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***Phlebotomus (Larroussius) kandelakii* the Principal and Proven Vector of Visceral Leishmaniasis in North West of Iran**

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Abstract: Visceral leishmaniasis is one of the most important health problems in Iran which is transmitted by sandflies species to human. In order to determine vector/s of the disease an investigation was carried out in Meshkinshahr district, the most important focus of the disease in Ardebil province, north west of Iran, during 2001-2005. Using sticky papers, CDC light traps and aspirators, totally 2500 sandflies were collected and identified at the species level. Host blood preference and natural infection of female specimens to promastigotes were tested, respectively using ELISA and dissection followed by PCR assay using species-specific kinetoplast minicircle primers. Results showed that six species of *Phlebotomus kandelakii*, *P. perfiliewi transcaucasicus*, *P. papatasi*, *P. jacusieli*, *P. caucasicus* and *P. sergenti* were present in the district, where *P. kandelakii* with 40% was the most prevalent species. Anthropophilic index of *P. kandelakii* species was calculated 32.8%, indicating a strong preference to human. Among the dissected female sandflies, only 11 out of 1002 (1.1%) of *P. kandelakii* was found naturally infected with promastigotes. Species-specific amplification of the Giemsa stained promastigote slides revealed specific PCR products of *Leishmania infantum* DNA in the infected *P. kandelakii* sand-flies. Having found high prevalence and anthropophilic index and natural infection to *Leishmania infantum* provide enough evidences to incriminate the species of *P. kandelakii* as the main and proven vector of visceral leishmaniasis for the first time in the region and in science.

Key words: *Phlebotomus kandelakii*, proven vector, *Leishmania infantum*, PCR, Iran

INTRODUCTION

Visceral leishmaniasis (kala-azar) which its causative agent is *Leishmania infantum* considered as a severe, often fatal disease common in Mediterranean region^[1]. In these areas domestic dogs (*Canis familiaris*) are the principle reservoir host and some species of sand-flies belong to subgenus *Larroussius* are the primary vectors of disease^[2]. The annual occurrence of visceral leishmaniasis (VL) is estimated around 500000 cases throughout the world^[3]. More than 700 species of phlebotomine sand-flies (Diptera: Psychodidae) have been described so far. Genus of *Lutzomyia* in the new world and *Phlebotomus* in the old world are known as the vectors of *Leishmania* parasites and other pathogens to human.

Three important endemic foci of visceral leishmaniasis have been reported from Iran including; Ardebil, East Azerbaijan (north west of Iran) and Fars (southern Iran) provinces. The disease occurs sporadically in other parts of Iran as well^[4]. Ardebil district comprises more than 50% of total cases of visceral leishmaniasis, so that this area is the most important focus of the disease in the country (unpublished). More than 90% of VL cases are seen under 10 years old and domestic dogs are primary reservoir host of disease^[5]. On the basis of entomological studies, two species, *P. kandelakii* and *P. perfiliewi*, have been found naturally infected with promastigote and they have been reported the probable vectors of VL in northwest of Iran^[6].

The main purpose of present study was to determine the main and proven vector(s) by employing of

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Nested-PCR technique as well as anthropophilic index of them.

MATERIALS AND METHODS

Study areas: The study was carried out in three villages of Ahmad-Abad, Gourt-Tapeh and Parikhan, Meshkin Shahr district Ardebil province, north-west of Iran during 2001-2005 (Fig. 1). Its Altitude was 1490 m above the sea level. The total population of the Meshkin Shahr was about 158581 in year 2002. The climatic condition was very hot (up to 40°C) in the summer and quite cold (-27°C) during the winter. Warm season is short and lasting from mid May to mid September. The main activities of the people are agriculture and veterinary farming.

Identification of host preference: The smear of blood meals of each engorged female sand-flies was prepared on Whatman No.1 filter paper. The number of sand-flies, date and place of collection were noted. The prepared papers were sent to the Parasitology Unit, Department of Medical Parasitology and Mycology in Tehran University of Medical Sciences for ELISA testing^[7].

