

Identification of Species and Characteristics of an Outbreak of Cutaneous Leishmaniasis in a New Focus of Iran

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ABSTRACT

Both urban and rural cutaneous leishmaniasis (CL) is endemic in different parts of Iran and has long been recognized in most provinces. However, there is no report of endemicity of CL in rural areas of Kashan, 200 km north of Isfahan and 260 km south of Tehran, Iran. To our knowledge, this is the first report of outbreak of cutaneous leishmaniasis in this area. To study and identify the nature of infection in this area, a survey was conducted in a borough, 30 Km south-east of Kashan on 3,530 people, using questionnaire for obtaining clinical status, and leishmanin skin test as an indicator of the prevalence of infection. In addition, 42 lesion samples from patients residing in this area were cultured, which twenty eight of them were positive. By enzyme immunoassay, using three species-specific monoclonal antibodies against common species of *Leishmania* in Iran, species of isolates were identified. Some of these isolates were also analyzed by PCR. The results showed the prevalence of 6% patients with lesion(s) in the population and 3.1% of people displayed lesion scar(s). Also 12% of population showed sensitivity to leishmanin. In this study the *Leishmania major* strain was identified in majority of cases, which showed two different patterns when analyzed by PCR. Both patterns belonged to different endemic strains of *L. Major* in Isfahan, which indicates the possible transmission of infection from Isfahan to this area. *Iran. Biomed. J.* 3(1 & 2): 31-39, 1999

Keywords: Identification of species, New Outbreak, Cutaneous leishmaniasis, *Leishmania major*

INTRODUCTION

Leishmaniases are intracellular protozoan infections with a broad spectrum of clinical manifestations ranging from a self-cured cutaneous lesion (oriental sore) to fatal disseminated and visceral disease (kala-azar). The outcome of disease in leishmaniasis depends on both host cellular immune responses and species of organism [1]. Cutaneous leishmaniasis (CL) also displays different clinical forms in various regions of the world, reflecting the different species of parasite and the genetically determined immune responses of the patients. Three species involve in CL of the Old World: *Leishmania tropica*, *L. major* and *L. aethiopica* [2], each of them are responsible for different forms of diseases. CL is endemic in many parts of Mediterranean region [3, 4], some countries

of Africa [5, 6], and almost all countries of Middle East and west of India [2].

In CL, there is a strong correlation between clinical forms of disease and cell-mediated immune (CMI) responses. After recovery from infection a long term protection develops against re-infection, which is contributed to expansion of type 1 subset of T-cells [7]. Leishmanin skin test (LST), the only *in vivo* test of CMI, is positive during active phase of disease and remains reactive for life-long [8] in almost 100% of recovered cases [9, 10]. Therefore, LST is an important indicator for evaluation of the exposure to leishmanial infection, especially in epidemiological studies of leishmaniasis [11].

CL caused by *L. tropica* and *L. major* is an important endemic disease in different parts of Iran [12]. The prevalence of infection is high in some provinces, including Isfahan [13], Fars [14],

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Khorasan [15], Khozestan, Boushehr, Hormozgan and Kerman. Isfahan is a well-known endemic area of Zoonotic cutaneous leishmaniasis (ZCL). In north-east of Isfahan, the incidence of disease is high, especially in suburb and rural areas [13]. In spite of some reports about increased rate of CL in north area of Isfahan province, however, there have been no previous reports of endemicity of ZCL around Kashan, 230 km north of Isfahan. In 1994, visiting students to the area reported an outbreak of CL. Inquiry from local health authorities and people and a preliminary survey using leishmanin skin test, showed that probably during the summer of 1994 and 1995, an outbreak of leishmaniasis has occurred in the southern part of Kashan. Thus, this study was undertaken to define two important points: a) to determine the prevalence of cutaneous leishmaniasis, by clinical finding and LST test in the area. b) to identify the species of *Leishmania* that has caused the infection using different immunological and molecular methods.

