) are in agreement with results of *L. major* lesions (12-14).

The highest prevalence was seen in the age group of 30-50 yr old, as 50% of the patients were in this category; this can be caused by more contact of this age group with vector of the disease, because they spend more time outdoors. These findings are in consistent with results of studies in Gonbad-e-Qabus, in Kermanshah, and Kashan (9, 15, 16).

The main vector of leishmaniasis is *P. papatasi* in Kalaleh County (8). One study determined *L. major* as major causative agent of CL by PCR method in Gonbad-e-Qavus (9). *Ph. papatasi* is predominant species of sandflies in Marave Tappe County, Golestan Province (10). Kalaleh County has high frequency of rural CL. Identifi-

cation of exact causative agent of disease help physicians for choosing appropriate treatment regimen.

The large scale entrance of non-immune individuals (such as emigrants, tourists and soldiers) to epidemic regions result in significant increase in the disease incidence and epidemic leishmaniasis (17, 18). Interestingly, an epidemic event occurs in 2011, 85% of patients were emigrants (labors of border villages and soldiers). On the other hand, there was a decrease in the cutaneous leishmaniasis in 2012 in Kalaleh County. The main reason for decrease of the cases in 2012, was preventive measures of County and province health centers, including awareness of the people about transmission of the disease and health education, vector control (spraying of insecticide particularly in husbandries) and rodent control using zinc phosphide grain baits (8).

In this study, stained smears were used for DNA extraction. DNA extraction for all smears was done successfully, in accordance with another study (19). Thus, molecular identification could be done on stained smears.

However, the limitation of our study was lack of DNA extraction on negative smears, which should be done in future. However, smears without parasites should also submit to molecular identification, as they may be positive. After adding HaeIII endonuclease (carrying out RFLP) to PCR products of LITSR and L5.8S primers, and running electrophoresis, 200 and 140 bp bands, and 200 and 60 bp bands are detectable under UV light for *L. major* and *L. tropica*, respectively (19). Small fragments are not visible under UV light. Researchers used ITS-PCR-RFLP and introduced as an appropriate method to distinguish species of *Leishmania* (20, 21).

Conclusion

Zoonotic cutaneous leishmaniasis is endemic in Kalaleh County, Golestan province. *L. major* is found causative agent of CL in this case, however, more studies on investigation of reservoir of the parasite and natural infection in vectors is highly recommended.

Ethical considerations

Acknowledgments

References

1. Nadim A, Javadian E, Mohebali M, A ZM (2008). *Leishmania Parasite and leishmaniasis.* 3rd ed. Tehran University Publication Center, Tehran.

2. Mohebali M, Hajjaran H, Hamzavi Y, Mobedi I, Arshi S, Zarei Z, Akhoundi B, Naeini KM, Avizeh R, Fakhar M (2005). Epidemiological aspects of canine visceral leishmaniosis in the Islamic Republic of Iran. *Vet Parasitol,* 129(3-4):243-251.

3. Leishmaniases WECotCot, Meeting WECotCotL, Organization WH (1990). *Control of the Leishmaniases: Report of a WHO Expert Committee.* ed. World Health Organization.

4. Ardehali S RH, Nadim A (1994). *Leishmania parasite and leishmaniosis.* 2nd ed. Academic Publication Center, Tehran, Iran.

5. Berman J (1997). Human leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last 10 years. *Clin Infect Dis,* 24(4):684-703.

6. Al-Jawabreh A, Schoenian G, Hamarsheh O, Presber W (2006). Clinical diagnosis of cutaneous leishmaniasis: a comparison study between standardized graded direct microscopy and ITS1-PCR of Giemsa-stained smears. *Acta Trop,* 99(1):55-61.

7. Marques MJ, Volpini ÂC, Machado-Coelho GL, Machado-Pinto J, da Costa CA, Mayrink W, Genaro O, Romanha AJ (2006). Comparison of polymerase chain reaction with other laboratory methods for the diagnosis of American cutaneous leishmaniasis: diagnosis of cutaneous leishmaniasis by polymerase chain reaction. *Diagn Microbiol Infect Dis,* 54(1):37-43.

