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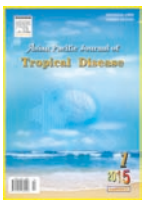

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A new focus of zoonotic cutaneous leishmaniasis in Isfahan Province, Central Iran

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ABSTRACT

Objective: To determine the epidemiological features of cutaneous leishmaniasis including human infection, reservoirs and vectors in the city of Aran o Bidgol.

Methods: This cross-sectional study was carried out on *Leishmania* spp. isolated from rodents, sandflies and patients with cutaneous leishmaniasis in Aran o Bidgol. Parasites were identified by random amplified polymorphic DNA-PCR technique and data were reported by using descriptive statistics and frequency percent.

Results: Random amplified polymorphic DNA-PCR showed that 71.4% of human isolates were *Leishmania major* (*L. major*) and the rest were *Leishmania tropica.* In addition, 17.8% of *Rhombomys opimus* and 1.9% of female *Phlebotomus papatasi* were infected with *L. major*.

Conclusions: The results indicate that *L. major* parasite is the causative agent of the disease among patients. And *Rhombomys opimus* and *Phlebotomus papatasi* are the main reservoir host and vector in the dissemination of *L. major* in the city. Therefore Aran o Bidgol is introduced as a new focus of zoonotic cutaneous leishmaniasis in Central Iran in order to prevent zoonotic cutaneous leishmaniasis, and control of the rodents and sandflies are suggested.

1. Introduction

Leishmaniasis is still one of the world's most neglected diseases, affecting largely the poorest people, mainly in developing countries. Three hundred and fifty million people are considered at risk of contacting leishmaniasis, and some 2 million new cases occur yearly (0.5 million of visceral leishmaniasis and 1.5 million of cutaneous leishmaniasis)[1]. Leishmaniasis is caused by about 20 distinct species, subspecies and strains of *Leishmania* parasites in approximately 90 countries and is transferred by adult female sandflies[2]. Cutaneous and visceral leishmaniasis are mainly seen in 14 of the 22 countries in the Eastern Mediterranean Region. Foci of zoonotic and anthroponotic cutaneous leishmaniasis (CL) are Afghanistan, Iran, Iraq, Morocco, Pakistan, Saudi Arabia, Syria and Yemen[3]. CL is the first most important vector borne disease at present in Iran[4]. CL is seen in rural (wet) and urban (dry) forms in Iran. Although, about 20 000 cases of CL in various parts of Iran are reported annually, probably the true number is four or five times more. Rural type is common in most rural areas of 15 provinces and urban type is endemic in many parts of cities[5]. Isfahan Province is one of the main foci of the disease. Currently, several foci of CL such as Isfahan City, Natanz and Ardestan are identified in the province. *Phlebotomus papatasi* (*P. papatasi*) and *Rhombomys opimus* (*R. opimus*) are the main vector and reservoir host of rural CL in Iran and Isfahan Province respectively[6-8]. In the past years, the limited cases of CL were seen in Aran o Bidgol City. In recent years, due to the population growth, expanding of the city, construction of settlements and established residential areas near the nests of reservoir, rodents have changed the status

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of the disease. Reports from the health center of Aran o Bidgol on increasing CL cases led us to perform molecular studies on parasites isolated from patients, rodents and sandflies caught in this area to identify the *Leishmania* species that causes CL in Aran o Bidgol, Central Iran, during 2007-2008.

2. Materials and methods

This cross-sectional study included 42 patients, 54 rats and 1 531 sandflies from the study area.

2.1. Study area

Aran o Bidgol City is located in the southwestern margin of the central desert of Iran and restricted from north by Salt Lake of Aran o Bidgol, Qom and Semnan provinces, from west by the city of Kashan, from south by Natanz and from east by the city of Ardestan. Aran o Bidgol is one of the cities of Isfahan Province and is situated between 51°29' E and 34°14' N with an elevation of 912 m above the sea level. Aran o Bidgol with an area of 6 051 km^2 is located in the central part of Iran; it lies 235 km by road southwest of the capital Tehran and 210 km from the province center (Figure 1). The climate of Aran o Bidgol is desert conditions; the annual rainfall is 100-150 mm, the minimum and maximum temperatures recorded were 5 ℃ in winter and 48 ℃ in summer.