MATERIALS AND METHODS

Study area and population. The study area was the borough of Abozeidabad, 30 km south-east of Kashan, close to the desert. Kashan is an ancient city located 230 kilometer north of Isfahan, and 230 kilometer south of Tehran. The study site covers about 6 km, is divided into a central village and 4 sub-villages. From residents of this area, 3,530 volunteers (55.4% female and 44.6% male) were interviewed and skin tested in January-February 1996, in the local health center of Abozeidabad. Almost 3,099 individuals, 1,733 females (56%) and 1,366 males (44%), returned to the center for reading the test results. Majority of population (56.8 %) were under 20 years old, and 37.0 % of them were between 11 to 19 years old. The follow up of disease, healing and sampling continued for two years. The adult volunteers gave their consent to the participation in a written form, and for the children the consent was given by their legal guardians.

Skin test antigen and method. The reference leishmanin of TDR/WHO [16] was used throughout the study. This reagent is routinely produced in our lab according to the procedure described elsewhere [17]. DTH reactions to leishmanin were measured according to the method described previously [17] using ball-point technique for reading the results [18]. Indurations equal or more than 5 mm were

considered as positive.

Sample collection, culture and storage of parasite suspensions. Forty-two patients with skin lesions suspected of cutaneous leishmaniasis from 5 villages at the area of Abozeidabad were selected for study. After obtaining a complete clinical history, the samples were aspirated from the edge of skin lesion, using disposable punches. A direct smear for microscopical examination was made, and the samples were also cultured into liquid phase of Novy-MacNeal-Nicole-(NNN) medium. After growth, the parasites were seeded in medium 199 or RPMI 1640 (both from Sigma), supplemented with 2 mM L-glutamine and 15% fetal bovine serum (Gibco), and 200 U/ml penicillin and 200 µg/ml streptomycin. When sufficient number of parasites (at the end of logarithmic or at the beginning of stationary growth phase) was obtained, the promastigotes were harvested, and washed three times with PBS, pH 7.2. The pellet, was suspended into cryoprotectant medium (RPMI plus 45% FBS and 8% glycerol), and aliquots were frozen gradually at -20°C, 70°C and subsequently in liquid nitrogen.

Inoculation of BALB/c mice. The parasites from 24 isolates were harvested at the stationary phase of growth, and washed once with PBS, pH 7.2. Then, 2×10^6 live parasites from each sample were injected subcutaneously into the base tail of three BALB/c mice. The mice were examined weekly for appearance of lesion in the injection site. After ulceration, they were sacrificed and their spleens were removed and screened for disseminated parasites by direct examination and culture.

Identification of *Leishmania* species by immunoassay (Preparation of antigens). The frozen parasites were washed three times with PBS, pH 7.2, and adjusted to 5×10^7 parasites per ml. Wells of 96-well flat-bottom microtiter plates were coated with 50 µl of parasite's suspension and incubated overnight at 4° C. The standard strains of *L. major* (MHOM/SU/73/5ASKH), *L. tropica* (MHOM /SU /74/k27), and *L. donovani infantum* (MHOM/TN/ 80/IPT1) were used as controls. These strains were obtained from Dept. Medical Parasitology, London School of Hygiene and Tropical Medicine.

ELISA. Identification of *Leishmania* species was carried out by standard method of indirect ELISA

[19], using peroxidase-conjugated anti-mouse IgG, and substrate of *o*-phenylenediamine-2HCl in phosphate-citrate buffer. Three species-specific monoclonal antibodies (Mabs) against three dominant species of *Leishmania* in Iran were used for identification of isolates: anti-*L. major* (XLVI-5B8-B3 (T-1)), anti-*L. tropica* (IS2-2B4-A11), and anti-*L. Donovanii/L. infantum* (LXXVIII-2E5-A8 (D-2)). These Mabs were kindly provided by Dr. F. Modabber (TDR, WHO, Geneva, Switzerland), and was a gift from Dr. D. McMahon-Pratt (Department of Epidemiology and Public Health, School of Medicine, Yale University, New Haven, USA). In addition, a monoclonal antibody against *L. major*, Iran vaccine strain (MRHO/IR/75/ER), generated in our laboratory and used in identification of the isolates.

