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PCR Based on Identification of Vectors of Zoonotic Cutaneous Leishmaniasis in Shahrood District, Central of Iran

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Abstract: A study was made in rural region of Shahrood city, Semnan province in the central of Iran during 2005 to investigate of vectors of zoonotic cutaneous leishmaniasis. Sticky traps and an aspirator were used for collecting of sandflies. Three species of *Phlebotomus papatasi*, *P. caucasicus* and *Sergentomyia sintoni* were collected and identified and the first species was dominant (53%). Nested PCR method were employed for identifying of isolated parasites of dissected female of sandflies. Among the dissected sand flies 3 out of 24 (12.5%) *Phlebotomus papatasi* and 2 out of 48 (4.2%) *Phlebotomus caucasicus* were found naturally infected with promastigotes. Species-specific amplification of Giemsa staind promastigote slides revealed specific PCR production of *Leishmania major* DNA in the infected *P. papatasi and P. caucasicus* sand flies. Having high prevalence and infection rate provide enough evidence to incriminate of *P. papatasi* as the main and proven vector of cutaneous leishmaniasis to human in the region and the species of *P. caucasicus* play the second role for maintenance of disease between rodents.

Key words: Vectors, cutaneous leishmaniasis, PCR, Iran

INTRODUCTION

Cutaneous Leishmaniasis (CL) is still considered as an important health problem in many parts of the world especially the Mediterranean regions, Africa and almost all countries of the Middle East (William *et al*., 2004, Khoury *et al*., 1996).

In Iran cutaneous leishmaniasis is the most important health problem after malaria and there are at least two species of old world Leishmania (*L. tropica* and *L. major*) which are responsible for self healing ulcerative diseae and occasionally metastatic Cutaneous Leishmaniasis (Nadim *et al*., 1994).

Cutanous leishmaniasis due L. major (ZCL) has been widespread in several parts (14 of the 28 provinces) of Iran including: rural areas of Isfahan, Shahrood, Turkman Sahra, Arsanjan, Marvdasht, Neiriz, Semnan, Abardezh of Varamin, Khorasan, Baluchistan, Kusistan, Ilam and Bandar Abbas region (Yaghoobi-Ershadi *et al*., 2004; Nadim *et al*., 1968a, b; Seyedi-Rashti and Nadim., 1967; Nadim and Faghih., 1968; Nadim, 1977; Rassi *et al*., 2006, 2004 and 2001).

Zoonotic Cutaneous Leishmaniasis is reported from at least 13 villages of Shahrood district and recently the number of new foci as well as new cases is going to be increased. (unpublished data).

The main aim of the present study was to identify the sand fly species responsible for most transmission of L. major to humans using NESTED-PCR in Shahrood district.

MATERIALS AND METHODS

Study areas: The study was carried out in two villages of Bekran (eastern Kalates district) and Ahmad Abad (Khartouran district) in shahrood county (36°25'N,

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055°01'E, alttitude 1345 m). There was about 200 km distance between two foci of eastern Kalates and Khartouran. The population of Shahrood was 134,920 in 2006 and their main activities are agriculture and farming.

Collection of sand flies: Sand flies were collected from indoors (bedroom, guestroom, toilet, stable) as well as outdoors (rodent burrow, wall cracks) biweekly using sticky traps and an aspirator during the sand flies activity period. Sand flies were rinsed from the sticky traps and mounted in a drop of Puri's medium. Species identification was conducted using a standard key (Seyedi-Rashti and Nadim, 1992; Theodor and Mesghali, 1964).

Dissection of sand flies: Blood fed, semi gravid and gravid sand flies were dissected in a drop of normal saline. Infected sand flies with promastigote was fixed and stained by methanol and Giemsa, respectively.

DNA extraction: DNA was extracted as described by Motazedian (Motazedian *et al*., 2002). 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 microliters μL 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.

