Clinical Features of Cutaneous Leishmaniasis and Direct PCR-Based Identification of Parasite Species in A New Focus in Southeast of Iran

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Abstract

Background: To clinically characterize the cutaneous leishmaniasis and identify the causative parasite species in Mirjaveh, an important geographical region across the border of Iran-Pakistan at Southeast of Iran.
Methods: A number of 116 patients during a year since March 2005 to April 2006, subjected to the study. Clinical information collected and scrapings were taken from cutaneous lesions and used for microscopic examination, NNN cultivation and kinetoplast DNA-PCR amplification.
Results: The cases comprised of 48 males and 68 females, 84 (72.4%) Iranians and 32 (27.6%) non-Iranians. They aged between 2 months to 68 years with the most affection of children, 0-10 years (55.2%). The patients presented a total of 248 active lesions with an average of 2.14. The ulcers distributed mostly on upper extremity (42.3%) then on face (32.7%), followed by lower extremity (20.6%) and other parts (4.4%). The majority of ulcers stated to be developed rapidly, <1 month (40.3%) or 1-2 months (45.2%). However, from 248 ulcers, only 19 (7.7%) found to be wet and the remaining were dry or moderately wet, 45 (18.1%) and 184 (74.2%), respectively. kDNA-PCR assay detected 51 out of 73 samples, all of which were identified as *L. major*, the causative agent of zoonotic cutaneous leishmaniasis.
Conclusion: *L. major* is the species responsible for cutaneous leishmaniasis in Mirjaveh, however the pattern of clinical findings, does not completely resemble the ZCL characteristics. These indicate that the manifestation of the lesions may not necessarily correspond to the *Leishmania* species and may be unreliable to conclude the speciation of parasite without laboratory identification.

Keywords: *Cutaneous leishmaniasis,* Leishmania major*, Iran*

Introduction

Cutaneous leishmaniasis (CL) is endemic in 88 countries including Iran (1) and has been emerged or re-emerged in a number of endemic countries in recent years (2-5). CL has also been increased in several areas of Iran during recent decade and there is evidence that this infection is emerging in new foci (6). Two *Leishmania* parasite species of the Old World localised cutaneous leishmaniasis, *L. tropica* and *L. major*, are endemic in many foci in Iran, located in several provinces.

CL has also been emerged during recent years in rural areas of Mirjaveh, Sistan va Baluchestan province at Southeast of Iran, situated in border areas between Iran and Pakistan. Following an epidemiological investigation, we have recently reported this area to the health authorities, as a new focus of CL (unpublished data). It is one of the important geographical regions with different points of views, in particular cultural and economical purposes as well as cross-border movements for different bilateral or multilateral purposes, i.e. trade and busi-

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ness, tourism, exchange visiting of neighbouring countries, Pakistan and Afghanistan.

The causative *Leishmania* species was unknown in this region and the local health authorities and physicians have only relied on the clinical observation to consider the disease as zoonotic or otherwise. Meanwhile, the clinical symptoms are variable and may be confused with other etiological agents (7). Microscopic examination of direct smears is the traditional method for the detection of parasite in clinical samples, but is not sensitive enough; besides, it can not differentiate between zoonotic and anthroponotic cutaneous leishmaniasis. To treat patients more effectively and organize a control program, basic information is needed and the infecting parasite species is required to be determined. PCR-based assays, utilizing different DNA targets, such as SSU rRNA (8), ITS1 (7, 9), repetitive sequences (10), Tubulin gene (11), gp63 gene (12), mini-exon (13-14), microsatellite DNA (15), have been widely used for detection or identification of the leishmania species. Most of these require a complement technique, like RFLP or sequencing. Kinetoplast DNA (kDNA) is another target with over 10,000 copies in *Leishmania* genome that attracted notices for developing markers to be used in clinical diagnosis and/or species identification (16-20). A marker of this target, based on species-specific nucleotide variations, has been developed and packed in a kit (Cinna-Gen, Iran). This marker that can detect and differentiate *L. major* and *L. tropica* by a single PCR amplification has been used in the present study.