Identification of isolates by PCR. Cultured parasites were centrifuged at 8,500 rpm for 3 min. and washed 2× by NET100 buffer (0.1M EDTA, 0.1 M NaCl, 0.01 M Tris base, pH 8.0), followed by addition of 0.7 ml and 0.3 ml of 10% Sarcosyl (Sigma) for 10⁹ parasites. The mixture was then incubated at 60° C for 1 hr., and used for kinetoplast DNA (kDNA) extraction.

Extraction and amplification of kDNA. Proteinase K, 100 µg/ml, (Sigma) was added to tubes containing the parasite's suspension, followed by incubation at 56° C for 30 min. Tubes were filled with distilled water (DW) and centrifuged at 16,000 × g for 1 hr. After discarding the supernatant, the pellet was suspended in 200 µl of DW and kDNA was extracted using 200 µl Tris saturated phenol (Merck) at pH 8.0. An additional extraction was carried out by phenol/chloroform/isoamyl alcohol (Merck). The suspension was transferred to a 1.5 ml tube, filled with DW, centrifuged at 16,000 × g for 1 hr and the pellet was resuspended in 100 µl of DW [20, 21]. kDNA was amplified using a pair of primers 5'-TCGCAGAACGCCCTACC3' and 5'-AGGGG TTGGTGTAATAAGGC3' which were synthesized by Applied Biosystems 391 DNA synthesizer PCR-Mate (kindly provided by Dr. Barker) [21]. The PCR reaction mixture (20 µl) contained 1 × PCR buffer (1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl pH 8.3), 0.2 mM of dNTP (deoxynucleoside triphosphates, Pharmacia), 1 mM spermidine (Sigma), 0.5 unit of *Taq* DNA polymerase (BRL) and 15 pmol of each primer.

Primary denaturing was performed at 93°C for 4 min., followed by 35 cycles of denaturing at 93°C for 30 seconds, annealing at 64°C for 1 min., extension at 72°C for 1.5 min. The last cycle was carried out at annealing temperature of 64°C for 1 min., and an extension at 72°C for 5 min. Two reference strains of *L. major* (P-strain), and *L. tropica* were used as control. The PCR products were analyzed on a 2% agarose gel.

Statistical analysis. Collected information of the survey was analyzed by statistical packages Epi-Info. version 5.

RESULTS

Detection of parasite. Direct examination of smears stained by Giemsa stain showed the presence of *Leishmania* amastigotes inside the macrophages. Likewise, 28 from 42 isolates showed fast growth and proliferation of flagellated parasites in the cultures.

Clinical findings in volunteers. Data obtained from questionnaires and physical examination of population showed a prevalence of ulcerative localized CL (LCL) of 6 % (212 cases from 3530 individuals), and cases with scar of 3.1 % (111 cases), from which 0.3 % with history of leishmanization, in February 1996. Single lesion was seen in 55.2 % of patients, appearing as a round popular plaque with a diameter of 5-60 mm. Double lesions were observed in 24.1% of patients and 20.7% of patients showed multiple (3-10) lesions. Likewise, 45.41 % of those tested showed lesions on their hands, 24.86 % on their feet and 15.68 % above the neck. Detailed distribution of lesion and scar according to the number of lesion (s) and scar (s) were shown in Table 1. In addition, grading of lesion percentage in different age groups was demonstrated in Figure 1.

DTH responsiveness. A positive reaction to leishmanin skin test (LST) was observed in 12.04 % (373 individuals, 170 females, and 203 males) of volunteers. 59.8 % of LST positive cases showed symptoms including lesion (s) (37.8 %) and scar(s) (22.0 %); and 40.2% of LST positive cases (150 individuals) were asymptomatic. The prevalence of LST positivity according to the sex was studied by

Table 1. Distribution and localization of lesion (s) and scar (s) in patients with cutaneous lesion and cases with scar, respectively.

