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Emergence of cutaneous leishmaniasis in a border area at south-east of Iran: an epidemiological survey

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Abstract

Background & objectives: Cutaneous leishmaniasis (CL) has been recently emerged in new foci, posing a public health problem. Increasing cases of CL have been reported during recent years from a border area between Iran and Pakistan, a previously non-endemic area. The present study was designed for epidemiological and parasitological characterization of the disease for the first time in this area.

Methods: A total of 3100 individuals from the city of Mirjaveh and its four rural districts were randomly selected and surveyed from March 2005 to February 2006. Microscopic examination, *in vitro* culture, mouse inoculations and species-specific kDNA-PCR assay were carried out for *Leishmania* detection and species identification.

Results: CL was endemic in an important rural district of Mirjaveh, presenting active lesions and scars in 6.6 and 9.5%, respectively. The highest rates of both active lesions and scars were found in the age group of 10 years or under with significant differences ($p < 0.05$) comparing to the older age groups. No association between genders and the rate of leishmaniasis was observed ($p > 0.05$). The most affected location was upper limb, 39.2% of ulcers and 41.7% of scars. Inoculation of the clinical isolates on Balb/c mice, led to the development of ulcers in the animals, implying that the causative parasite is *Leishmania major*. The PCR amplification also generated amplicons specific to *L. major*.

Conclusion: It can be concluded that Mirjaveh is an endemic region of cutaneous leishmaniasis as a new focus due to the recent emergence in this border area of south-east of Iran with a major contribution of *L. major*, as the causative parasite species.

Key words Cutaneous leishmaniasis – epidemiology – Iran – *Leishmania major* – new focus

Introduction

Emergence or re-emergence of cutaneous leishmaniasis (CL) has recently occurred in many countries[1–4]. Both zoonotic and anthroponotic cutaneous leishmaniasis (ZCL and ACL) have been prevalent in a number of rural and urban areas of Iran. They have also emerged in new foci during recent decades[5,6]. Mirjaveh is located along the border areas between Iran and Pakistan in eastern part of Sistan va Baluchestan Province, south-east of Iran. It is also close to the border area between Iran and Afghanistan. This town is important, particularly with regards to the economical activities and exchange between people of both sides of the border.

Sistan va Baluchestan Province basically has not previously been known as an important endemic area for

leishmaniasis. Frequent reports of CL are only recorded from a restricted rural area of Chabahar port in the southern part of the province which is hundreds of kilometres far from the present study area, Mirjaveh. Both *Leishmania major* and *L. tropica* are also prevalent in parts of the neighbouring countries, namely Pakistan[7–10] and Afghanistan[11–15].

There was no report of CL outbreak or epidemic in Mirjaveh before 1996. An outbreak of CL occurred in a couple of villages of Mirjaveh for the first time in 1996[16]. Then, the number of reported cases rose annually from a few in the early years to more than 400 in 2005 (unpublished data, provincial local health services) having caused a major public health problem in this area. However, the disease was not epidemiologically characterised. We investigated a number of CL cases with focus further on their clinical features and phenotypic categorization that was almost synchronous with the present study and the obtained results were published earlier[17]. But the present study is a population based survey, designed to determine the epidemiological measures in all parts of the study area to be used in planning a more accurate control programme.

Material & Methods

Study area: Mirjaveh with more than 40,000 population and 313 villages across the border between Iran and Pakistan and few kilometers distant from Iran-Afghanistan border, is situated 70 km south-east of Zahedan, the capital of Sistan va Baluchestan province, south-eastern Iran. Different ethnic groups including Baluchi, Fars, Afghan and Pakistani are settled in this Iranian border area, and several inhabitants usually move across the border with concern to their jobs, business or visiting their relatives in the neighbouring countries, Afghanistan and Pakistan. Mirjaveh is at an altitude of 1373 m above the mean sea level, with annual precipitation of 15.5 mm and a relative humidity of 24%. The average annual temperature is 21.7ºC. The study was carried out during a period of 12 months, since March 2005 to February 2006.

