



Molecular and parasitological study of cutaneous leishmaniasis in Bushehr province, southwest of the Islamic Republic of Iran: a cross-sectional study during 2009–2012

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Abstract Cutaneous leishmaniasis (CL) is one of the most important parasitic disease in Iran. CL is distributed among more than half of 31 provinces of Iran. Studies on epidemiological aspects of the disease and *Leishmania* species identification among infected humans are necessary for providing a comprehensive prevention and control program thus; this descriptive cross-sectional study was conducted on all CL suspected patients who referred to Health Centers of Bushehr province from 2009 to 2012. Physical examinations were carried out in suspected individuals and CL cases were confirmed by microscopical examinations. Prepared slides from suspicious cases of CL were fixed with absolute methanol and stained by Giemsa 10 %. All the Giemsa-stained slides examined under a light microscope with high magnification (1,000×) and classified them based on grading of

Leishmania parasites. DNA from each slide was extracted, separately. The ribosomal internal transcribed spacer 1 was amplified with specific primers and PCR products were digested by restrict enzymes (*Hae*III), run them in 3 % gel agarose for electrophoresis and visualized on a UV transilluminator after staining with ethidium bromide. SPSS version 21 was used for data analyses. A total of 726 suspected CL cases were referred to Health Centers of Bushehr province from 2009 to 2012 and samples were only prepared from 188 of the patients whereas 43 (5.9 %) of them were microscopy positive. The most frequent of CL was observed in November (14 %) and December (12 %). The most distribution of CL lesions were observed on hands (32 %), feet (26 %), and face (21 %), respectively. The highest frequency of CL was observed in 1–9 years old (30 %). Altogether, 50 % of the patients showed one skin lesion and 2–10 skin lesions were occurred in the remained CL patients. Totally, 27 out of 43 (63 %) of the Giemsa stained slides were positive by PCR–RFLP assay because all the PCR–RFLP negative slides were prepared 3–4 years ago and kept without cover slip, and also observed scarce amastigotes during microscopy observations. *Leishmania* species were identified in 21 desirable slides which 14 of them were *L. major* and 7 of the remained isolates were identified *L. tropica* using PCR–RFLP.

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Introduction

Leishmaniases are considered endemic in 98 countries on 5 continents: Africa, Asia, Europe, North America, and

South America (Grimaldi et al. 1987). It's estimated that 12 million are infected; furthermore above 90 % of cutaneous leishmaniasis (CL) cases are prevalent in 9 countries including Iran (WHO 2010). CL occurs in two clinical and epidemiological forms, zoonotic (ZCL) and anthroponotic (ACL). ZCL caused by *Leishmania major* is endemic in northeast, south and central areas of Iran (Nadim et al. 2008; Mohebbali et al. 2004). ACL is caused by *Leishmania tropica* and is prevalent in large and medium sized cities of Iran (Nadim et al. 2008; Hajjarian et al. 2004). Almost 20,000–30,000 cases of CL (both ACL and ZCL) are reported annually from different parts of Iran and almost 75 % of reported CL in Iran are considered being ZCL (Diseases Management Center of Iran 2009).

Multiple lesions up to more than 100 may be occurred between patients (Pearson et al. 2001). Different species of *Leishmania* are morphologically indistinguishable thus, it is often problematic using parasitological method (Marfurt et al. 2003) Identification of *Leishmania* species was performed by isoenzyme analysis, DNA sequence and monoclonal antibodies.

Using molecular and biochemical techniques, *L. infantum*, *L. major* and *L. tropica* were isolated and characterized among infected humans, animal reservoir hosts and vectors of different geographical locations of Iran (Mohebbali et al. 2002; Hajjarian et al. 2004; Alimoradi et al. 2009; Kazemi-Rad et al. 2008; Motazedian et al. 2002).

This study aimed to show the current distribution of CL in Bushehr province and identify *Leishmania* spp. which were circulating using two parasitological and molecular methods.