Characters	% cases with lesion	% cases with scar
Single form	55.2	82.9
Double form	24.1	9.0
Multiple form	20.7	8.1

Localization		
Hand	45.4	36.9
Foot	24.9	26.2
Above the neck	15.7	25.0
Hand + other parts	10.8	6.0
Foot + other parts	3.20	6.0

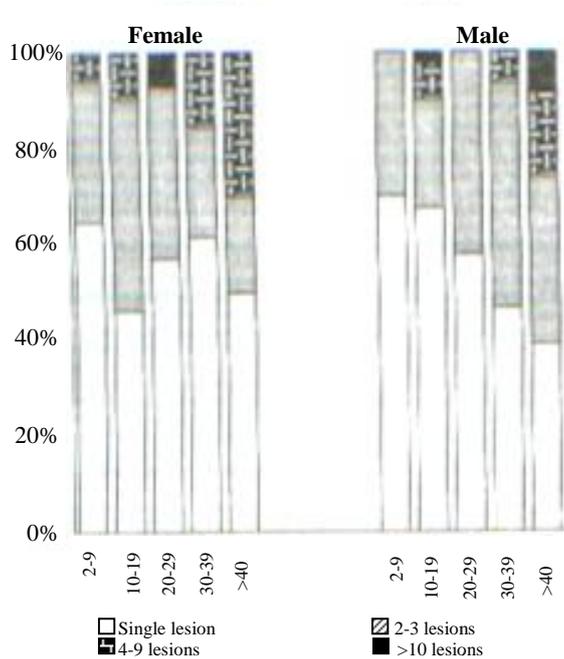


Fig. 1. Grading of lesion number by sex and age groups in patients with lesion (s), in Abozeidabad, 1995.

Z test and the results showed that the relative frequency of LST positive cases in the population under study were significantly ($p < 0.05$ and $Z = 4.16$) higher in males than females. Moreover, the comparison of the relative frequency of LST positive cases in different age groups and sex showed with 95% confidence interval ($p \pm 2SE$) significantly higher frequency of LST positivity in age group of 30-39 years than others, and higher percentage of LST positivity in males than females (Figure 2-A).

The size of induration in LST positive cases increased with age up to 40 years, and showed larger size in age group of 30-39, and the males had larger responses than females as shown in Figure 2B. Likewise, the mean diameter of induration was 11.40 ± 5.11 mm in cases above 20 years, and $9.39 \pm$

4.12 mm in cases below 20 years. Moreover, grading of induration in LST positive cases demonstrated the larger sizes in age group 30-39 in females and both age groups of 20-29 and 30-39 in males (Figure 3).