Sampling: A total of 3100 individuals, representative of the whole population, were randomly selected using systematic clustering. The study area was geographically divided into five parts, including the city of Mirjaveh and four rural regions, Tamin, Joonabad, Ladiz and Mil-e-72 and the samples were clustered and allocated according to the population distribution. Questionnaires were completed by house-to-house visit and interview for collecting epidemiological information including sex, age, education, occupation and the presence of ulcers or scars during March to April 2005. A follow-up visit was made in November 2005 to January 2006 for recording new cases. There was an arrangement for reporting possible new cases with active lesions that could appear amongst the selected sample, during the intervals between the two visits. Clinical information that was recorded in a separate form for each case, included site, size and number of the ulcers or scars as well as the course of the disease. Possible travelling experience and date of travelling to other endemic areas inside or outside the country were also questioned. The patients having ulcers were referred to the local clinics or health centres, to take scrapings by an experienced laboratory technician for light microscopic examination and *in vitro* inoculation. Informed and free consent was obtained from the participants and the parents of children before inclusion to the study. All patients with confirmed leishmaniasis were treated in a local clinic, accordingly. Statistical analysis of the epidemiological data was performed using Chi-square, Fisher's exact test, or Linear trend exact tests in the software SPSS 10, accordingly.

Parasitology and laboratory methods: For microscopic examination, at least two Giemsa-stained slides for each patient were prepared with smears from scrapping of the edge of ulcers. At least, 100 microscopic fields with x 1000 magnification under a light microscope were observed for the detection of *Leishmania* amastigotes before considering the samples as negative. *In vitro* culture was conducted using NNN media (Novy-MacNeal-Nicolle) as described previously[17]. The NNN promastigote isolates

from 14 samples were subjected to bioassay for parasite identification. They were inoculated into the tails of 28 Balb/c mice and the animals were periodically monitored for developing lesions. Their positive leishmanial lesions were subsequently confirmed by both microscopic examination and the NNN subculture. The inoculated mice were sacrificed after the tests. The experimental mice were maintained and cared according to the guidelines for use of laboratory animals in research, provided by the Research Ethics Committee at Zahedan University of Medical Sciences.

DNA extraction and PCR assay were performed on either NNN promastigote isolates or direct specimens (a total of 32 out of 50 samples) as described earlier[17]. The amplification target is a part of kinetoplast DNA (kDNA), designed for identification and differentiation between *L. major* (620 bp) and *L. tropica* (800 bp)[18]; the required materials were provided in a kit (Cinna-Gen, Iran). The DNA size marker for analysis of the PCR results was GeneRuller 100 bp DNA ladder (Fermentas).

Results

Cutaneous leishmaniasis was found to be considerably endemic in an important rural area, Mil-e-72. No cases with active lesion were found in the other four studied districts. Only a single case of scar was found in a rural settler in Tamin. Mil-e-72 is the most populated rural area of Mirjaveh with more than 70 villages distributed along the border. Out of the 762 visited persons in this area, 50 cases (6.6%) found to have active lesions and a number of 72 individuals (9.5 %) showed scars. Few cases of active lesions were found during the first visit from March to April, whereas, majority of the cases were found in the second visit, November 2005 to January 2006.

The participants included 394 (51.7%) males with 5.84% active lesions and 10.4% scars, respectively and 368 females with 7.39% active lesions and 8.4% scars. No significant differences were observed in the prevalence of ulcers or scars between two different genders ($p > 0.05$). It was found that the cutaneous leishmaniasis in this area was significantly more prevalent in the age group of 10 years or under (Table 1). The same age group was also shown with higher scar rate.

The studied individuals in Mil-e-72 comprised of 495 Iranian and 267 non-Iranian inhabitants. The proportion of infected cases (having active lesions) was 7.9% in Iranian *vs* 4.1% in non-Iranian inhabitants with significant differences ($p < 0.05$). However, the difference in the rate of scars in these two groups (8.3% in Iranians and 11.6% in non-Iranians) was not statistically significant. None of the patients showed a history of travelling to other endemic areas at least one year prior to their contraction of the ulcer.

Distribution of the active lesions and scars in groups with different occupation are detailed in Table 2. The highest prevalence was seen in the pre-school children, followed by the student group. This may be relevant further to the ageing rather than occupation. Other occupation groups were not significantly different in the prevalence rate of leishmaniasis. The scar rate also appeared more prevalent in the pre-school group; however, it was not statistically different from that of other groups. The education level of the people investigated in this study was not much variable; most of them were uneducated (85% of adults and 48% of all individuals) and the remaining were also at low education level. However, the sta-

Table 1. The prevalence of active lesions and scars in different age groups of CL patients in Mirjaveh rural areas, south-east of Iran (2006)

Age group (years)	No. investigated	Lesions*		Scars*	
		No.	%	No.	%
≤ 10	294	29	9.9	33	11.2
11–20	205	10	4.9	19	9.3
21–30	115	5	4.4	9	7.9
>30	148	6	4.1	11	7.5
Total	762	50	6.6	72	9.5

*($p < 0.05$).