Sand-flies collection: Sand-flies were collected from indoors (bedroom, guestroom, toilet and stable) as well as outdoors (wall cracks and crevices and animal burrows) biweekly using sticky papers, CDC light traps and aspirators. Three villages called Ahmad Abad, Gourt Tapeh and Parikhan were selected and sand-flies collected during August and September which is synchronized with the peak of activity of sand- flies during year 2001-2004. Sand-flies were dissected in a drop of normal saline. Systematic identification key was employed for species identification^[8].

DNA extraction: DNA was extracted as described by Motazedian^[9]. Firstly 50 µL of Lysis buffer [50 mM Tris-Hcl (pH, 7.6); 1 mM EDTA; 1% Tween 20] was added on promastigote infected slides and after a few minutes the content of each slide transferred in 0.5 mL micro tubes. Each slides was washed three times and the content of slides were transferred in to the same micro tube. Twelve micro liter of Proteinase K (19 µg mL⁻¹) were added and incubated in 37°C overnight prior to adding 300 µL Phenol:Chloroform: Isoamyl alcohol (25:24:1, by Vol.). After being shaken vigorously, the tube was centrifuged at 10000 rpm for 10 min and then the DNA in the supernatant

Solution was precipitated with 400 µL cold, pure ethanol, re- suspended in 50 µL double-distilled water and stored at -20°C before being used in PCR.

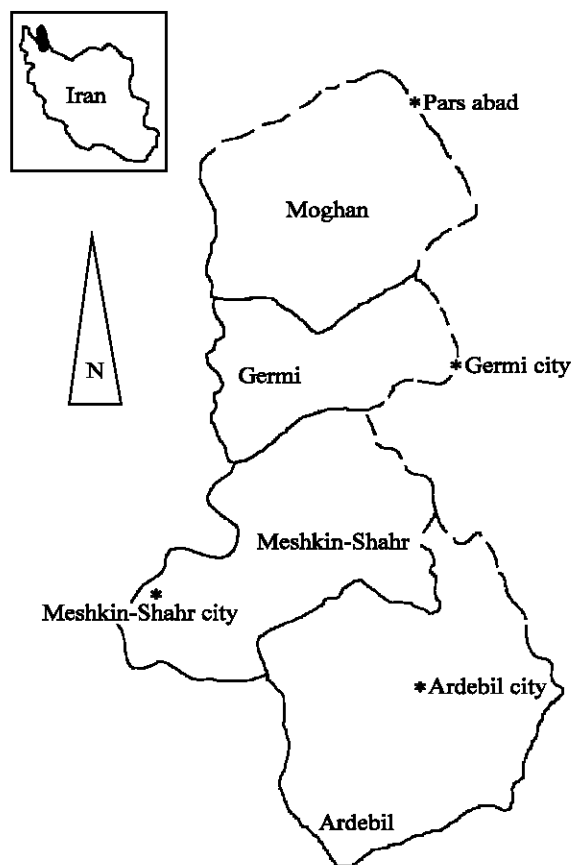


Fig. 1: Map of study area located in Ardebil province, Iran

PCR: Amplification of the variable area of the minicircle kinetoplast DNA of *Leishmania* was carried out with some modification as described elsewhere^[10]. Primers LINR4 (Forward) (5'-GGG GTT GGT GTA AAA TAG GG-3') and LIN17 (Reverse) (5'-TTT GAA CGG GAT TTC TG-3') have been designed within the conserved area of the kinetoplast minicircle and contained conserved sequence blocks (CSB), CSB3 and CSB2, respectively^[10]. Reference strains of *L. infantum* (MCAN/IR/96/Lon49), *L. tropica* (MHOM/IR/89/ARD2) and *L. major* (MHOM/IR/54/LV39) were used as standards. Amplification reaction was carried out in a total of 25 µL containing 250 µM deoxynucleoside triphosphate (dNTPs), 1.5 mM MgCl₂, 1 U Taq polymerase (5 u µL⁻¹) (Cinagene-Iran), 1 µM each of primers (LINR4 and LIN17), 1x PCR buffer (Boehringer Mannheim genbh-Germany) and 5 µL of DNA extract, overlaid with mineral oil. The DNA was amplified in a thermocycler (Corbett Research, CGI-96, Australia) at 94°C for 5 min followed by 30 cycles, each consisting of 30 sec at 94°C, 30 sec at 52°C and 1 min at 72°C. After the last cycle, the extension was continued for 5 min.