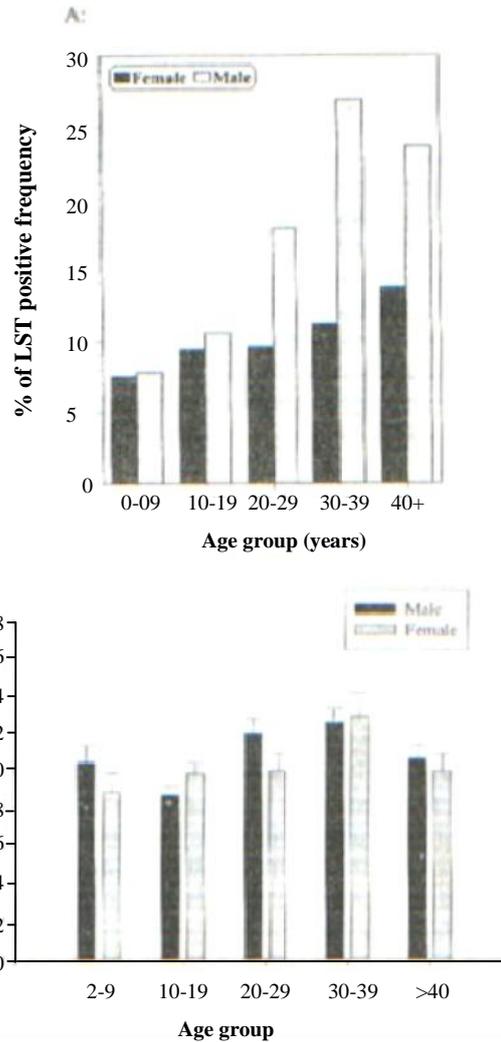


Fig. 2. (A) Relative frequency of leishmanin skin test positive cases by age groups and sex, in Abozeidabad, 1995. (B) Mean diameter of induration in LST positive cases by sex and age groups. Results are the mean \pm SME.

Susceptibility of BALB/c mice to parasite isolates.

24 groups of three BALB/c mice were inoculated with stationary growth phase of 24 isolates. After incubation time, 20 groups of mice showed lesions in the sites of inoculation. The weekly physical examination of mice demonstrated the progression of lesion size, which gradually disseminated to adjacent organs. Finally, direct examination and culture of spleen cells showed the presence of promastigotes in visceral organs of all 20 groups of mice.

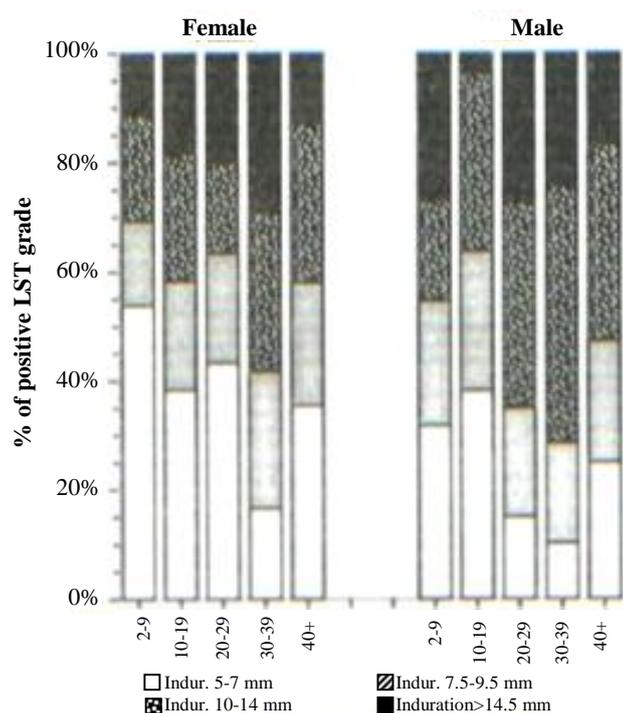


Fig. 3. Grading of induration in LST positive cases in different age groups of both sexes in population studied in Aboozaidabad, 1995

Table 2. Reactivity of different species-specific mabs with promastigotes of isolates from CL cases and standard strains.

Standard strains and No. of isolate	Ratio: OD-Bound Antibody/OD-Bound Control*		
	Anti- <i>L. major</i> Mab	Anti- <i>L. Tropica</i> Mab	Anti- <i>L.d.infantum</i> Mab
<i>L. major</i>	4.49	2.03	1.18
<i>L. tropica</i>	1.10	5.16	0.32
<i>L.d. infantum</i>	2.00	3.80	4.46
Sample 1	4.39	0.85	-
Sample 2	3.63	0.61	-
Sample 3	4.06	0.61	-
Sample 4	4.24	0.93	-

*An ELISA assay was used to determine the binding of the Mabs with promastigotes of isolates. Reactivity is presented as the ratio of OD of bound antibody / OD of bound control.

Identification of *Leishmania* species by Mabs.