Table 2. The prevalence of cutaneous leishmaniasis in different groups in Mirjaveh rural areas, south-east of Iran (2006)

Groups	No. investigated	Lesions*		Scars**	
		No.	%	No.	%
Pre-school	196	24	12.2	26	13.3
Student	134	11	8.2	11	8.2
Housewife	215	7	3.3	22	10.2
Labour	77	2	2.6	9	11.7
Farmer	52	3	5.8	1	1.9
Others	81	2	2.5	3	3.7
Total	755	49	6.5	72	9.5

*($p < 0.05$); **($p > 0.05$).

tistical analysis showed no correlation between education and leishmaniasis prevalence.

A total of 107 active lesions were observed in 50 patients, with average number of 2.14 per person and maximum number of seven in one patient. The majority of the patients (58%) showed to have more than one ulcer. They demonstrated 2, 3, 4, and ≥5 ulcers in 28, 14, 10 and 6%, respectively. The remaining 42% presented only one ulcer. A total number of 96 scars were observed in 72 inhabitants with the average of 1.3 per person and maximum number of three scars in six persons. Majority of the persons with scar exhibited to have only one scar (76.7%); 15.1 and 8.2% presented two and three scars, respectively.

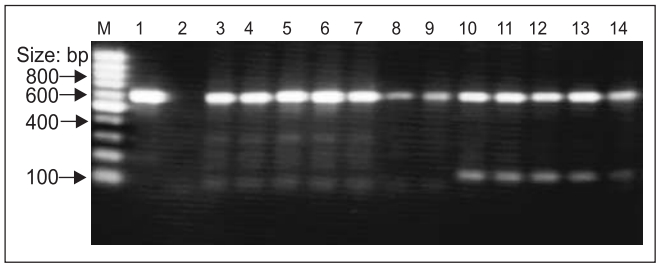

Fig. 1: Gel electrophoresis of the *Leishmania* species-specific PCR products (using kDNA marker) from the human isolates obtained from patients with cutaneous leishmaniasis in Mirjaveh, south-east of Iran. Lane M: 100 bp DNA ladder; Lane 1: *L. major* positive controls; Lane 2: negative control; and Lanes 3–14: amplicons of representative parasite isolates, IML51, IML52, IML53, IML54, IML55, IML56 IML57, IML58, IML59, IML60, IML61, IML12

Analysis of the ulcers distribution in the body showed 42 (39.2%) on hands as the most affected location, followed by face (29.9%), legs (24.3%) and on other parts of the body (6.6%). Similar distribution was observed for the scar locations, hands (41.7%), face (36.5%), legs (17.7%) and other parts (4.2%). Duration of the ulcers from onset until visiting time, based on interview statements, was less than one month in 53 (49.5 %), one to two months in 41 (38.3%) and more than two months in 13 (12.2%) ulcers.

Microscopic examination detected *Leishmania* amastigotes in 24 cases out of 50 (48%) and the NNN culture led to the growth of promastigotes in 32 samples (64%). Using the combination of both microscopic and culture techniques, we could detect parasites in most of the patients (82%). Mouse inoculation with the NNN-isolated promastigotes resulted in the occurrence and development of lesions in all inoculated animals and the microscopic examination of the animal lesions, all demonstrated the amastigote forms of the *Leishmania*. Further characterisation of the causative *Leishmania* species, using species-specific kDNA-PCR amplification on the isolates, resulted in the generation of a 620 bp DNA fragment in all amplicons, corresponding to that of *L. major* (Fig. 1).

Discussion

The results of the present study confirmed, for the first time, a new endemic focus of cutaneous leshmaniasis emerged recently in the border area of Sistan va Baluchestan, south-east of Iran and that the *L. major* is the causative agent of the disease in this area. A relatively high prevalence of CL (6.6%) was found in Mil-e-72 which is the most populated rural area in Mirjaveh with over 70 villages along the border. A scar rate of 9.5% was also found in this area. No case of CL disease was evident in the town of Mirjaveh and its other three rural districts. However, there may be a risk of potential transmission of CL from bordering villages to those non-endemic districts and needs consideration. Farming and agricul-

ture in the villages of Mil-e-72, is more traditional, compared to the other four districts which are distantly apart. Predominant colonization of rodents in this area was also observed during the visit. These probably provide a reservoir potential for the zoonotic CL type. We mentioned that the obtained results imply the presence of zoonotic leishmaniasis in this region, though further investigation is required for finding the particular possible reservoir.