Materials and methods

Study area

The study was conducted in all districts of Bushehr province, southwest of Iran. This province has a strategic importance due to trading potentials, gas and oil industries and neighborhood to Arab countries of Persian Gulf through a 700 km coastal line (Fig. 1).

Study population

This study was focused on the CL records at District Health Centers of Bushehr province which the diagnosis were done based on clinical manifestations and the positive microscopical smears. The passive case detections were carried out in the Rural Health Houses (RHCs), Rural Health Centers (DHCs) and District Health Centers (DHCs) or hospitals of different districts.

The early criteria for the selection of geographical localities for microscopical examinations were the annual records of CL among indigenous population at different districts of Bushehr province during 2009–2012. Demographic characteristics and information of CL were collected by a valid questionnaire. The microscopical examinations were carried out on clinically suspicious patients who were referred to laboratories of each Health Center at different districts of Bushehr province.

Microscopical examination

Patients who had the acute lesions with at least 2 weeks duration were considered for microscopically examinations. For each case having the acute skin lesion(s). A national standard questionnaire of CL was completed and the necessary information such as name, age, gender, living place, seasonal infection, history of disease and treatment, number of lesions, lesion site and lesion appearance were collected by a valid questionnaire.

Direct smears were prepared using scraping method with a flamed sterile vaccinostyle from edge of lesions of all suspected cases. At least, two smears were prepared from the serosity material of each lesion. All the prepared slides were transferred to RHCs/DHCS laboratories.

After drying the thin smears, they fixed with absolute methanol for 1 min stained with 10 % Giemsa for 25 min. For the detection of amastigote forms of *Leishmania* sp. and confirmation of CL, microscopical examinations were performed with light microscope at 100 microscopic fields with 1,000× magnification. All of suspected slides were examined by two microscopic experts before recording as negative results. The confirmed cases referred to Diseases Control Units of RHCS/DHCS for receiving the appropriate treatment.

PCR–RFLP assay

DNA extraction

All the Giemsa stained slides were washed with absolute ethanol and covered with 250 µl lysis buffer (50 mM NaCl, 50 mM Tris, 10 mM EDTA, pH 7.4, 1 % v/v Triton x-100 and 100 µg of proteinase k per ml). DNA extraction was carried out with QIAGEN Kit according to the manufacturer's instruction. DNA samples with A260/A280 ratios were selected and stored at –20 °C for further analysis.

DNA amplification by PCR

Two primers, LITSR (5-GTG CAG GAT CAT TTT CCG ATG) and L5.8s: 5-TGA TAC CAC TTA TCG CAC TT were designed for LTS1–PCR assays by identifying