We previously performed a community based epidemiological investigation that led to the initial understanding of a relatively high prevalence of CL (6.6% active lesions and 9.5% scars) in a rural area of Mirjaveh (unpublished data). However, the clinical characteristics of the disease and the causative *Leishmania* species in this area were not comprehensively understood. Although, mouse inoculation and PCR assay of a limited number of isolates in our previous study resulted in support of *L. major*

identification, a reasonable number of clinical isolates is required to be investigated. The present study proposed to clinically characterize cutaneous leishmaniasis and to identify the *Leishmania* species responsible for the disease in this newly endemic area by direct PCR.

Materials and Methods

Study area

Mirjaveh is situated 70 km Southeast of Zahedan, the capital of Sistan va Baluchestan Province, Southeast of Iran. It has more than 40,000 population and 313 villages across or near the border between Iran and Pakistan, also near to the border between Iran and Afghanistan. Mirjaveh altitude is 1373 meters above sea level with climatic condition of tropical or semi-tropical, annual precipitation of 15.5 mm, relative humidity of 24% and the average temperature of 21.7º C. The ethnic groups settled in this Iranian border area consisted of Baluch, Fars, Afghan and Pakistani.

Patients and samples

A number of 116 patients who were passively or actively referred to the health service centres of Mirjaveh during March 2005 to April 2006, subjected to the study. Informed consent obtained from patients before entering the study. They were recognised as having leishmaniasis, based on clinical features, microscopic detection of amastigotes in Giemsa-stained smears and/or PCR assay or NNN culture. Personal data and clinical information including site, size, duration and number of ulcers, were collected using questionnaires. Scrapings were taken from the border of cutaneous active lesions and each divided into three parts. One part placed in 200 μl of sterile PBS for direct PCR and another part was used for in vitro culture. Smears were also provided on glass slides for direct microscopy. The proven cases received meglumine antimoniate treatment that was provided by eligible physicians. Samples from 73 of the above patients were used for the PCR-based parasite identification.

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Culture

The specimens were inoculated in tubes containing Novy-Nicolle-MacNeal (NNN) medium, supplemented with 10% heat inactivated fetal bovine serum, added with phosphate buffer saline (as the liquid phase) and 200 IU/ml penicillin G (Roach, USA) and incubated at 25º C. The positive samples were sub-inoculated into either NNN or RPMI-1640 for further propagation and isolation of *Leishmania* promastigotes.

DNA extraction

DNA was extracted from both direct clinical specimens and NNN cultivated promastigotes. The clinical specimens were PBS washed three times and the pellets were kept frozen until use. From each culture tube, a number of 1×0^6 promastigotes were harvested and washed in PBS. Both specimen and promastigote pellets were re-suspended in lysis buffer including 50 mM Tric-HCl (pH 8.0), 50 mM NaCl, 50 mM EDTA, 1% sodium dodecyl sulphate) being added with 200 µl/ml Proteinase K (18) and incubated at 50°C for 3 h. The DNA was isolated using phenol-chloroform extraction method and precipitated in 2 volumes of cold absolute ethanol, then washed with 70% cold ethanol. The DNA pellet was air dried and re-suspended into TE buffer (pH, 8).

PCR amplification

The PCR assay was performed for parasite identification using a *Leishmania* species-specific detection kit (Cinna-Gen, Iran) according to the manufacturer instruction. The specific primer pair in the kit was previously designed (21). PCR reaction was provided for each sample, using 20 µl PCR mix (containing reagents and Leishmania kDNA primers), 1 unit (0.5 µl) Taq polymerase and 5 µl (containing 1 µg) DNA template in 0.2-ml microtubes, added each with a drop of mineral oil. A positive control reaction using standard *L. major* DNA and a negative control using distilled water were established in every round of PCR. Amplification of the target DNA was performed using Thermocycler Biometra (France) under the following conditions: one cycle of 3 min initial denatura-

tion at 95° C, followed by 35 cycles of 93° C for 40 s, 63° C for 30 s, 72° C for 60 s, ending by one cycle of final extension at 72° C for 5 min. Electrophoresis of the PCR products was performed using 10 µl of each amplicon, on 2% agarose gel in $0.5 \times$ TAE buffer for 80 min at 100 mA, followed by staining of the gels in 1 mg Ethidium bromide in 100 ml TAE buffer for 20 min and consequent observation of amplified DNAs by Gel grab under UV light. 1kb DNA Ladder was also used as the DNA size marker. Single fragments of 620 bp and 800 bp are indicative of the species *L. major* and *L. tropica*, respectively. Statistical data analysis was performed using Chi-square tests in the software SPSS 10.

