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Original paper

New foci of zoonotic cutaneous leishmaniosis due to *Leishmania major* in the northeastern Iran cities of Sabzevar and Neghaab

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ABSTRACT. Cutaneous leishmaniosis (CL) is a major public health challenge in Iran. Overall, 18 provinces out of 31 of the country's provinces are in the endemic areas. The objective of the present study was to determine the presence of CL species in Sabzevar and Neghaab cities, Khorasan Razavi province, northeastern Iran. Overall, 280 samples were taken from suspected individuals referred to Sabzevar's and Neghaab's health centers during 2014–2016. Smear preparation, case detection and further identification by partial amplification of 7SL RNA and high-resolution melting curve analysis were done for all samples, followed by randomly polymerase chain reaction (PCR)-sequencing confirmation. Based on findings, the most infection rate was found in males with the age groups of 20–30 in both districts. All samples were characterized as *Leishmania major*, except one isolate in Neghaab closely related to *Leishmania tropica*. Various risk factors play roles in creating new foci of zoonotic CL (ZCL) caused by *L. major* in Sabzevar and Neghaab in northeastern Iran. Reports of the prevalence of CL in new foci make serious concerns about the incidence of cases and expansion of disease to the neighboring areas. Further measures are essential to control the disease in the regions.

Keywords: zoonotic cutaneous leishmaniosis, *Leishmania major*, 7SL RNA, High-Resolution Melting, Iran

Introduction

Leishmaniosis is a neglected tropical disease that is widespread in many parts of developing countries. It comprises a group of diseases caused by protozoan parasites from more than 21 *Leishmania* species. They are transmitted to humans and animals by the female phlebotomine sandflies [1,2]. Cutaneous leishmaniosis (CL) is the predominant form that presents skin lesions. Ninety percent of CL cases are reported in Algeria, Brazil, Peru, and Middle East countries such as Iran [1–3]. An estimated at least 20,000 CL cases have been reported annually, although the actual number is estimated to be four to five-fold higher. Overall about 58% (18/31 provinces) of the provinces of the country are in the endemic areas [4,5].

Based on reports by WHO, there are two clinical-epidemiological forms of CL in Iran. Rural or zoonotic CL (ZCL) caused by *Leishmania major*, small gerbils are the main reservoir and *Phlebotomus papatasi* is the principal phlebotomine vector and urban or anthroponotic CL (ACL) form caused by *L. tropica* and humans and *Phlebotomus sergenti* are the major reservoir and vector [4]. The epidemiological features of CL depend on many factors such as population displacement, environmental modification, and climatic changes

Figure 1. Map of Iran displaying Khorasan Razavi province with two endemic areas, Neghaab and Sabzevar where new zoonotic cutaneous leishmaniosis foci emerged

[6–10]. Development of agricultural projects and population changes in the outskirts have been noted in disease transmission previously [8,11,12]. Therefore, the newly emerged foci of the disease are induced due to the aforementioned risk factors.

Khorasan Razavi province is located in the northwest of Iran, which demonstrates a high incidence of the disease [13–15]. Detection methods are diverse, but direct smear microscopy and culturing are conventional techniques with low sensitivity used frequently to diagnose the causative agent at the genus level. Identification of CL with similar clinical manifestation at the species level is difficult but essential for prognosis, epidemiological and therapeutic purposes, especially in endemic areas where more than one species, such as *L. tropica* and *L. major* are present [9,16,17]. In most countries, the microscopic examination is used as a primary diagnostic tool and cultivation is only performed in superior laboratories. However, comparison of diagnostic methods for CL over the past years emphasizes the high sensitivity and the more accuracy of polymerase chain reaction (PCR)-based techniques [18,19]. These molecular methods are highly efficient for field-based identification purposes because of their high specificity and sensitivity [16,20,21]. Recently, High-Resolution Melting (HRM) assay was introduced as an ideal technique for quick and precise characterization of *Leishmania* species [9,22,23].

