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ORIGINAL ARTICLE

Molecular detection of *Leishmania* species in northeast of Iran

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Abstract

Two known types of cutaneous leishmaniasis (CL) including zoonotic CL due to *Leishmania major* and anthroponotic CL due to *Leishmania tropica* are prevalent in 14 of 22 countries located in the Eastern Mediterranean region including Iran. According to existing data, CL is endemic in Sabzevar City (northeast of Iran) and, because of the climatic conditions in this semi-desert region, is suitable for living vector/reservoir hosts of infection. The aim of our study was to identify the recent status of CL causative species in rural areas of Sabzevar County. Suspected patients of CL who were referred to health centers in suburban areas of Sabzevar and confirmed via microscopic observation of amastigotes were included in the study. Molecular identification of *Leishmania* species was done via nested PCR assay, based on amplification of kinetoplast minicircle fragments of *L. major* and *L. tropica*. In total, 153 patients including 89 males and 64 females were enrolled in this study. A high infection rate was reported in the autumn season (with a peak in October). Our findings revealed that *L. major* is responsible for 100% of infections. In addition, there was no association between CL and risk factors after statistical analysis. It seems that the infection pattern of CL is changing predominantly to *L. major* in most regions of Iran, which may be due to environmental changes, or ecological amendment and their effects on (vector/reservoir) host distribution in rural parts. Finally, controlling programs as well as promotion in public health systems should be considered in this area.

Keywords Cutaneous leishmaniasis · *Leishmania major* · *Leishmania tropica* · Nested PCR

Introduction

Leishmaniasis is an obligate intracellular protozoan parasitic disease caused by *Leishmania* species (Mahmoudvand et al. 2011). Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis and known as a public health and social problem in all tropical and subtropical areas of the world (Murray et al. 2015; Kavarizadeh et al. 2017).

The distribution of this disease is very tightly linked to geographical regions so that villages even 15 miles apart can have very different rates of cutaneous leishmaniasis (Mathers et al. 2007). An estimated 0.7 million to 1.2 million new cases occur worldwide annually and over two thirds of new cases occur in 10 countries, namely Afghanistan, Algeria, Brazil, Colombia, Syria, Costa Rica, Ethiopia, Peru, Sudan, and Iran (Alvar et al. 2012).

According to the reports of the World Health Organization (WHO), the number of reported CL cases increased from 12,727 in 2000 to more than 21,148 in 2010 in Iran. In addition, the incidence rate of CL was 2.68 cases per 10,000 inhabitants in endemic areas of Iran. This report shows CL disease prevalence is increasing in Iran (Postigo 2016).

There are two forms of CL including anthroponotic cutaneous leishmaniasis (ACL) due to *Leishmania tropica* and zoonotic cutaneous leishmaniasis (ZCL) caused by *Leishmania major* in Iran (Mirahmadi et al. 2016;

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Vazirianzadeh et al. 2013). The different species are morphologically indistinguishable and sometimes cause similar clinical signs. Since treatment regimen is different in the two forms of CL, thus accurate identification of *Leishmania* species is essential for the control and prevention of the disease (Sharifi et al. 2012; Schwarz et al. 2015).

Direct microscopic examinations and culture method are the simplest means of diagnosing CL, but they do not discriminate the organism in the species level (Asgari Nezhad et al. 2012; Gillespie et al. 2016). Therefore, differentiation of *Leishmania* species requires diagnostic techniques with reliable sensitivity and specificity like molecular methods (Pouresmaeliyan et al. 2011; Shirian et al. 2014). For the genetic diversity in leishmania, the methods commonly used are Mini Exon RFLP and ITS RFLP and markers of microsatellite. The conserved area of *Leishmania* kinetoplast DNA (kDNA) minicircles has been used as a particular target for PCR assays. The kDNA is arranged at the base of the flagellum and contains a huge number of minicircles and many maxicircles. The minicircles, which encode for guide RNAs needed for editing the mRNA from maxicircles, have now been reported to be within about 10,000–20,000 copies per parasite (Shlomai 2004; Zarean et al. 2017).