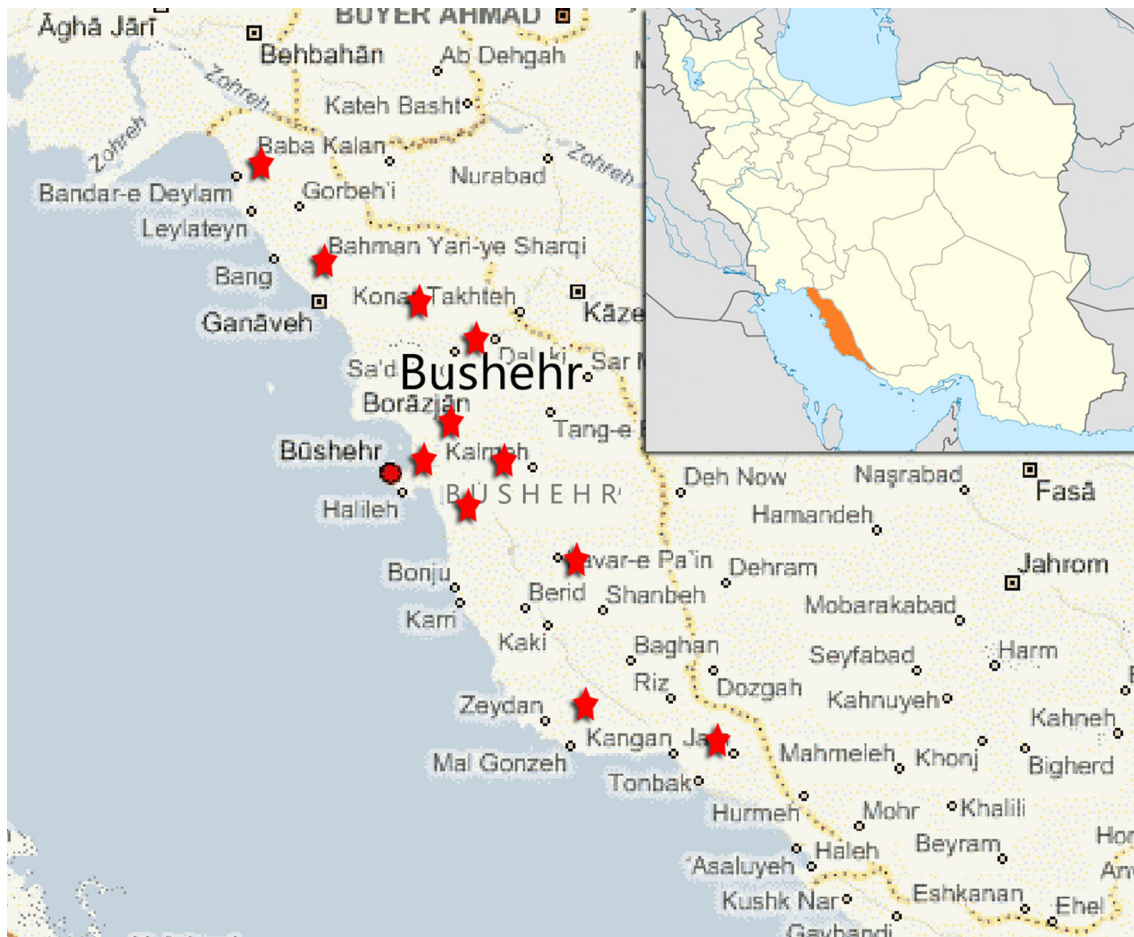


Fig. 1 Study areas from Bushehr province in southwest of Iran where this study was carried out

suitable regions described by Schonian et al. (2003) for the diagnosis and characterization of *Leishmania* in clinical samples.

PCR product analysis

Restriction fragment length polymorphism (RFLP) PCR was carried out on amplified region. Amplified products were separated by 1.5 % agarose gel electrophoresis, stained with ethidium bromide and visualized by ultraviolet (UV) transilluminator and then digital photographs were prepared. Extraction efficiencies and qualities were checked on 1 % agarose gel and DNA concentration and purity were estimated measuring the optical densities at 260 and 280 nm. We added 2 μ l *Hae*III to ITS1 PCR products (20 μ l) at 37 C for 12 h with conditions recommended by the supplier (Fermentas, Germany). Positive specimens against kDNA were tested against the ribosomal internal transcribed spacer 1 (ITS1) region using the—GTG CAT GATCAT TTTCCG ATG) and L5.8s :5-TGA TAC CAC TTA TCG CAC TT followed by digestion by *Hae*III. A Gene Ruler TM 50 bp (base pair) DNA ladder was used

as a marker. Samples were considered as positive when a PCR product of 565 bp was detected. Positive controls containing DNA of *L. infantum*, *L. major*, *L. tropica* and a negative control containing distilled water, were included. PCR products were sequenced (MWG Company, Germany) for confirmation of PCR–RFLP results.

Data analysis

Chi squared, Mac Nemar and Fisher exact tests were used to compare prevalence values relative to gender, age, and monthly distribution. Analyses were performed with SPSS (version 21), with a probability (*p*) value of <0.05 were considered as statistically significant.

Results

Altogether, 726 suspected CL cases were referred to Health Centers of Bushehr province from 2009 to 2012 and samples were only prepared from 188 of the patients whereas 43(5.9 %) of them were microscopy positive.