PCR: Amplification of the variable area of the minicircle kinetoplast DNA of *Leishmania* was carried out with some modification as described elsewhere (Aransay *et al*., 2000). 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. 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 $MgCl_2$, 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, CG1-96, 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 microliter of each PCR products were resolved in a 1.5% agarose LE gel and visualized under Ultraviolet transluminator with ethidium bromide. Parasites were identified by comparison with reference strains of *L. major*, *L. infantum* and *L. tropica* and molecular weight markers.

RESULTS

Eastern kalate focus

Bekran village: Overall, 777 sand flies (348 females and 429 males) were caught and identified in 2005. Adult sand flies were only collected between the middle May and the end of December, with the highest numbers per collection-night, caught at the middle August. The total collection included 2 species of *Phlebotous papatasi* and *Sergentomyia sintoni* (Table 1). *Phlebotomus papatasi* was the first common species in the collection, representing over 71% of sand flies caught.

Altogether 59 female sand flies were collected and dissected in the middle August (the peak of sandflies activity) of 2005. All parous sand flies were caught from rodents burrows less than 100 m distance to human places. They were *Phlebotous papatasi* (15%) *P. caucasicus* (80%) and *Sergentomyia sintoni* (5%). Only 1 out of 9 *Phlebotous papatasi* (11.1%) and 2 out of 47 *P. caucasicus* (4.3%) were found naturally infected with promastigotes under light microscope 40 X magnification. 8 slides of promastigote from infected sand-flies prepared and stained with Giemsa (Fig. 2). Two infected slides were checked for detection of *Leishmania major* by Nested-PCR. They were identified *L. major* using standard PCR with primers LINR4 and LIN17. The visualized obtained bands were similar to standard *L. major* and infected *P. papatasi* and *P. caucasicus* specimens, which was equal to 560 bp (Fig. 1). The obtained bands of standards *L. infantum* and *L. tropica* were 720 and ~760 bp, respectively (Fig. 1).

Table 1: The 2 species of sand flies collected from Bekran village, Shahrood district, 2005

	No. caught		
Species	Males	Females	Total
Phlebotomus (Phlebotomus) papatasi	345	209	554
Sergentomyia (Sergentomyia) sintoni	84	139	223

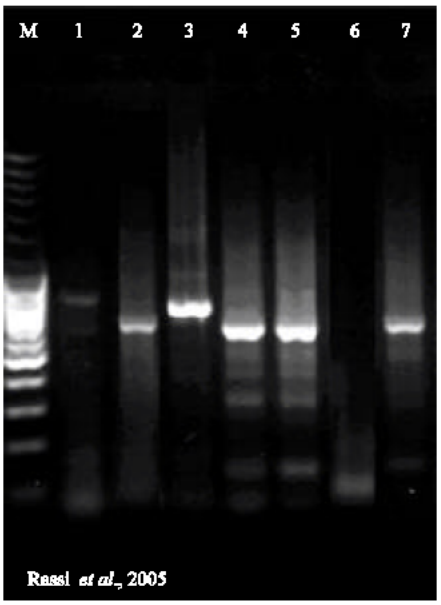

Fig. 1: The results of PCR-based on DNA extracted from Giemsa stained promastigote slides. The bands shown, on 1.5% agarose gel stained with ethidium bromide, correspond to molecular weight markers (Lane M), Reference strains of *Leishmania tropica* (Lane 1), *L. major* (Lane 2), *L. infantum* (Lane 3), Sample from one specimen of *Phlebotomus papatasi* (lane 4), Sample from one specimen of *Phlebotomus caucasicus* (Lane 5) negative control from a male *P. papatasi* (Lane 6) and another sample from one specimen of *Phlebotomus papatasi* (Lane 7)

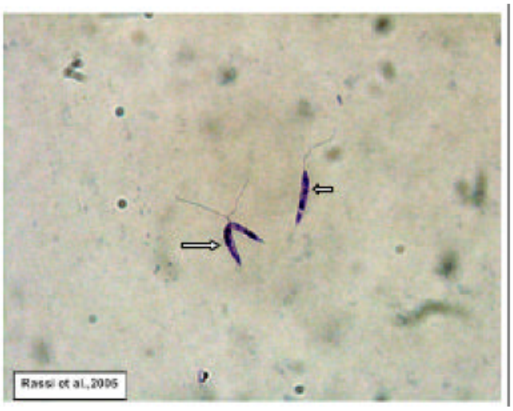

Fig. 2: Giemsa stained of promastigote of *Leishmania major* from naturally infected of *P. papatasi*