Agarose-gel electrophoresis: Five micro liter of each PCR products were resolved in a 1.5% agarose LE gel and visualized under Ultraviolet transilluminator with ethidium bromide. Parasites were identified by comparison with reference strains of *L. infantum*, *L. major* and *L. tropica* and molecular weight markers.

RESULTS

Altogether 2500 female sand-flies were collected and they were *P. kandelakii* (40%), *P. perfiliewi transcaucasicus* (7%), *P. papatasi* 20.5%), *P. jacusieli* 0.5%), *P. caucasicus* (20.5%) and *P. segenti* (12%). Ninety percent of them were parous and remaining were nulliparous. Among the collected sand flies 72% were unfed, 5% gravid, 10% semi gravid and 13% blood fed. *P. kandelakii* was dominant species. Only 11 out of 1002 *P. kandelakii* (1.1%) were observed naturally infected with promastigotes under light microscope 40 X magnification. Five infected sand flies had been caught by sticky papers and six of them using CDC light traps. Two infected *P. kandelakii* were collected from indoors (kala azar case dwellings) and nine of them from outdoors less than 4 m distance to positive cases houses. Eleven slides of promastigote from infected sand-flies prepared and stained with Giemsa (Fig. 2). Four infected slides were checked for detection of *Leishmania infantum* by Nested-PCR. They were identified *L. infantum* using standard PCR with primers LINR4 and LIN17. The visualized obtained bands were similar to standard *L. infantum* and infected *P. kandelakii* specimens, which was equal to 720 bp (Fig. 3). The obtained bands of standards *L. major* and *L. tropica* were 560 and 760 bp, respectively (Fig. 3).

According to the ELISA test, among 241 blood meals of *P. kandelakii*, the proportion giving a positive reaction from (+) to (+++) with alkalie phosphate antihuman conjugate, ranged between 21.1-35.7% with an average 32.8% indicating strong preference for human. The positive proportions for dogs, the main animal reservoir of the disease, were between 16.3-35% with an average of 21.2% (Table 1).

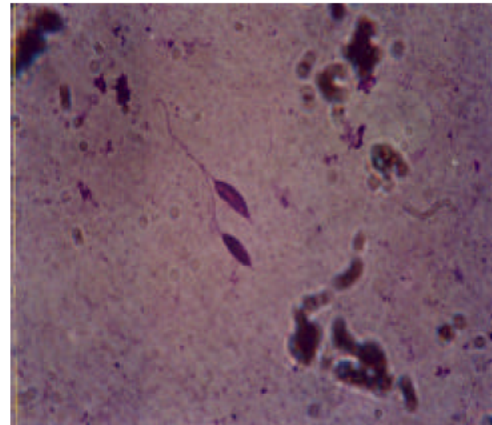


Fig. 2: Giemsa stained of promastigotes of *Leishmania infantum* from naturally infected *P. kandelakii*

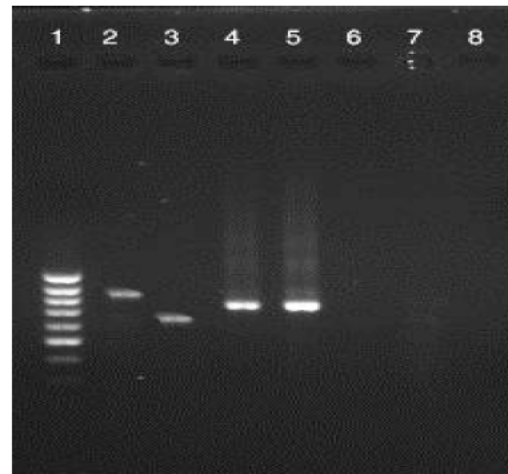


Fig. 3: The results of PCR -based of DNA extracted from Giemsa stained promastigote slides. The bands shown, on 1.5% agarose gel stained with Ethidium bromide, correspond to molecular weight markers (Lanes 1), Reference strains of *Leishmania tropica* (Lane 2), *L. major* (Lane 3), *L. infantum* (Lane 4), Samples from one specimen of *Phlebotomus (Larroussius) P. kandelakii* (Lane 5), negative control from a male *P. kandelakii* (Lane 6) and blanks (Lanes 7-8)