Figure 1. Geographical location of Aran o Bidgol and its districts, showing the region of study.

2.2. Human samples

The study was performed on 42 patients, who were referred to the health centers in city of Aran o Bidgol (including urban and rural areas) between 2 to 65 years of age residing in Aran o Bidgol City (57% male and 43% female). Medical ethics were considered and all patients were satisfied to participate in this work. Fourty-two patients with skin lesions were suspected of CL and 14 isolates of *Leishmania* species were collected. Samples were obtained from the edge of skin lesion of patients by using vaccinostyle and were fixed with pure methanol. Following the staining of samples by Giemsa, CL was confirmed by observation of the parasite (amastigotes) by light microscopy. They were cultivated in Novy-MacNeal-Nicole (NNN) medium and maintained for 7 days and then subcultured in RPMI 1640 medium supplemented with 10% of fetal bovine serum[9].

2.3. Rodent samples

During the activity seasons of rodents in study area in years 2007-2008[10], wild rodents were captured by Sherman live trap baited with cucumber in rural areas (around the infected villages). The traps were placed at the end of each day and checked the next morning for captured rodents. The captured animals were anesthetized and identified based on morphological characteristics[11,12]. After washing and disinfecting rat ears, internal and external surface partition of ears were scratched and discharged from the blood, and thin layers were spread over microscopic slides[13]. Slides were studied by microscope after being stained with Giemsa. If Leishman body was observed in direct samples, the rat ear blood was cultured in the NNN medium.

2.4. Sandflies samples

By using sticky paper (castor oil-coated white papers 20 cm × 30 cm) every two weeks per month from June to October, aspirator and funnel traps from inside and outside of the locations (wall cracks and rodent nests) sandflies were captured[14]. Sandflies were identified through diagnostic key[15]. After sandflies were washed in 1% detergent and then in sterile distilled water, they were dissected in fresh sterile normal saline under the stereomicroscope. In case there were any move and flagellates bodies were observed, some were cultured into liquid phase of NNN medium and some were inoculated in tails of BALB/c mice. If sandfly was infected with *Leishmania major* (*L. major*), the mice were also infected with CL after 4-12 weeks when parasites were collected from the mice wound and cultured under sterile conditions in the NNN medium.

2.5. Sample culture and DNA extraction from positive smears

Rodents, sandflies and humans' wounds samples were cultured into liquid phase of NNN medium and Schneider's medium (Sigma) supplemented with 10% fetal bovine serum and 200 IU/mL penicillin G at 26 ℃. Promastigotes were harvested from a 15 mL stationary phase of mass culture by centrifugation (4 000 r/min at 4 ℃ for 10 min) and washed for 3 times in cold sterile phosphate buffer saline (pH = 7.2). The sediment was resuspended in 500 μL of cell lysis buffer consisting of 50 mmol/L ethylene diamine tetraacetic acid, 1% sodium dodecyl sulfonate, 50 mmol/L NaCl and 50 mmol/L Tris-HCl, pH = 8.0 with 100 μg/mL proteinase K, and incubated at 55 ℃ overnight. The lysate was extracted by phenol/chloroform followed by ethanol precipitation. The DNA was resuspended in double distilled water and stored at 4 ℃. Working solutions were adjusted to 10 ng/μL in double distilled water[9].