According to our knowledge, over the past decades, no comprehensive investigations have been carried out on the prevalence and molecular characterization of CL in the province, especially in Neghaab and Sabzevar areas. In the present study, for the first time, we aimed to assess new emerging foci of CL in northeastern Iran with HRM assay.

Materials and Methods

Study area

Khorasan Razavi Province in northeastern Iran has plains, foothills, and mountainous areas that cover 118,884 km^2. The province is located between 36.2980°N and 59.6057°E. Afghanistan borders this region in the East and Tajikistan in the North. It has 28 counties, including Neghaab and Sabzevar, located between foci of ACL and ZCL caused by *L. tropica* and *L. major*, respectively (Fig. 1).

Sampling and data collection

Sampling was done among 170 and 110 suspected individuals referred to Sabzevar's and Neghaab's health centers in 2014–2016, respectively. For each patient, a questionnaire was completed by face-to-face interview along with clinical observation of the lesions. Demographic and clinical data were recorded. A skin scraping was obtained from the edge of each lesion by a scalpel and blade and then smeared on a microscopic glass slide. Smear preparation slides were air-dried, fixed with methanol, and stained by Giemsa for direct microscopic examination. Each case was defined by the clinical evaluation of the lesion and observation

Table 1. Sex and age distribution of the infected patients in Sabzevar and Neghaab districts, Khorasan Razavi province, Iran

Characteristics		Sabzevar no. (%)	Neghaab no. (%)	Total no. (%)
Age (year)	<20	14 (23.3)	8 (20)	22 (22)
	20–39	33 (55)	18 (45)	51 (51)
	40–59	9 (15)	10 (25)	19 (19)
	≤60	4 (6.7)	4 (10)	8 (8)
Sex	Male	44 (73.3)	26 (65)	70 (70)
	Female	16 (26.7)	14 (35)	30 (30)
Total		60 (60)	40 (40)	100 (100)

of amastigotes (leishman bodies) [24]. Simultaneously, another glass slide was prepared for each case for further identification by HRM analysis.

To prevent unwanted results, CL patients with a history of traveling to other endemic areas within one year prior to sampling were excluded. All the CL patients were registered and examined by direct smear preparation. Positive smear preparations were the source for further PCR assays. The parasite burden was classified as the number of amastigotes per field according to the method initially proposed by Ridely [25].

Molecular characterization

DNA was extracted from the direct smear preparations using ExgeneTM Clinic SV kit (Geneall, Korea) following the manufacturer's instructions and was stored at −20ºC until used. To avoid errors caused by the number of parasites on each slide, the extracted DNA was quantified with a spectrophotometer (Nano Drop-2000c; Thermo Scientific) and diluted to the same concentration according to the study by Mohammadi et al. [26]

The partial amplification of the 7SL RNA gene was amplified with specific primers according to the method described by Nasereddin and Jaffe [27]. PCR was carried out with primer pairs forward (5'-ACGTGGACCAGCGAGGGT-3') and reverse (5'-CGGTTCCCTCGCTTCA AC-3'), which amplified a 119-bp polymorphic internal region of 7SL RNA, with the following mixture: 4 µl master mix (5x HOT FIREPol® EvaGreen®HRM Mix), 40 ng/reaction of DNA extract and 1 µl of 2.5 mM of each primer in final 20 µl PCR reaction. The mixture was incubated at 94°C for 15 min followed by 40 cycles; each was consisting of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C. The process was immediately followed by a melt program ramping from 72 to 90°C, in 0.1°C increments pausing for 2 s per step for reaction and a melt program ramping from 78 to 94°C, 0.2°C increments pausing for 2s per step. Normalization regions were selected between 84 and 93°C. Amplification and melting curve analysis was carried out in a RotorGeneQ (Qiagen, Hilden, Germany) system, and HRM analysis was performed using the RotorGeneQ software. In order to examine the reproducibility, DNA from each sample was tested in triplicates and repeated on two different days by keeping the same conditions.