Table 1: Results of ELISA test on blood meals of *P. kandelakii*

Name of Villages	Date of sand fly collection (Jul-Aug)	Total No. of blood-meals	Human		Dog		Unknown	
			No. (+)	% (+)	No. (+)	% (+)	No.	%
Ahmadabad	2002	62	21	33.9	14	22.6	27	43.5
	2003	98	35	35.7	16	16.3	47	48.0
Gourt-tapeh	2002	20	7	35.0	4	20.0	9	45.0
	2003	22	6	27.3	5	22.7	11	50.0
Parikhan	2002	19	4	21.1	5	26.3	10	52.6
	2003	20	6	30.0	7	35.0	7	35.0
Total No. of blood meal		241	79	32.8	51	21.2	111	46.0

DISCUSSION

Information and knowledge on ecology and epidemiology of leishmaniasis is very important for control of the disease. These knowledge could be focused on identification of reservoir host(s) as well as identification and detection of agents in the vector(s). The suggested criteria for incrimination of a vector are anthropophily and common infection with the same leishmania parasite are found in man in the same places^[11]. Isoenzyme detection method provide the gold-standard characters for identifying species and reference strain of leishmania, but this method has disadvantage due to requiring the culture of large number of parasites and primary isolates can easily become contaminated or in a mixed infection only the strain that grows fast in laboratory conditions can be yield^[12]. At the present time the applicability of molecular techniques (PCR) including κDNA for detection and identification of leishmania within sand-flies by DNA hybridization have been proved^[13-15].

The high sensitive technique of PCR has been used formerly for detecting of leishmania in sand-flies of new world^[16] and India^[17]. Some species of sand-flies belong to subgenus *Larroussius* are potential vectors of visceral leishmaniasis in Mediterranean basin. Aransay *et al.*^[10] used the primers LINR4, LIN17 and LIN19- (which we have used in this study) for detection of leishmania infection in *P. (lar.) neglectus*, *P. (lar.) tobbi*, *P. (lar.) simici* and *P. (para.) alexandri* using highly semi-nested PCR technique in Greece. In Iran, according to surveying on vectors of VL in two important endemic foci of the disease (north west and south) four species of *Larroussius* group sandflies including: *P. (lar.) kandelakii*, *P. (lar.) perfiliewi transcausicus*, *P. (lar.) keshishiani* and *P. (lar.) major* have been observed naturally infected with amastigotes, but due to lack of availability of molecular methods at that time, identification of parasite was difficult, therefore, the mentioned above sand-flies were reported as the probable vectors of VL in Iran^[6,18].

In this study Giemsa stained promastigote slides prepared from infected sand-flies were used for DNA extraction^[9]. All infected specimens were parous so this females longevity was enough to complete parasite cycle in its body.

According to the finding of present investigation, including observation of naturally promastigote infected of *P. kandelakii* (which were collected from patients houses or from out doors near the kala azar cases habitats) among wild population of sand-flies; detection of parasite as *L. infantum*; high anthropophily and density

of *P. kandelakii*, we confirm that this species could be introduced as the primary and proven vector of visceral leishmaniasis in north west of Iran. From the literature review It postulated that this is the first report on *P. kandelakii* as the proven vector of VL in the world.