2.6. Random amplified polymorphic DNA (RAPD)-PCR procedure

RAPD-PCR was employed for detection and identification of *L. major* in rodent, sandfly and humans' ulcers by the method explained by Mauricio *et al*.[16]. Each 20 μL of RAPD reaction,

contained 20 ng genomic DNA, 2 mmol/L $MgCl_2$, 0.2 mmol/L dNTP (Roche Biotech), 20 pmol of each primer, 1 IU *Tag* polymerase (Roche Biotech) in the PCR buffer. Reactions were overlaid with 30 μL of mineral oil and amplified in a thermocycler (Techne USA) programmed for one cycle at 94 ℃ for 5 min followed by 35 cycles of 94 ℃, 36 ℃, 72 ℃ for 1 min each, and 1 cycle of 72 ℃ for 10 min. About 12 μL of PCR products were run along with a 100 bp ladder on a 1.2% agarose gel containing ethidium bromide for 4 h at 50 V. The gel was observed on a UV transilluminator and then, digital photographs were prepared[16]. In this study, two primers A4 and A8 were used [A4, 5′ AATCGGGCTG and A8, 5′ GTGACGTAGG (Roche Biotech)]. Two primers were valuated with two *Leishmania* reference strains (positive controls). Primer A4 was the optimal primer having clear and consistent bond pattern for the separation of *Leishmania* species. Then the bands created by the samples were compared with bands created by the standard: *Leishmania tropica* (MHOM/IR/IR/99) (*L. tropica*) and *L. major* (MHOM/IR/75/ER) and marker (XIV) (Roche) and the results were obtained. Positive controls were obtained from the Parasitology Laboratory, Pasteur Institute, Iran. The data collected related to the patients, reservoirs and vectors were analyzed by descriptive statistics and bands of PCR product were compared to the standard marker strains.

3. Results

In 42 lesion samples of patients that were cultured (Figure 2), 14 grew and were evaluated by RAPD-PCR technique. Experiments showed that 71.4% of isolates were *L. major* and the rest were *L. tropica*. As can be seen in Figure 3, Lanes 1 and 2 were of the standard species *L. tropica* and *L. major* respectively and the other unidentified *Leishmania* isolates were related to human samples. The results obtained with the primer A4 (Figure 3) showed bands of about 700 bp to 850 bp size and bands with size of about 300 to 2400 bp for *L. tropica* and *L. major* stock respectively. So by comparing unidentified *Leishmania* isolated with those obtained from reference strains, isolates No. 5 and 6 were *L. tropica* and No. 3 and 4 were *L. major*.

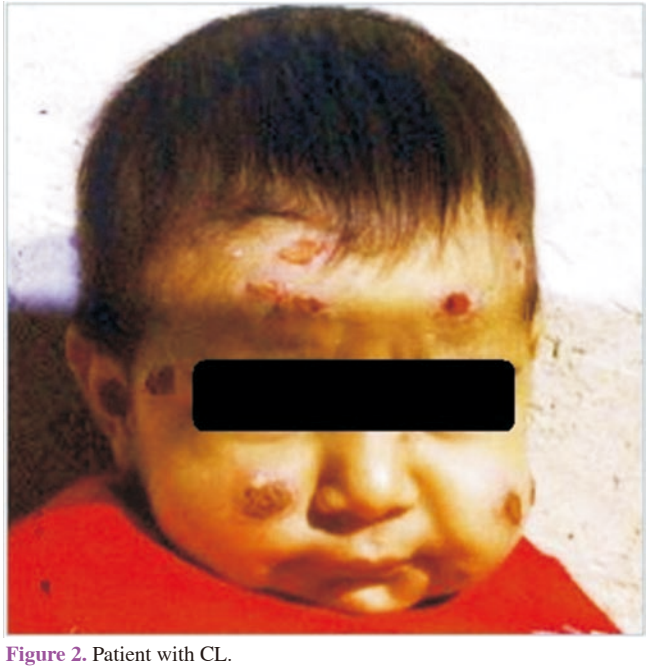

Figure 2. Patient with CL.