Forty-two samples from infected cases were cultured on NNN medium, from which twenty-eight isolates showed sufficient growth in the cultures. Majority of isolates with positive culture had been aspirated from patients who had lesion (s) in their hand (s). Parasite suspensions prepared from the isolates were used for identification of *Leishmania* species using an indirect ELISA. The relative reactivities of isolates promastigotes with monoclonal antibodies are expressed as a ratio of bound antibody to bound control. The reactivities of three monoclonal

antibodies against 28 samples and the WHO reference strains are shown in Table 2 and 3. As demonstrated in the tables, monoclonal antibodies against *L. major* showed strong reactivities with majority of isolates. No obvious binding (ratio more than 2) was seen to monoclonal antibodies against *L. tropica*, and *L. d. infantum*. On the basis of the results, the species of isolates were identified as *L. major*.

Table 3. Reactivity of different species-specific mabs with promastigotes of isolates from different cutaneous leishmania patients.

No. of samples	Ratio: OD-bound anti- <i>L. major</i> /OD-bound control*	No. of samples	Ratio: OD-bound anti- <i>L.major</i> /OD-bound control*
5	8.00	17	9.00
6	5.33	18	6.43
7	6.56	19	3.03
8	7.00	20	3.94
9	2.20	21	9.05
10	3.11	22	3.56
11	3.33	23	5.53
12	3.03	24	2.95
13	2.87	25	3.26
14	2.10	26	2.66
15	8.20	27	2.80
16	2.90	28	2.10

*An ELISA assay was used to determine the binding of the anti-*L. major* Mab with promastigotes of isolates. Reactivity is expressed as the ratio of OD of bound antibody/OD of bound control.

Amplified kDNA. Eight isolates from patients in Kashan were analyzed by PCR and the results showed the presence of two different patterns of *L. major* strains. As shown in Figure 4-A, isolates No. 3, 5, 6, and 7 displayed one fragment of 620 bp, which was similar to WHO standard strain (P-strain). However, isolates 1, 2, 4 and 8 showed two fragments of 620 and 830 bp, similar to the results obtained for standard Isfahan strain (MRHO/IR/64/Nadim-1) as demonstrated in Figure 4-B.

DISCUSSION

The presence of intra-macrophage amastigotes in direct examination and the growth of promastigotes in the isolate cultures, initially indicated that the cutaneous lesions of new outbreak in south-east of Kashan are due to leishmaniasis. On the other hand, increased sensitivity to leishmanin skin test was demonstrated in patients with lesion (s) and cases with scar (s) residing in the area. The prevalence rate of infection was determined by LST in almost 70% of resident population. Considering that, the