In order to accelerate progress towards the strategies of WHO for leishmaniasis prevention, control, and monitoring programs in highly endemic regions, we aimed to determine the recent status of causative CL species in rural areas of Sabzevar County, northeast of Iran.

Materials and methods

Suspected patients of CL who were referred to local health centers in suburban areas of Sabzevar City during the study period were defined as the target population. Slide samples from scars and ulcers of patients were obtained by experienced staffs of laboratories and evaluated for leishmania amastigotes via direct microscopic examination. Giemsa-stained slides were sent to the research center laboratory in Sabzevar University of Medical Sciences and prepared for molecular tests. Briefly, slide surfaces were scratched with a scalpel and precipitated in a 1.5-ml microtube through ethanol wash (Hosseininasab et al. 2014; Pour et al. 2011).

The Ethical Committee of Sabzevar University of Medical Sciences approved this project. Consent forms were signed by participants and a questionnaire form fulfilled by each patient in this study.

DNA extraction

DNA was extracted by DNP extraction kit (Cinnagen Ltd. Co., Iran) according to the manufacturer's instruction and eluted in 50 μl of TE buffer.

PCR

Variable regions of minicircle fragments of kinetoplast DNA (kDNA) were amplified by nested PCR assay using two sets of previously described primers (Table 1) (Noyes et al. 1998; Zarean et al. 2015). First round of nested PCR was performed in a final volume of 25 μl (12 μl of Master mix PCR solution 1X) (Ampliqon, Denmark), 1 μl of primers (20 pmol) CSB1XR and CSB2XF, 3 μl DNA (50 ng), and 8 μl deionized distilled water, over 30 cycles including denaturation at 94 °C for 30 s, annealing at 55 °C for 60 s, and extension at 72 °C for 90 s, followed by a final extension step at 72 °C for 8 min (Bio-Rad thermocycler). One microliter of the first-round product diluted in 9 μl of distilled water was used as template for the second-round PCR in a total volume of 25 μl under the same conditions as those for the first round, except with primers 13Z and LiR. A negative control (water) and two positive controls [*L. tropica* (MHOM/Sudan 158/OD strain) and *L. major* (MRHO/IR/75/ER strain)] yielding 750- and 560-bp fragments, respectively, were used in each round of PCR. Finally, PCR products were analyzed on 1.5% agarose gel (Roche, Germany) through 0.5% TBE buffer (Sigma-Aldrich) and were visualized by DNA safe stain (Cinnagen Ltd. Co., Iran) under a UV transiluminator.

Data analysis

Statistical analyses were estimated using SPSS for windows (version 20. Chicago, Ltd., USA). Chi-square test with the level of significance defined as $P < 0.05$ and common odds ratio (95% CI) were used for possible association of variables.

Results

One hundred fifty-three patients diagnosed with CL were enrolled in this study. In total, 89 (58.2%) out of 153 cases were male and 64 (41.8%) were female. Patient's ages ranged from 3 to 88 years and the highest rate of infection was recorded in the 20–30 years age group (23.5%). Figure 2 shows the infection rate of participants in different age groups in this study. The maximum number of CL was reported in autumn (with a peak in October) and there was no infection in winter.

Table 1 Primer sequences used for amplification of variable minicircles kDNA

Primers		Primer sequence (5′–3′)
External	CSB1XR	CGAGTAGCAGAAACTCCCGTTCA
	CSB2XF	ATTTTTCGCGATTTTCGCAGAACG
Internal	13Z	ACTGGGGGTTGGTGTAAAATAG
	LiR	TCGCAGAACGCCCCT

Thirty-eight percent of infected people had a single lesion and multiple lesions were seen on 33.3% of patients. Furthermore, the most common lesion sites were observed on patients' hand and arm (59.4%) (Table 2).

Our findings revealed that all patients in this study are suffering from zoonotic cutaneous leishmaniasis form and *L. major* is responsible for 100% of infection in this geographical region (Fig. 1).