Results

The 116 patients included 48 males and 68 females, consisting of 84 (72.4%) Iranian nationals and 32 (27.6%) non-Iranian settlers, mostly Afghan. One hundred eight patients stated to have not travelled in other endemic areas at least one year before the appearance of their ulcers. Only 8 patients (4 Iranians, 4 Afghans) had travelled to Pakistan few months before their visit. A total of 248 active lesions with an average of 2.14 per person were observed; it was 1.85 in males and 2.34 in females. A considerable number of patients (44%) showed to have only one ulcer, but the others demonstrated 2, 3, 4, and ≥5 ulcers with the percentages of 28.4%, 13%, 8.6% and 6%, respectively. A maximum number of 7 ulcers observed in three patients.

The patients aged between 2 months to 68 yr. The most proportion of the cases (37.1%) grouped with the age of 0-5 yr (Table 1). Only 17.2% of the patients were older than 20 yr.

The site distribution of the lesions in total cases, showed the most affection of upper extremity (105 lesions, 42.3%), followed by face (81 lesions, 32.7%), (Table 2). However, in female, face and upper extremity were equally affected (38.4%). In male, this was significantly differ-

ent; 49.4% of ulcers appeared on upper extremity and only 22.5% observed on face. Chi-square test showed that the face in male was significantly less affected, comparing with that in female.

The size of active lesions ranged between ≤5mm to 45mm in diameter (Table 3). The maximum percentage of the ulcers (33.9%) was observed in the range of 11-20 mm; only 6 ulcers (4.4%) observed to have >40 mm sizes.

Duration of the disease from onset to the visiting time, according to the patients' statements, was relatively short regarding most of the lesions. It was less than a month in 100 ulcers (40.3 %), one to two months in 112 (45.2%) and more than two months in 36 (14.2%) ulcers. From 248 ulcers, only 19 (7.7%) found to be highly exudative and the remaining showed to be dry (45, 18.1%) or moderately wet (184, 74.2%). The leishmanial ulcers occurred mostly during November to February with a maximum number of 42 on January (Fig. 1). The PCR amplification of samples from 73 patients detected parasite DNA in 50 samples (68.5%). It generated species-specific DNA fragment of 620 bp (Fig. 2), corresponding to *L. major* in all samples. None of the amplicons showed DNA band corresponding to *L. tropica* species. All isolates in the study area, therefore, identified to be *L. major*, the causative agent of zoonotinc CL.

Table 1: Age and sex distribution of cutaneous leishmaniasis in 116 patients in Mirjaveh, Southeast of Iran

| Age group(yr)	Male (%)	Female (%)	Total (%)
0-5	14 (29.2)	29 (42.6)	43 (37.1)
6-10	12 (25.0)	9 (13.2)	21 (18.1)
11-15	6 (12.5)	5 (7.4)	11 (9.5)
16-20	9 (18.7)	12 (17.6)	21 (18.1)
> 20	7 (14.6)	13 (19.1)	20 (17.2)
Total	48 (100)	68 (100)	116 (100)

Table 2: Distribution of ulcers in the body of patients with cutaneous leishmaniasis in rural areas of Mirjaveh, Southeast of Iran

| Site	Male		Female		Male+Female	
	n	%	n	%	n	%
Face	20	22.5*	61	38.4*	81	32.7
Upper limbs	44	49.4*	61	38.4	105	42.34
Lower limbs	22	24.7	29	18.24	51	20.56
Neck, shoulder & abdomen	3	3.4	8	5	11	4.4
Total	89	100	159	100	248	100

*P< 0.05

Table 3: The size of ulcers in the cases of leishmaniasis in Mirjaveh, Southeast of Iran