According to the interview statements, the subjects had never left their residential villages for more than a year, indicating local transmission. This further confirms that the CL has emerged in Mirjaveh county and led to establishing a new endemic focus in this area. The trans-border movement of people in such areas provides the capacity, in which parasite can be introduced to non-endemic areas, leading to the emergence of new CL foci, where ecological conditions such as the presence of potential vectors and reservoir hosts, are in favour of the parasite life-cycle.

Comparison of the active lesion rate as well as the scar rate between males and females showed no significant differences; indicating that both genders were equally affected, which is in consistent with the results of studies reported elsewhere[2,5,19–22]. The age group with the highest prevalence of CL was ≤10 years. Other studies showed that most highly infected age groups with both ACL and ZCL are either ≤20[22,23] or ≤10[3,19,20,24–26]. One reason is that the immunity against *Leishmania* parasite in adults is more than that in children due to their previous exposure to the parasite, however, this is the case in older endemic areas. In our study area, as a new focus, one may expect that the lesions would be observed in all age groups without considerable differences. Higher rate of lesions in children in the study area is not clearly known, but their more outdoor activities may be one of the reasons, as proposed previously[3].

The highest rate of scars was also found in children with 10 years age or under and this rate was lower in the adults. This indicates further that the CL had not previously been endemic in the study area and has been recently emerged. In areas with a long history of CL endemicity, the scar rate in the older age groups is usually higher than that of the younger groups. Job association of the CL in this area is not remarkable. Despite the significant differences of lesion rate in Table 2, it does not indicate reasonable correlation between CL prevalence and occupation, because the most prevalent group, as is shown in Table 2, is children who are not involved with job and can not be considered as occupation group. This difference is actually associated with age rather than occupation. Further, there was no significant correlation between scar rate and occupation.

Similar to the findings of some other studies[5], the most affected part of the body was upper limb (39.2%) followed by the face (29.9%). Similarly, the upper limb was the most affected site by the scars. This is one of the usual characteristics of the ZCL; whereas, in the ACL, the most commonly involved site is face and the upper limb is the second most affected site[3,19,22]. However, this is not always consistent, and there are some reports indicating predominant involvement of the face in ZCL[6,23]. Single lesion was shown only in 42% of the patients; whereas, single scar was demonstrated in the majority of the persons with scars (76.7%). This can be due to the fact that some ulcers do not necessarily lead to the appearance of scars for several possible reasons, i.e. immune system interference or early healing of the ulcers, spontaneously or due to treatment. Duration of the disease until visiting time, in the majority of the patients (87.8%) was stated to be two months or less; only 12.2% had duration longer than two months. This is similar to the clinical duration of the acute form of CL that is usually short[1]. In the clinical analysis of active lesions in 116 cases, similar results were presented earlier[17]; (a few cases overlapped in two studies).

The prevalence of the infection was found to be higher in the second visit, predominantly between November to January. It was decreased thereafter and

disappeared by the end of winter. This seasonal endemicity in the study area is also in accordance with the ZCL characteristics as was observed in other ZCL foci[5]. The rate of scars between Iranian and non-Iranian settlers was statistically insignificant. However, the rate of active lesion in Iranian inhabitants was significantly more than that of non-Iranians (mainly Afghan). The reasons are not clearly known; possible annual movements of a number of non-Iranians, in particular seasons, to other areas that are probably non-endemic, may be one of the reasons.

Combination of two detection techniques, microscopy and NNN culture, resulted in diagnosis of 82% of the suspected cases. The negative samples may have belonged to the lesions that have received anti-leishmanial treatment or the old lesions with spontaneous healing progress. The occurrence and development of lesions in all inoculated mice with the NNN promastigote isolates, is in favour of identification of *L. major* in the evaluated samples. In addition, the species-specific kDNA-PCR confirmed *L. major* as a causative parasite species in this area.

We propose that the emergence of CL in this part of the country has been originated from other foci either inside or outside the country. ZCL, as well as the ACL are endemic in Pakistan[7–10]. In Afghanistan, it is mostly due to *L. tropica*[11–15]. Several ZCL and ACL endemic areas are known in Iran but they are far distant from the study area. To find the original source of the parasite, further studies such as genotype analysis of the *Leishmania* isolates in comparison with isolates from other CL endemic areas are required. The vector in the study area has never been investigated, but majority of sandflies, captured previously, were identified as *Phlebotomus papatasi*[16].

It is concluded that Mirjaveh rural district is an endemic region of the cutaneous leishmaniasis as a new focus in south-east of Iran, being recently emerged. Further, *L. major* is a causative parasite species in this area. Further studies are required to obtain more information about the vectors and to determine possible reservoir species in this region.

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