Table 1 Comparison of microscopy and PCR–RFLP results on Giemsa stained slides based on skin lesion appearance in CL patients referred to Health Centers of Bushehr province from 2009 to 2012

Clinical appearance of CL	Experiment results (positive)			
	PCR–RFLP		Microscopic	
	Percentage	Number	Percentage	Number
Wet	52	14	54	23
Dry	48	13	46	20
Total	100	27	100	43

Table 2 Comparison of microscopy and PCR–RFLP results with age groups in CL patients referred to Health Centers of Bushehr province from 2009 to 2012

Age groups	Experiment results (positive)			
	PCR–RFLP		Microscopic	
	Percentage	Number	Percentage	Number
≤9	15	4	19	8
10–19	26	7	23	10
20–29	15	4	16	7
30–39	18	5	21	9
≥40	26	7	21	9
Total	100	27	100	43

Totally, 27 out of 43 (63 %) of the Giemsa stained slides were positive by PCR–RFLP assay because all the PCR–RFLP negative slides were prepared 3–4 years ago and kept without cover slip, and also observed scarce amastigotes during microscopy observations.

Leishmania species were identified in 20 desirable slides which 14 of them were *L. major* and 7 of the remained isolates were identified *L. tropica* using PCR–RFLP (Table 1).

Altogether, 62.5 % of CL cases were male and 37.5 % of them were female. Age distribution of the confirmed CL patients showed in Table 2. Distribution of scar rates were 25.6 % on hands, 16.3 % on legs, 27.9 % on face, 11.6 % on hands and legs and 18.6 % on hands and face.

Totally, 46.5 % cases lived in urban areas while, 53.5 % of the patients lived in rural areas.

The number of scars on each patient varied from one to ten. Distribution of number of skin lesions were 44.2 % had one, 20.9 % had 2, 13.9 % had 3, 9.3 % had 4, and 11.6 % of remained patients had more than 4 skin lesions on their bodies.

Statistical analysis on the disease cases using SPSS program and conducting Chi square test, showed that there's not a meaningful difference between the scar numbers and the age of the patients (p value >0.05). Chi square test showed a meaningful difference between the age and permanent address and the scarred part of the cases (p value <0.05).

Chi square test didn't show statistically differences between the gender and the number of lesions (p value >0.05). This test also showed a statistically significance

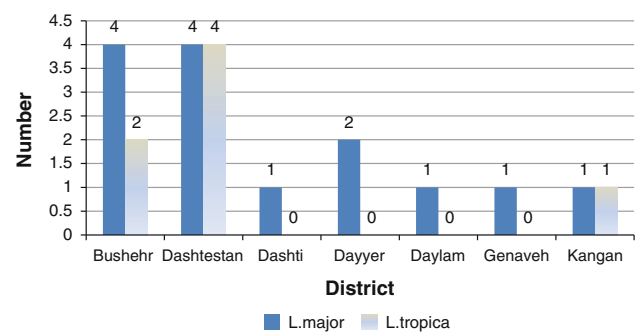


Fig. 2 Distribution *Leishmania* species in Bushehr province using the PCR–RFLP method

between the gender and living place of the patients (p value <0.05) (Fig. 2).

Results of PCR–RFLP showed that 27 out of 43 collected samples were positive by PCR–RFLP (63 %), and 43 of microscopical positive samples were showed a parasitemia from 2 to 4 grades. Twenty-one out of 27 (77.8 %) of the samples were *Leishmania* species identified that 14 of them were *L. major* and 7 of the remained isolates were *L. tropica* (Fig. 2).

Discussion

Bushehr province is one of the endemic regions for CL and VL (Hamzavi et al. 2000; Mohebbali et al. 2001). An appropriate control method for CL depends on the disease prevalence and environmental changes (WHO 2013).

Characterization of *Leishmania* species is important, because different species may require distinct treatment regimens. Furthermore, such information is also valuable in epidemiologic studies where the distribution of *Leishmania* species in human and animal hosts, as well as in insect vectors, is a prerequisite for designing appropriate control measures (Schonian et al. 2003; El Tai et al. 2000). Various biochemical, immunological, and molecular methods are invented for differential diagnosis of *Leishmania* species. Among biochemical methods, the method of analyzing iso enzymes is the most appropriate method for specifying the parasite taxonomy, but this method is time consuming and requires estimating 10–20 enzymes and also requires aseptic isolating the parasite in the specific media (Grimaldi et al. 1987). Since molecular methods such as PCR assays have been reported as desirable methods for *Leishmania* species identification (Gadisa et al. 2007).