Diameter (mm)	No.	%
≤ 5	58	23.4
6-10	67	27
11-20	84	33.9
21-30	29	11.7
31-40	4	1.6
> 40	6	4.4
Total	248	100
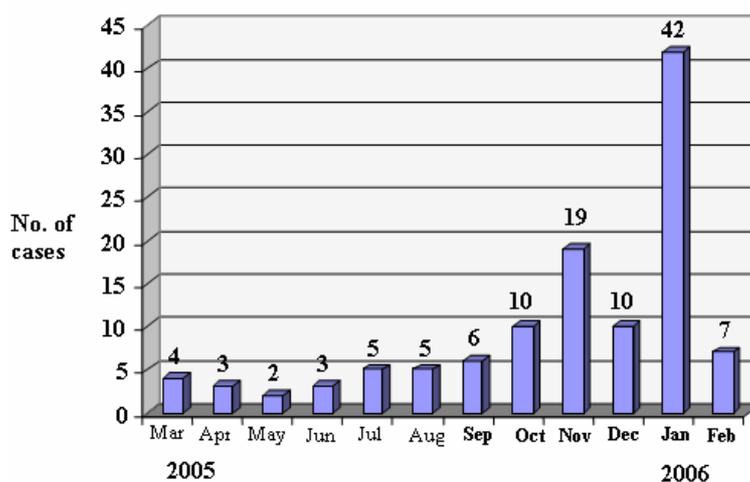
Fig. 1: Seasonal distribution of cutaneous leismaniasis in Mirjaveh, Southeast of Iran, March 2005 - February 2006

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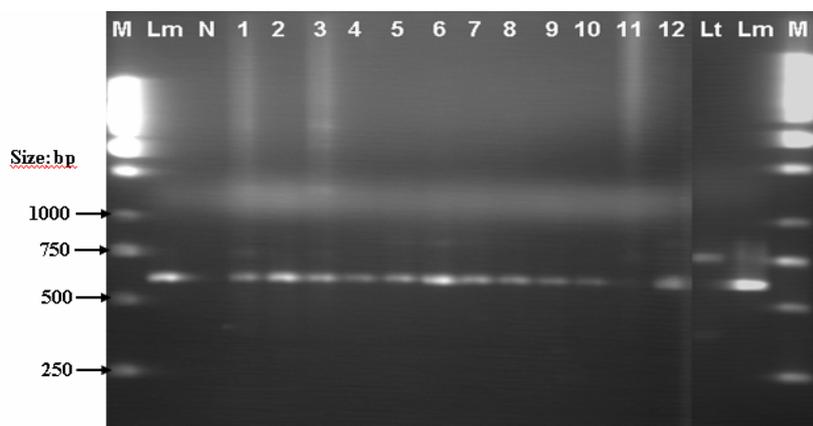
Fig. 2: Gel electrophoresis of the *Leishmania* PCR products from clinical isolates of patients from Mirjaveh, Southeast of Iran, using species specific kDNA marker in Cinna-Gen kit. Lanes M: 1kb DNA size marker. Lanes Lm: *L. major* positive controls. Lane N: negative control. Lane Lt: *L. tropica* positive control. Lanes 1-12: clinical isolates, labelled as 20IM, 21IM, 22IM, 23IM, 24IM, 25IM, 26IM, 27IM, 28IM, 29IM, 30IM, 31IM. They are representative of the PCR results of 50 isolates that were successfully amplified and all showed DNA pattern identical to that of *L. major*

Discussion

In our previous study (unpublished data), mouse inoculation of 16 isolates resulted in the development of leishmanial lesions, implying the presence of zoonotic CL. The patients included 84(72.4%) Iranian and 32(27.6%) non-Iranian settlers. Only 8 cases (4 Iranians and 4 Afghans) had travelled to Pakistan during few months before the investigation. The others stated to have not been in other endemic areas inside or outside the country at least one year before contraction of the disease, reflecting the local transmission.