Validity and conformation of characterized samples were prepared by additional PCR-sequencing for tree random selected isolates. The PCR processing and sequencing for 7SL RNA amplicons were prepared by the specific primers, which are previously described [28]. The products of positive samples were purified and sequenced by Macrogen Company (South Korea). Sequence similarity searching for three random selected isolates was performed using the NCBI basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Ethical consideration

This project (no 93/526) was reviewed and approved by the Ethics Committee of the Kerman University of Medical Sciences and Kerman Leishmaniasis Research Center. Before initiating the project, relevant health authorities were notified, and complete cooperation in various aspects of the work was obtained. Participation was voluntary upon signing the written informed consent of the subjects. All the demographic information was kept confidential. The patients were given drugs free of charge, accordingly.

Results

Descriptive study

Overall, 100 positive specimens (60/170 Sabzevar and 40/110 Neghaab) out of 280 samples were taken randomly for further examination. In Sabzevar, the males were have the most infection frequency (n=44, 73.3%) and females were have the lowest (n=16, 26.7%), Likewise, in Neghaab the highest and lowest rate of infection was observed in males (n=26, 65%) and females (n=14, 35%) respectively. In the present study, the most infected patients were observed in age groups 20–30 years in Sabzevar and Neghaab districts (Tab. 1).

Molecular identification

Melting analysis of 7SL RNA amplicons specified *L. major* and *L. tropica* as the main species of cutaneous leishmaniosis in Sabzevar and Neghaab. The discrimination between three major old-world *Leishmania* species as positive controls samples in HRM curve is shown in figure 2. The average melting point (Tm) ± standard deviation (SD) for 7SL RNA amplicons was calculated and summarized in table 2.

HRM analysis of Sabzevar clinical samples showed strong similarity patterns to *L. major* positive control with minor SD. In the same way, most of Neghaab clinical samples were characterized as *L. major* species except one sample that isolated from a 6-year-old girl, which was shown a close relation with *L. tropica* (Fig. 2). In addition, some deviations from the standard sample were observed as *L. major* samples in both Neghaab and Sabzevar isolates. Nucleotide sequence analysis of three random selected isolate was confirmed the HRM characterization results (Fig. 3).

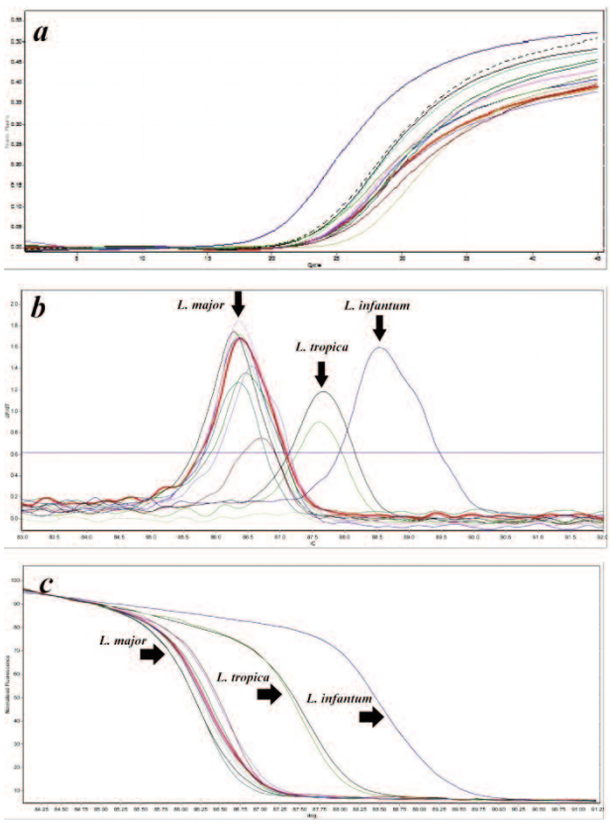

Figure 2. Genotyping of cutaneous *Leishmania* isolates with high resolution melting curve analysis of partial 7SL RNA amplicons, a: amplification curve of samples (determine base line), b: melting curve analysis of samples, c: normalized graph of high regulation melting curve analysis of samples (curves was not shown for all samples)