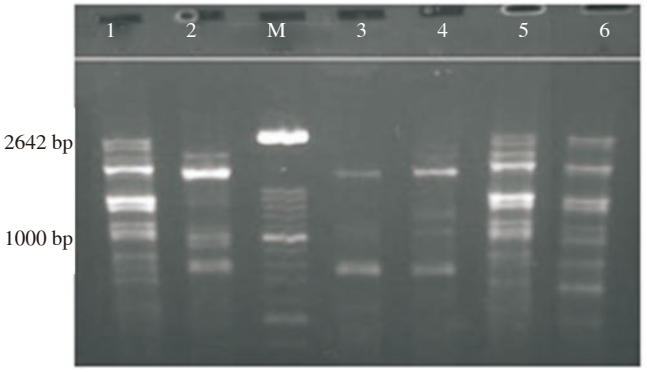

Figure 3. RAPD-PCR technique results in a few human isolated in Aran o Bidgol, 2007-2008.
Lane 1: *L. tropica* (standard species); Lane 2: *L. major* (standard species); Lane M: Marker (XIV 100 bp); Lanes 3 and 4: *L. major*; Lanes 5 and 6: *L. tropica.*

A total of 54 small rodents were caught and examined (Figure 4). The great majority were *R. opimus*, the great gerbil (Cricetidae; Gerbillinae) (83.3%), *Meriones libycus* (*M. libycus*), the Libyan jird (Cricetidae; Gerbillinae) (9.3%), *Rattusr rattus*, the black rat(Muridae; Murinae) (3.7%) and *Gerbillus nanus*, the Balochistan gerbil (Muridae; Gerbillinae) (3.7%). Experiments showed that only the great gerbil was infected. Leishman body was observed in 17.8% of the *R. opimus*. As can be seen in Figure 5, Lanes No.3 and 4 were of the standard species *L. tropica* and *L. major* respectively and the other unidentified *Leishmania* isolates related to *R. opimus* samples. So by comparing *R. opimus* isolated with those obtained from stock, isolates No.1 and 2 were *L. major*.

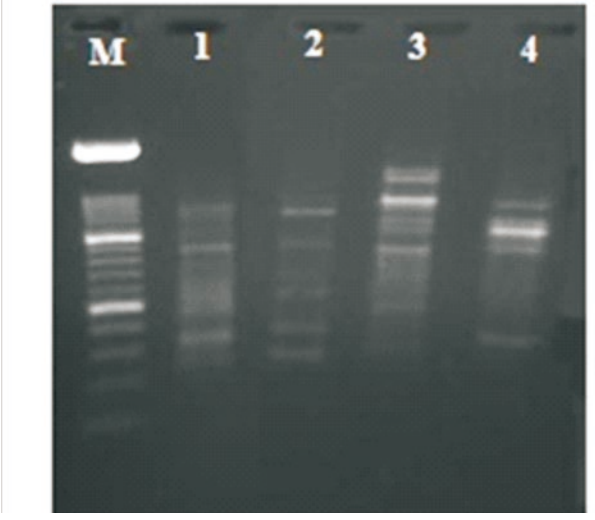

Figure 4. RAPD-PCR technique results in a few *Rhombomys* isolated in Aran o Bidgol, 2007-2008.
Lane M: Marker (XIV 100 bp); Lane 3: *L. tropica* (standard species); Lane 4: *L. major* (standard species); Lanes 1 and 2: *L. major.*

A total of 1531 sandflies were collected. Among 315 female *P. papatasi*, six (1.9%) were found to be infected with *L. major*. With A4 primer (Figure 5) bands No. 2 were observed for *L. major* stock. So by comparing unidentified *Leishmania* isolated of *P. papatasi* with those obtained from stock, isolates No.1 were *L. major*.

Leishmania isolates from humans, rodents and sandflies identified by RAPD-PCR technique, were inoculated at the base of the tail of BALB/c mice susceptible to parasite. After incubation period (4 to 12 weeks), mice showed lesions in the site of

inoculation (Figure 6). These isolates were diagnosed previously by RAPD-PCR technique as *L. major*. Isolates of *L. tropica* inoculated with same procedure did not cause any lesion or infection on the BALB/c mice.