In endemic areas where more than one *Leishmania* species is present, diagnostic tools are required for the detection of parasites directly in samples and distinguish all relevant *Leishmania* species (Schonian et al. 2003; Al-Jawabreh et al. 2006).

A Study was carried out between standardized graded direct microscopy and ITS1–PCR–RFLP on Giemsa stained smears (Al-Jawabreh et al. 2004). Also, PCR–RFLP method was used for diagnosing and typing cutaneous and visceral leishmaniasis and finally PCR–RFLP was reported as a sensitive and suitable method for routine diagnosis of leishmaniasis (Serin et al. 2007).

We used PCR–RFLP methods for diagnosis and characterization of *Leishmania* species on Giemsa stained slides without the need for cultivation them. Giemsa-stained slides are appropriate for field condition as such samples can be easily stored and sent to the diagnostic laboratory (Kazemi-Rad et al. 2008). This study showed that *Leishmania* DNA could be efficiently extracted and amplified even from old Giemsa-stained microscopic slides that were stored for 4 years with relatively high *Leishmania* spp. load and if these were protected by a cover slip. In this study, the Giemsa-stained slides were examined by both microscopy and ITS1–PCR–RFLP. Most of the slides that were high scored amastigote numbers as microscopy-positive were also positive by PCR–RFLP. This study also showed that Giemsa-stained slides used for the diagnosis with microscopy, could readily be used as samples for identifying of *Leishmania* species with PCR–RFLP. Although the costs for PCR–RFLP diagnosis are higher and its concordance is lower than microscopic examination but this method can identify *Leishmania* species without need for cultivation them. In conclusion, the PCR–RFLP method seems to be applied as a suitable tool for direct diagnosis and characterization of *Leishmania* species in Giemsa-stained slides.

Overall there were 726 CL patients who referred to the Health Centers of Bushehr province from 2009 to 2012.

Most refers were recorded during the November (14 %) and December (12 %). Seasonal changes in CL incidence are consistent with the pattern of rural type CL (Nadim 2000). Unlike the urban type which most skin lesions are seen on the face, in this study, most lesions have been observed on hands and legs and most CL cases were ≤ 9 (30 %).

In a study was surveyed CL epidemiologic situation in plains and cities of Bushehr province and detected three isolates—which were taken from patients using RAPD–PCR method—as *L. major* (Hamzavi et al. 2000).

In conclusion, differential diagnosis of CL referred to Health centers of the disease is highly recommended. Since PCR–RFLP is an accurate, sensitive, and fast method for *Leishmania* spp. characterization even on (Giemsa stained slides). Thus, we recommended this method in endemic areas of CL.

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References

- Alimoradi S, Hajjarian H, Mohebbali M, Mansouri F (2009) Molecular identification of *Leishmania* species isolated from human cutaneous leishmaniasis by RAPD–PCR. Iran J Public Health 38:44–50
- Al-Jawabreh A, Schnur LF, Nasereddin A, Schwenkenbecher JM, Abdeen Z, Barghuthy F, Khanfar H, Presber W, Schonian G (2004) The recent emergence of *Leishmania tropica* in Jericho (A'riha) and its environs, a classical focus of *L. major*. Trop Med Int Health 9:812–816
- 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
- El Tai NO, Osman OF, EL Fari M, Presber W, Schönian G (2000) Genetic heterogeneity of ribosomal internal transcribed spacer in clinical samples of *Leishmania donovani* spotted on filter paper as revealed by single-strand conformation polymorphisms and sequencing. Trans R Soc Trop Med Hyg 94(5):575–579
- Gadisa E, Genetu A, Kuru T, Jirata D, Dagne K, Aseffa A, Gedamu L (2007) *Leishmania* (Kinetoplastida): species typing with isoenzyme and PCR–RFLP from cutaneous leishmaniasis patients in Ethiopia. Exp Parasitol 115:339–343
- Grimaldi G Jr, David JR, McMahon-Pratt D (1987) Identification and distribution of new world *Leishmania* species characterized by serodeme analysis using monoclonal antibodies. Am J Trop Med Hyg 36:270–287
- Hajjarian H, Mohebbali M, Razavi MR, Rezaei S, Kazemi B, Edrissian GhH, Mojtavavi J, Hooshmand B (2004) Identification of *Leishmania* species isolated from human cutaneous leishmaniasis, using random amplified polymorphic DNA (RAPD–PCR). Iran J Public Health 33:8–15