Discussion

CL is one of the endemic transmittable diseases in most provinces of Iran and presented as one of the major health problems in these regions [1,5]. The aim of this study was to determine the species

Table 2. Results of HRM analysis of 7SL RNA amplicons in patient isolates

Sample	TM*	(SD**)
*L. tropica***	87.63	0.18
*L. major***	86.41	0.26
*L. infantum***	88.55	0.13
Sabzevar isolates (*L. major*)	86.41	0.28
Neghaab isolates (*L. major*)	86.45	0.23
Neghaab isolate (*L. tropica*)	87.49	N/A

*TM: temperature of melting, **SD: standard deviation, ***positive control, N/A: not available

Figure 3. Alignment and comparison of nucleotide sequences of *Leishmania* species (*L. tropica*, *L. major*, *L. donovani*, *L. infantum*) with the isolates from Neghaab and Sabzevar

of *Leishmania* parasites in stained smear preparation slides, which were taken at the health centers of Sabzevar and Neghaab in newly emerged foci. Additionally, we tried to develop a precise molecular HRM-analysis method to differentiate *Leishmania* species isolated from CL patients.

CL can be divided as zoonotic or anthroponotic related to whether the normal reservoir of the *Leishmania* is human or rodents [29]. The reservoir of ACL is human and incidentally dogs. However, in most of cases the transmission occurs from human to human. Simultaneously, the reservoir of ZCL is rodents which makes the possibility of transmission from animal to human. Hence, changes in the environment can be a major factor for the propagation of the disease cycle. The most important of these factors are probably agricultural development and widespread suburbanization [8,30]. This could grow and provide a food source for gerbils, the main reservoir and attract them to the area, where the biological vector is present and in turn transmission of the cases occurred.

The emergence of new foci of leishmaniosis that were close to endemic areas are highly imaginable. The prevalence of leishmaniosis in the Khorasan Razavi province, especially in Sabzevar and Neghaab cities, can be attributed to several factors such as environmental conditions. Environmental changes can affect the rate of infection. Prevalence and increase in its incidence have been related to travel, urbanization, climatic changes, and socio-economic situation in several parts of the endemic area [5,31,32]. Akbari et al. [33] demonstrated that the prevalence of *Leishmania* in the past decade had been related to the reduction of seasonal rainfall in the second half of the year. Probably, these factors, along with the development of agricultural, irrigation (water supply), construction projects, and disease transition in understudied regions, played roles in convincing reasons for the occurrence and establishment of new emerging foci of the disease in the province.

PCR is the most commonly used method for *Leishmania* identification and several targets have been described, but no direct and straightforward protocol has emerged [26]. In the recent study, Real-time PCR with HRM curve analysis was used for detection and characterization of *Leishmania* species because of its simplicity, cost-effectiveness, high sensitivity, and easy automation [26,27,34].

On the other hand, there are several target genes for PCR-based methods, including kinetoplast or ribosomal DNA genes used for the identification of *Leishmania* species in the Old or New World. However, recently special PCR-based processes were optimized for ITS1, Hsp70, and 7SL RNA genes for HRM techniques to identify the species [16,19]. Therefore, in this study, the HRM analysis of the 7SL RNA gene was used, and it could effectively be detected and identified the parasite species. The average temperature of melting for *L. tropica* and *L. major* isolates were 86.4°C and 87.4°C, respectively. The temperature differences were similar to other HRM analysis studies for the 7SL RNA region [27,35]. Although differences in DNA extraction method, PCR-HRM Kit, and fluorescent dyes can cause differences in results, the similar differential melting curve patterns in

comparison to positive controls could be beneficial. Besides, confirmation of the suspected sample by sequencing is always in touch.

Our study demonstrated new foci of ZCL due to *L. major* in Sabzevar and Neghaab in Khorasan Razavi province. Reports of CL make serious concerns about the incidence of cases and expansion of disease to the neighboring areas. Further measures are essential to control the disease in the areas.

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