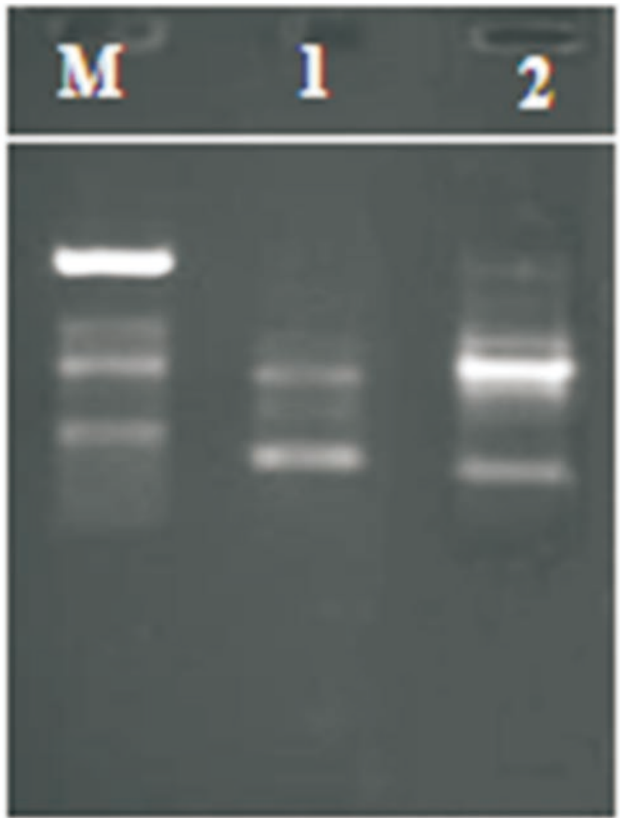

Figure 5. RAPD-PCR technique results in a sandflies isolated in Aran o Bidgol, 2007-2008.
Lane M: Marker (XIV 100 bp); Lane 1: *L. major* ; Lane 2: *L. major* (standard species).

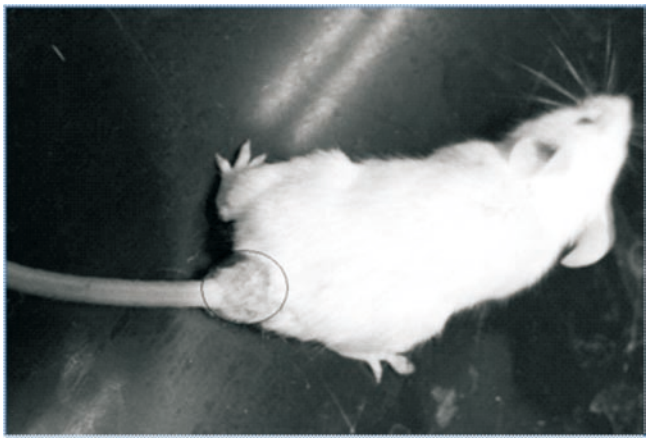

Figure 6. Wound was created after inoculation with isolates of *L. major* in the tail of BALB/c mouse.