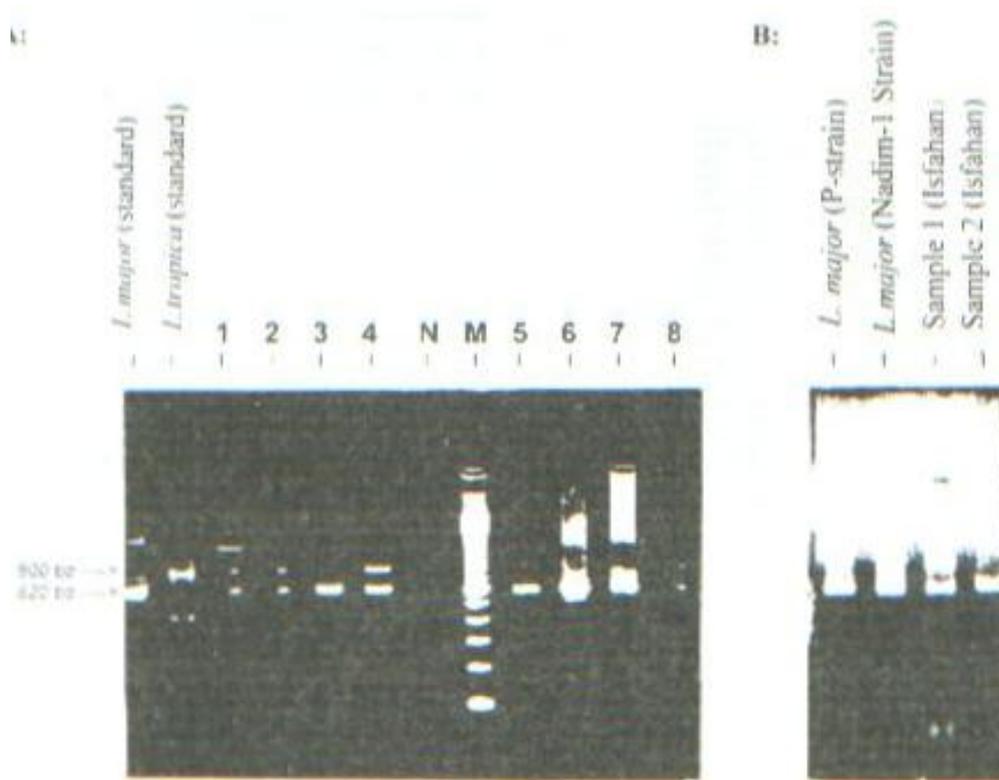


Fig. 4. Products obtained on amplification of kDNA from cultured isolates of patients with cutaneous leishmaniasis. (A) The pattern of eight isolates indicates that the species of case 1, 2, 4 and 8 are double fragments *L. major* (Nadim-1 strain), and the species of case 3, 5, 6 and 7 are single band *L. major* (P-strain). (B) Standard strains of *L. major* and two samples from Isfahan isolates with single and double bands.

prevalence rate of 12 % reported in this study seems to be rather high, and could reflect the occurrence of an almost severe outbreak of CL in the area. In clinical study, 6 % of people under survey showed single or multiple ulcers and 3.1 % of them showed scar(s) in their hands, feet, face and other parts of the body. The frequency of lesion was almost twice as high as scars, indicating the ascending progression of outbreak during past two years. Majority of ulcers or scars was seen on hands and upper parts of the body, which may be due to sleeping out of covered area on the terraces or house yards without using bed-nets during the summer. Duration of active lesion was restricted to less than one year in most cases. While healing occurred in about 98% of patients within months, in a few cases (about 2-4%), the cured individuals experienced a new secondary lesion(s).

Skin testing with leishmanin showed a markedly higher prevalence of positive skin test in males than females. It seems that females were less exposed to sand fly bites than males, because, they use bed-net and spend most of their times at home and protect themselves by clothing more than males according

to their religious and social habits. Moreover, the degree of positivity was correlated with increasing age up to 40 years (Figure 2-A). The increase in frequency of LST positivity with age, and increase of lesion number with age in males (Figure 1) may reflect the permanent working of adults, especially male adults in field and house with bare hands or feet. The LST positivity reached to a plateau with low frequency in both genders at the ages below 20 years, suggesting that the prevalence of LST positivity in young individuals or students are lower than other people. The probable reasons for this situation may be due to using more hygienic and protecting measures, and their training by school and health authorities. Likewise, the size of induration in LST positive individuals increased with age up to 40 years as shown in Figure 2-B and 3, suggesting the multiple exposures of adults to clinical or subclinical challenge with parasites via possible mechanisms mentioned above. However, about 4.8 % of healthy individuals without previous history of CL showed positive reaction to LST. It seems that reactions in asymptomatic individuals have been probably induced by subclinical infection

of CL without onset of apparent lesion, as mentioned also in other studies [22, 3].