8. Sofizadeh A. Study on vectors and reservoir hosts of cutaneous leishmaniasis by using molecular methods in kalaleh district, Golestan province [MS thesis]. School of Public Health, Tehran University of Medical Sciences, Iran; 2007. [In Persian]

9. Mesgarian F, Rahbarian N, Hajaran H, Shahbaz F, Mesgarian Z, Taghipour N (2010). Identification of Leishmania species isolated from human cutaneous Leishmaniasis in Gonbad-e-Qabus city using a PCR method during 2006-2007. *Teharn Univ Med J ,* 68(4): 250-6. [In Persian]

10. Sofizadeh A GA, Gorganli Davaji A , Gharemeshk Gharavi A (2015). Epidemiological Status of Cutaneous Leishmaniasis and Ecological Characteristics of Sandflies in Maraveh-Tapeh County, Golestan Province, 2011-2012, Iran. *Qom Univ Med J,* 41(6): 53-65. [Text in Persian]

11. Sambrook J, Fritsch EF, Maniatis T (1989). *Molecular cloning.* 2nd ed. Cold spring harbor laboratory press New York.

12. Rafati N, Shapori-Moghadam A, Ghorbani R (2004). Epidemiological survey of cutaneous leishmaniasis in Damghan (1999-2005). *J Semnan Univ Med Sci,* 2(1):247-53.

13. Karami M, Doudi M, Setorki M (2013). Assessing epidemiology of cutaneous leishmaniasis in Isfahan, Iran. *J Vector Borne Dis,* 50(1):30-7.

14. Abbasi AE, Ghanbary M, Kazem NK (2004). The epidemiology of cutaneous leishmaniasis in Gorgan (1998-2001). Ann *Med* Health Sci *Res,* 2(1):275-8.

15. Hajjaran H, Vasigheh F, Mohebali M, Rezaei S, Mamishi S, Charedar S (2011). Direct diagnosis of Leishmania species on serosity materials punctured from cutaneous leishmaniasis patients using PCR-RFLP. *J Clin Lab Anal,* 25(1):20-24.

16. Doroodgar A, Mahbobi S, Nemetian M, Sayyah M, Doroodgar M (2009). An epidemiological study of cutaneous leishmaniasis in Kashan (2007-2008). *Koomesh,* 10(3):177-184. [In persian]

17. WHO Expert Committee on the Control of the Leishmaniases (2010). *First WHO report on neglected tropical diseases: working to overcome the global impact of neglected tropical diseases.* In: *First WHO report on neglected tropical diseases: Working to overcome the global impact of neglected tropical diseases.* Ed(s): WHO.

18. Yaghoobi-Ershadi M, Zahraei-Ramazani A, Akhavan A, Jalali-Zand A, Abdoli H, Nadim A (2005). Rodent control operations against zoonotic cutaneous leishmaniasis in rural Iran. *Ann Saudi Med,* 25(4):309-312.

19. Kazemi-Rad E, Mohebali M, Hajjaran H, Rezaei S, Mamishi S (2008). Diagnosis and characterization of Leishmania species in Giemsa-stained slides by PCR-RFLP. *Iran J Public Health,* 37(1):54-60.

20. Dweik A, Schönian G, Mosleh I, Karanis P (2007). Evaluation of PCR-RFLP (based on ITS-1 and Hae III) for the detection of Leishmania species, using Greek canine isolates and Jordanian clinical material. *Ann Trop Med Parasitol,* 101(50):399-407.

21. Dilmec F, Matur A, Alhan E, Uzun S, Karakas M, Alabaz D, Aksoy N, Akkafa F, Aksaray F, Memisoglu H (2008). Investigation of Leishmania parasites from clinical samples using polymerase chain reaction technique in Cukurova Region, Turkey. *J Harran Univ Med Fac,* 5(1):10-4.