- Hamzavi Y, Mohebbali M, Edrissian A, Foruzani A (2000) Epidemiology of cutaneous leishmaniasis (human and animal reservoirs of infection) of the city of Bushehr Dashtestan. *J Public Health* 2:177–179
- Kazemi-Rad E, Mohebbali 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* 37:54–60
- Marfurt J, Niederwieser I, Makia ND, Beck HP, Felger I (2003) Diagnostic genotyping of old and new world *Leishmania* species by PCR–RFLP. *Diagn Microbiol Infect Dis* 46:115–124
- Ministry of Health and Medical Education, Center for Disease Control (2009) Leishmaniasis control instructions
- Mohebbali M, Hamzavi Y, Edrissian GH, Forouzani A (2001) Seroepidemiological study of visceral leishmaniasis among humans and animal reservoirs in Bushehr province, Islamic Republic of Iran. *East Mediterr Health J* 7(6):912–917
- Mohebbali M, Motazedian MH, Parsa F, Hajjaran H, Yaghoobi-Ershadi M (2002) Identification of *Leishmania* species from different parts of Iran using a random amplified polymorphic DNA in humans, animal reservoirs and vectors. *Iran J Public Health* 15:243–246
- Mohebbali M, Javadian E, Yaghoobi-Ershadi MR, Akhavan AA, Hajjaran H, Abaei MR (2004) Characterization of *Leishmania* infection in rodents from endemic areas of the Islamic Republic of Iran. *East Mediterr Health J* 10:591–599
- Motazedian H, Karamian M, Noyes HA, Ardehali S (2002) DNA extraction and amplification of leishmania from archived, Giemsa-stained slides, for the diagnosis of cutaneous leishmaniasis by PCR. *Ann Trop Med Parasitol* 96:31–34
- Nadim A (2000) Leishmaniasis. In: Aziz F, Janghorbani M, Hatami H (eds) Epidemiology and control of common disorders in Iran. Nashr-e-Tehran University, Tehran, pp 527–534
- Nadim A, Javadiyan A, Mohebbali M, Zamen Momeni A (2008) *Leishmania* and leishmaniasis, 3rd edn. Nashr-e-Daneshgahi Press, Tehran, p 11
- Pearson RD, Queiroz Sousa A, De Mandell GL, Douglas RG Jr, Bennett JE (2001) *Leishmania* species: visceral (kala-azar), cutaneous and mucosal leishmaniasis. Principle and practice of infectious disease. Churchill Livingstone, New York, pp 2831–2845
- Schonian G, Nasereddin A, Dinse N, Schweynoch C, Schallig HD, Presber W, Jaffe CL (2003) PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. *Diagn Microbiol Infect Dis* 47:349–358
- Serin MS, Waki K, Chang KP, Aslan G, Direkel S, Otag F, Kayar B, Koksall F, Emekdas G (2007) Consistence of miniexon polymerase chain reaction–restriction fragment length polymorphism and single-copy gene sequence analyses in discriminating *Leishmania* genotypes. *Diagn Microbiol Infect Dis* 57:295–299
- [www.who.int](http://www.who.int/entity/mediacentre/factsheets/fs375/en/index.html) (2013) /entity/mediacentre/factsheets/fs375/en/index.html
- www.who.org (2010) Control of leishmaniasis. Technical report series 793 of WHO Expert Committee, Geneva