To characterize the species of *Leishmania* involved in this outbreak, three major procedures were performed. Firstly, the susceptible mice to *L. major*, were inoculated with virulent promastigotes. This procedure was used to identify and prove the involvement of *L. major* infection in the ulcerative cases. The results obtained from this experiment showed the dissemination and visceralization of the infection in majority of mice. Secondly, identification of species was carried out by ELISA, using species-specific monoclonal antibodies. This method is a well known and useful method for precise identification of species and subspecies of New and Old World *Leishmania* genus [23-25]. There is evidence that, the results of ELISA parallel those obtained from isoenzyme electrophoresis and kinetoplast DNA analysis [26, 27]. Moreover, several studies have shown that by use of species-specific monoclonal antibodies that react only with *L. tropica* [28] or recognize a determinant specific for *L. major* [29] distinguishing the species of *Leishmania* in the isolates would easily be possible. On the other hand, it is well known that both anthroponotic CL caused by *L. tropica*, and ZCL caused by *L. major* are endemic in different parts of Iran, and there are also several endemic areas of visceral leishmaniasis in Iran. Therefore, to identify precisely the *Leishmania* species of isolates, three specific Mabs against common species of *Leishmania* in Iran, were used for identification by immunoassay. Almost all isolates examined with this procedure showed positive reactivity with anti-*L. major* monoclonal antibodies. However, the reactivity of isolates defined by the rate of bound anti-*L. major* antibodies to bound control was different in various cases and ranged from 2.1 to 9.05 (Table 2 and 3). This discrepancy may be due to different antigenicity of isolates or probably variations have occurred in different strains of *L. major* during its transmission to a new area. Thirdly, to further characterize species, a supplementary approach was applied using PCR procedure. PCR is a method with high sensitivity and specificity, which has been used in recent years for detection and identification of different leishmanial species [30-32]. Amplification of extracted kDNA of isolates by PCR confirmed the presence of *L. major* promastigotes, but two different patterns were observed in PCR products. almost 50% of isolates showed single band of 620 base pair, that was identical with control strain of WHO (p-strain), and the rest of isolates showed a

different two bands of 800 and 620 bp, which were similar to pattern of Nadim-1 strain, which had been isolated from a patient from Isfahan area. Both patterns were similar to the patterns seen in isolates of Isfahan area.

Together, these data indicated that species of *L. major*, the causative agent of ZCL, are involved in outbreak of CL in this area. Transmission of infection from Isfahan area to this area may be explained by ecological status of the region. It has been well documented that the infection rate is high among the people living at the edge of desert and forest [2]. This suggests that rural area of Kashan and other areas near the desert are at risk of ZCL infection. Some other factors have also caused and promoted the rapid spread of infection to this area, including development of an artificial forest from a famous holy shrine named Aga-Ali-Abbas to the Kashan's surroundings since 1975. Aga-Ali-Abbas is a hyperendemic area of ZCL in the north part of Isfahan province, and is an important focus for transmission of ZCL infection. Since, colonies of *Rhombomys* live in the desert of Dasht-e-Kavir [12], probably, breeding and migration of these gerbils, along the secondary jungle is an explanation for transmission of leishmaniasis. These animals are interested in the roots of artificial forest trees and might be reproduced and served as reservoir of parasite along the area beside desert and provided convenient conditions for sandfly vectors. On the other hand, it seems that non-immune travelers from Abozeidabad to the Aga-ali-abbas have been bitten by infected sandflies during their stay in this area and served as reservoir after return to their residential place. Moreover, the infection has also been seen in the Aran-Bidgol city, 5 Km of Kashan as sporadic state. Since the secondary forest has continued to this area, this region is also at risk of wide transmission. Therefore, the greater frequency of transmission in the rural area of Kashan implies a greater public health concern. Both hygienic measures and especially immunoprophylaxis are needful for prevention from dissemination of infection in endemic and sporadic regions around Kashan.

ACKNOWLEDGMENT

The authors would like to thank Dr. F. Modabber, Dr. D. McMahon-pratt and Dr. C.L. Jaffe for the gift of Mabs. We are grateful to Dr. Y. Dowlati and Dr. A. Khamesi-pour for their suggestion and administrative collaborations. We thank Dr.

Bahonar, Dr. Chavoshi, Dr. Mofarrah, Dr. Dibaj and their colleagues, for assistance in field trial. We also deeply thank Dr. A. Jafari and Dr. M. Abolhasani, for their careful review of the manuscript. We express our gratitude to our colleagues in Pasteur Institute of Iran Mr. D. Irvani, Mrs. M. Vaziri, Mr. S. Faraj-nia, Mr. F. Allahyari, Mr. A. Nahvi, and Mr. A. Javadi for their technical and administrative helps.

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