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REFERENCES

1. Lachaud, L., S. Marchergui-Hammami, E. Chabbert, J. Dereure, J. Dedet and P. Bastien, 2002. Comparison of six PCR methods using peripheral blood for detection of canine visceral leishmaniasis. *J. Clin. Microbiol.*, 40: 210-215.
2. World Health Organization, 1990. Control of the leishmaniasis. Report of a WHO expert committee. Technical Report Series No. 793. Geneva: WHO., pp: 159.
3. World Health Organization, 2000. *Leishmaniasis* and *Leishmania* /HIV co-infection. WHO/CDC/CSR/ISR., pp: 1-2.
4. Rassi, Y., F. Kaverizadeh, E. Javadian and M. Mohebbali, 2004. First report on natural promastigote infection of *Phlebotomus caucasicus* in a new focus visceral leishmaniasis in north west of Iran. *Iranian J. Publ. Health*, 33: 70-72.
5. Mohebbali, M., Y. Hamzavi, G.H. Edrissian and A. Forouzani, 2001. Seroepidemiological study of visceral leishmaniasis among humans and animal reservoirs in Bushehr province, Islamic Republic of Iran. *Eastern Mediterranean Health J.*, 7: 912-917.
6. Rassi, Y., E. Javadian and A. Nadim, 1997. Natural Promastigote Infection of sandflies and its first occurrence in *S. dentata* in Ardebil province, north west of Iran. *Iranian J. Public Health*, 6: 7-12.
7. Edrissian, G., A.V. Manochehri and A. Hafizi, 1985. Application of an Enzyme-Linked Immunosorbent Assay (ELISA) for determination of the human blood index in anopheline mosquitoes collected in Iran. *J. Am. Mosq. Cont. Assoc.*, 1: 349-352.

8. Theodor, O. and A. Mesghali, 1964. On the phlebotomine of Iran. J. Med. Entomol., pp: 285-300.
9. Motazedian, M.H., M. Karamian, H.A. Noyes and S. Ardehali, 2002. DNA extraction and amplification of *Leishmania* from archived, Giemsa stained slides, for the diagnosis of cutaneous leishmaniasis by PCR. Ann. Trop. Med. Parasitol., 96: 31-34.
10. Aransay, A.M., E. Scoulica and Y. Tselentis, 2000. Detection and identification of *Leishmania* DNA within naturally infected sand flies by semi-nested PCR on minicircle kinetoplast DNA. Applied Environ., Microbiol., 66: 1933-1938.
11. Killick-kendrick, R., 1990. Phlebotomus vectors of visceral leishmaniasis: A review. Med. Vet. Entomol., 4: 1-24.
12. Alvar, J. and J.R. Barker, 2002. Molecular tools for epidemiological studies and diagnosis of leishmaniasis and selected other parasitic disease. Trans. Royal Soc. Trop. Med. Hygiene, 96: S1-S250.
13. Ready, P.D., D.F. Smith and R. Killick-kendrick, 1988. DNA hybridization on squash-blotted sand flies to identify both *Phlebotomus papatasi* and infecting *Leishmania major*. Med. Vet. Entomol., 2: 109-116.
14. Rodriguez, N., C.M. Aguilar and M.A. Barrios, 1999. Detection of *Leishmania braziliensis* in naturally infected individual sand flies by the polymerase chain reaction. Trans. Royal Soc. Trop. Med. Hygiene., 93: 47-49.
15. Rogers, W.O., P. F. Burnheim and D.F. Wirth, 1988. Detection of *Leishmania* within sand flies by kinetoplast DNA hybridization. Am. J. Trop. Med. Hygiene, 93: 47-49.
16. Mukherjee, S., M.Q. Hassan, A. Ghosh, A. Bhattacharya and S. Adhya, 1997. Short report: *Leishmania* DNA in *Phlebotomus* and *Sergentomyia* species during a kala-azar epidemic. Am. J. Trop. Med. Hygiene, 57: 423-425.
17. De Bruijn, M.H. and D.C. Barker, 1992. Diagnosis of new world leishmaniasis: specific detection of species of the *Leishmania braziliensis* complex by amplification of kinetoplast DNA. Acta Tropica, 52: 45-58.
18. Sahabi, Z., M.A. Seyedi Rashti, A. Nadim, E. Javadian, M. Kazemini and M.R. Abai, 1992. A preliminary report on the natural leishmaniasis infection of *Phlebotomus major* in an endemic focus of V.L. in Fars province, south of Iran. Iranian J. Public Health, 21: 87-93.