Only 41.4% of the cases were male versus 58.6% females. This probably can be due to the fear of cosmetic disfiguration in female cases more than males, that urges them to refer for a clinic visit and treatment. The oldest patient had 68 yr of age, however, the majority of the cases (82.8%) aged between 2 months to 20 yr. This is similar to many other studies in Iran (22-25) and some other countries (5, 26). Although, in areas with longer history of leishmaniasis endemicity, one reason can be higher sensitivity of children versus adults due to acquiring immunity following past parasite exposure, in the study area that is a new endemic focus, this can not be the case. One possible reason, instead, can be outdoor activity of children and teen-agers and more exposure to the sand fly bites. In ZCL cases, affection of upper extremity is usually common. However, there are some reports of predominant involvement of face in this type (22, 27) like the ACL lesions that are known to be presented mostly on face and then on upper extremity (3, 5, 23). In our study, the site distribution of the lesions in total cases, were similar to the typical ZCL, consistent with some other studies (6), with the most affection of upper extremity (42.3%), followed by face (32.7%). However, in female, face and upper extremity were equally affected (38.4% each). In male, this was significantly different; 49.4% of ulcers appeared on upper extremity and only 22.5% observed on face. The reason of this difference between male and female is not clearly known. One may propose that bearded face in male may avoid the sand fly biting, but this seems not to be the case here, as the majority of the patients were in pre-school or primary school ages.

L. major usually develops rapid necrosis, leading to open lesion that is large and wet (2) while in the ACL caused by *L. tropica*, ulcers are small and dry, although up to 200 mm lesion has been reported (5). In the present study, most of the patients presented dry (18.1%) or moderately wet lesions (74.2%) with typically small size, mostly less than 20 mm in diameter

(Table 3), that is different from that of ZCL type. It seems that the size of active lesions may be inaccurate categorizing criterion, as it usually depends on the course and developmental stages of the lesions. The appearance of multiple lesions is also reported to be more frequent with *L. major* infection (2, 22), though single lesion in some endemic areas has been predominant (27). We observed the majority of the patients having either single lesion (51 cases, 44%) or double lesions (33 cases, 28.4%); only 27.6% showed multiple lesions. Duration of the ulcers from onset to the visiting time, as stated by the patients, was relatively short (85.5% less than 2 months). This corresponds to the duration of lesions created by *L. major*, which is usually short (2) in contrast with those of *L. tropica* that is known to be 6 months to 1 yr.

The above characteristics showed that the CL in the study area is partially similar to the typical ZCL which is caused by *L. major*; however, it is similar to the ACL in some characteristics. Uncommon clinical presentations have been reported elsewhere (28). This implies that the clinical and epidemiological features are not completely inclusive and should not be addressed merely to a particular CL type, i.e. ZCL or ACL. Classification of the disease based on the clinical presentations and theoretical corresponding to the *Leishmania* species, has been made in several studies (4, 29). The pattern of clinical findings in the present study does not completely resemble the described characteristics of ZCL. These indicate that the manifestation of the lesions may not necessarily correspond to the *Leishmania* species and the clinical features alone may be unreliable to be concluded for the speciation of the parasite without laboratory identification. Clinical variation of infections with the same parasite species may be attributed to possible genetic variation of the *Leishmania* parasite populations that attracts molecular investigations. Occurrence of the leishmanial ulcers increased in fall and winter. This is of characteristics of rural cutaneous leishmaniasis due to involved factors such as seasonal activity of its parasite vectors (30) and the prepatent period of infection with *L. major*. *Leihmania* minicircle Kinetoplast DNA is one of the genetic targets that has been applied and proved to be useful for detection of parasites in clinical specimens and isolate characterisation by many researchers (16-20). Further studies found that kDNA is more sensitive than other DNA markers such as internal transcribed spacer 1 (ITS1), glucose-6-phosphate (G6P), mini-exon, gp63, for detection and/or identification of both Old and New World CL (31-33). Here, we used a kDNA-PCR approach, using a single PCR amplification that can generate single bands of 620 bp and 800 bp specific to *L. major* and *L. tropica*, respectively. This species-specific PCR resulted in amplification of DNA fragment, corresponding to a 620 bp amplicon in all reactions, belonging to *L. major*. None of the samples showed to be infected with *L. tropica*. This indicates that the CL in the study area is most probably zoonotic type and is in agreement with several areas of Iran and Pakistan with zoonotic type of CL, recommended to be considered in control programs. The animal reservoirs and sand fly species remain to be investigated for further understanding of the parasite life cycle in this newly endemic area.

Acknowledgements

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