4. Discussion

In this study, RAPD-PCR technique showed that cutaneous lesions in Aran o Bidgol was due to rural leishmaniasis and 71.4% isolates which caused human CL were *L. major*. These patients had no positive history of traveling to leishmaniasis endemic areas during this study. They were infected in their residential place. Aran o Bidgol is located between cities of Isfahan and Qom. In two studies conducted in Isfahan, 89.51% and 100% patients with CL were infected by *L. major*[17,18]. Considering the endemicity of CL in Isfahan and Qom, transmission of infection from both cities to this area is possible[18,19]. However, Aran o Bidgol is added to other zoonotic cutaneous leishmaniasis (ZCL) foci in Iran. One study has reported a list of rodents' species caught in Kashan and Aran o Bidgol districts. They consisted of *R. opimus* (60.3%), *M. libycus* (33.9%), *Meriones vinogradovi* (3.31%), *Gerbillus cheesmani* (0.83%), *Gerbillus nanus* (0.83%) and *Rattus rattus* (0.83%)[20]. Natural Leishmanial infection caused by *L. major* in *R. opimus*, the great gerbil, was confirmed by using RAPD-PCR technique. This rodent plays an important role as the main reservoir host in the epidemiology of ZCL in this area. *R. opimus* is the primary reservoir host of *L. major* in central, southeast and northeastern part of Iran[21-23]. *M. libycus* is the primary reservoir of ZCL in some areas of Central and Southern Iran[24]. But this rodent was not reservoir of ZCL in area of this study. Doroodgar *et al.* reported a list of sandfly species caught in the mountains and plains of Kashan and Aran o Bidgol districts[10]. They consisted of 12 species of *Phlebotomus* and 8 *Sergentomyia*. *P. papatasi* was the major species captured from houses and rodent burrows and had the most frequency (62%) among other sandflies[10].

In this study, 1.9% of female *P. papatasi* sandflies were found to be infected by *L. major* by using the standard RAPD-PCR method. Several studies show various ratios of *P. papatasi* infected by *L. major*, for example: 0.3% in Kalaleh District, Golestan Province, 2.1% in Chabahar, 1.8% in Natanz (nearby Aran o Bidgol) and 6% in Sarbisheh East of Iran, 12.7% in Rafsanjan[22,24-27]. *P. papatasi* sandfly is the primary vector of *L. major* in many parts of Iran. This sandfly is the main vector of the disease in Aran o Bidgol. Leishmaniasis is in 14 countries in the Middle East. ZCL foci affected by *L. major* factor are Libya, Morocco, Palestine, Pakistan, Afghanistan, Egypt, Iran, Iraq, Jordan, Saudi Arabia, Syria and Yemen[5].

Although CL is usually not associated with a high mortality rate, it is very considerable because it causes the patient's persecution and creates skin deformation in some cases that will remain for more than a year and scar will be left after healing for a lifetime. Therefore disease's care and control is very important.

Before performing any ZCL controlling measures, *Leishmania* species from human, mammalian reservoir hosts and sandfly vectors should carefully be identified.

The following factors can prevent the spread of disease: factors related to the management and execution, health education and community knowledge, environmental factors, factors related to the vectors, factors related to reservoirs and treatment and personal protection. Although the proper coordination and implementation of all these factors are difficult, seriousness in controlling the disease and increasing intersectoral coordination are very important in the disease control particularly. Community education, active patients screening, appropriate treatment, dressing of location of lesion are important to fight against the main vectors and reservoir hosts[5].

Based on this survey, *L. major* is the causative agent of the disease among patients and mammalian hosts. On the one hand, *R. opimus* plays the main role in the dissemination of *L. major* in the Aran o Bidgol City. It seems that the current situation is affected by some factors such as urbanization, development of the city, construction of buildings nearby or the rodent colonies

and traveling non-immune travelers during the active season of sandflies to other ZCL foci of the country. Another important factor is geographical location and neighboring of Isfahan and Qom that are important foci for transmission of ZCL infection. Thus, organizations of health care providers should pay attention to appropriate measures of disease control. Recognitions of the parasite, vector and reservoir host are the primary requirements to determine the proper way for controlling disease. Based on this survey, a CL focus is detected with *L. major* as the agent in Aran o Bidgol area. Since the ZCL is included in zoonoses diseases, controlling measures should be based on the control of *P. papatasi* as the main vector and *R. opimus* as the main reservoir. In addition, RAPD-PCR technique is more accurate compared with the microscopic observations and is a powerful method for identification of *Leishmania* species.

Conflict of interest statement

We declare that we have no conflict of interest.

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