In addition, there were no statistically significant differences between CL infection and related risk factors in patients.

Discussion

In this study, *L. major* was the only strain isolated from patients living in the suburban area of Sabzevar. This finding is in agreement with that of a previous study conducted in this region which reported *L. major* as the main cause of CL (73%) in rural areas (Mohajery et al. 2010). Likewise in our results, *L. major* is reported as the only pathogen isolated from patients suffering from CL in different areas of Iran like Varzaneh, Mehran, Gonbad-e-Qabus, Esfahan, and Qom (Arjmand et al. 2014; Feiz Haddad et al. 2016; Mesgarian et al. 2010; Mohammadi et al. 2011; Nateghi Rostami et al. 2013). Moreover, *L. major* was responsible as the main cause of infection in some other studies conducted in Tehran (64%), Shiraz (90.7%), Khuzestan (94.5%), and Kashan (92.1%)

Table 2 Characteristics of features of patients with cutaneous leishmaniasis in Sabzevar County, northeast of Iran

Feature	Classification	No. of cases	Percent
Location of lesions	Hand	53	35.1
	Arm	37	24.3
	Foot	32	21
	Neck	17	11
	Face	9	6
	Others	5	2.6
	Total	153	100
Gender	Male	89	58.2
	Female	64	41.8
	Total	153	100
Age	< 10	15	9.5
	10–19	33	22
	20–29	35	23.5
	30–39	22	14.5
	40–49	15	9.5
	50–59	18	12
	60–69	4	2.5
	70–79	6	3.5
	> 80	5	3
	Total	153	100

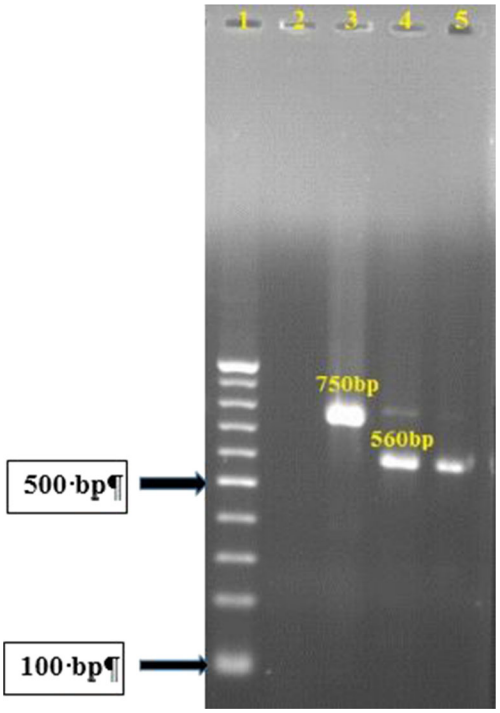

Fig. 1 Agarose gel electrophoresis of *Leishmania* isolates. Lane 1: DNA size marker 100 bp. Lane 2: negative control. Lane 3: *L. tropica* (positive control 750 bp). Lane 4: *L. major* (positive control 560 bp). Lane 5: *L. major* isolates obtained from skin lesions of the patients in Sabzevar, northeast of Iran

(Farahmand et al. 2011; Izadi et al. 2016; Maraghi et al. 2013; Shiee et al. 2012). Based on these findings, it seems that a changing profile of *Leishmania* species is happening in different areas of Iran (Zarean et al. 2017), which may be due to implications on treatment and control strategies for ACL like patient monitoring systems, as well as combating with another reservoir host (dogs), and consequently breaking the cycle of transmission.

Our work indicated that the most highly infected age groups are 21–30 years and 10–19 years (23.5 and 22%, consecutively). Based on data published from Borojerd, Ilam, and Shoushtar, individuals between 21 to 30 years are the most highly infected age group (Ahmadi et al. 2013; Kassiri et al. 2012, 2014). In addition, some other studies imply that CL is more prevalent in people younger than 20 years old (Azizi et al. 2013; Khosravi et al. 2013). Generally, it could be related to pre-exposure to sand flies and infection in people > 30 years old.

In the present study, single lesions were recorded as more common than double or multiple lesions and uncovered parts of patients' bodies including the hand and arm, legs, neck, and face were documented as the most bitten sites, respectively. These results are consistent with those of several studies in other endemic areas of ZCL in Iran (Kermanjani et al. 2016; Tolouei et al. 2014). It seems that this model is mostly related to single biting habits of sand flies and the fact that they cannot drink the blood over clothes.

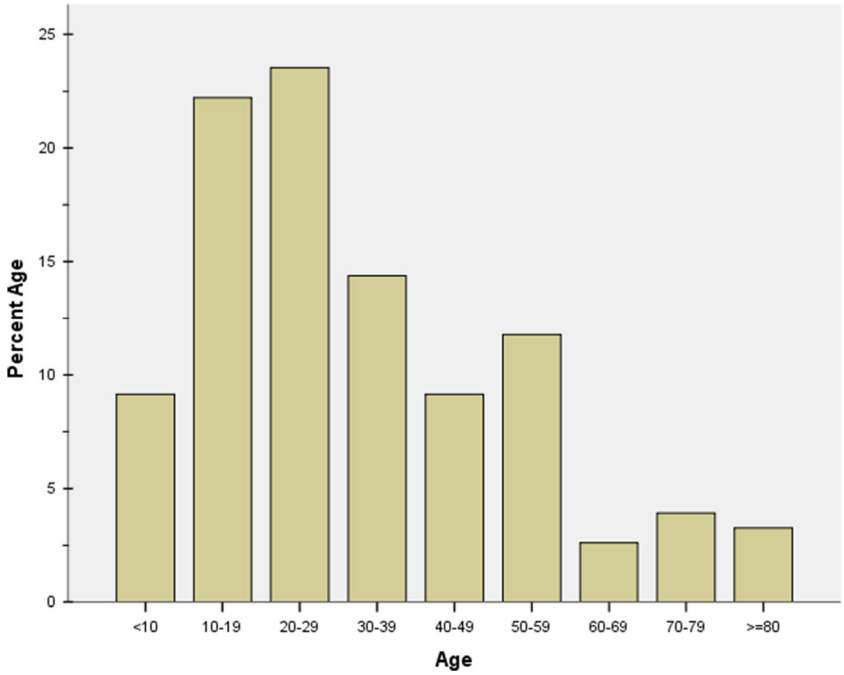

Fig. 2 Infection rates of patients based on age groups

Based on data reported from other cities like Kashan and Kermanshah, a high incidence rate of infection happens in the last summer and first autumn annually (Doroodgar et al. 2012; Hamzavi and Khademi 2015). Accordingly, almost 50% of infections in this study were documented just in October. Except weather condition, possibly the most likely reason of that is occupational contact (as a farmer) with sand flies.

Conclusion

Leishmania infection is still a main problem in semi-desert areas and the present study shows that *L. major* is the main causative agent of CL in our region (Sabzevar) which is located northeast of Iran. Furthermore, in view of the fact that different species of *Leishmania* require different treatment regimens, molecular assays like nested PCR are precise tools for diagnosing the epidemiological pattern of this disease. Finally, we suggest further studies for identification of vector/reservoir hosts and their role in *Leishmania* infection in each endemic area.

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Authors' contributions M.J. Namazi conceived and designed the study; A. Balooti Dehkordi collaborated in the preparation of samples; F. Haghighi drafted the manuscript; M. Mohammadzadeh contributed to the design of the study and was involved in all steps of the experiment; M. Zarean critically revised the manuscript and approved the final study; M. Haghighi Hasanabad interpreted data and PCR performance. All authors have read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This study was reviewed and approved by the Ethics Committees of Sabzevar University of Medical Sciences, Sabzevar, Iran. Consent forms were signed by participants and a questionnaire form fulfilled by each patient in this study.

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