Khartoran focus

Ahmad Abad village: During the end of May-middle of December of 2005, totally, 326 sand flies (148 females and 178 females) were collected and identified. They were

Table 2: The 2 species of sand flies collected from Ahmad Abad village, Shahrood district, 2005

	No. caught		
Species	Males	Females	Total
Phlebotomus (Phlebotomus) papatasi	90	60	150
Sergentomyia (Sergentomyia) sintoni	88	88	176

P. papatasi and *S. sintoni* (Table 2). The activity peak of *P. papatasi* was observed in the middle of August.

Twenty female and parous sand flies including, *P. papatasi* (75%), *P. caucasicus* (5%) and *S. sintoni* were collected from rodents burrows in the middle August of 2005. Only 2 out of 15 *P. papatasi* (13.3%) were observed naturally infected with *L. major* identified by PCR assay using species-specific kinetoplast primers. (Fig. 1).

DISCUSSION

In endemic areas, the effective control of the human leishmaniases requires a thorough knowledge of the ecology and epidemiology of the parasites causing the diseases and their vertebrate and invertebrate hosts (Aransay *et al*., 2000). In many areas, however, despite considerable research on these diseases, the main reservoir hosts and the species of sandfly responsible for most transmission have still to be identified. In many foci of CL there is at least one species of sandfly that is sufficiently common and anthropophilic to be considered a probable vector and detection of parasites in wild caught females of this species support this belief.

At the present time, the applicability of molecular techniques (PCR) including kDNA for detection and identification of leishmania within sand-flies by DNA hybrizidation have been proved (Ready *et al*., 1988; Rodriguez *et al*., 1999; Rogers *et al*., 1988). The high sensitive technique of PCR has been used formerly for detecting of leishmania in sand-flies of new world (Mukherjee *et al*., 1997) and India (De Bruijn and Baiker, 1992).

In this study, Giemsa stained promastigote slides prepared from infected sand-flies were used for DNA extraction (Motazedian *et al*., 2002). All infected specimens were parous so this females longevity was enough to complete parasite cycle in its body.

In Iran, the main vector of disease to human is *Phlebotomus* (*Phlebotomus*) *papatasi* Scopoli (Diptera: Psychodidae) and other vectors among rodents in rural areas are *P.* (*Paraphlebotomus*) *caucasicus*, *P.* (*Para*) *mongolensis*, *P.* (*Para*) *and rejevi*. *P.* (*Para*) *alexandri* and *P.* (*Synphlebotomus*) *ansarii* (Nadim *et al*., 1994, 1968a,b; Yaghoobi-Ershadi *et al*., 2004, 1995, 1994; Parvizi *et al*., 2004.)

In the central parts of Iran (Isfahan province) as well as south east the country, the-females of *Phlebotomus papatasi* have been reported as the main vector of cutaneous leishmanias is due to *Leishmania major* (Parvizi *et al*., 2004; Yaghoobi-Ershadi *et al*., 1995). leishmanial infection of *Phlebotomus papatasi* from rodent burrows has been reported from other ZCL foci of Iran with ranges. 2-10.9% during 1967-1991 (Mesghali *et al*., 1967; Javadian *et al*., 1976; Seyedi-Rashti and Salehzadeh, 1990).

Comparison of our finding in this study (12.5% infection) with those reported from Iran and other countries indicate the highest infection rate of *Phlebotomus papatasi* that occurred in middle August, to coincide with the activity peak of this species.

Observation of *L. major* in wild population of *Phlebotomus papatasi*, high density of its population in the study region, anthropophilic index (12.7-44.4%) of this species (Yaghoobi-Ershadi *et al*., 2004), to be near of rodents burrow (less than 100 m) to human places, farming and veterinary of people near rodents burrow during the active period of sand flies, make the best condition for transmitting of the disease to human and incriminating of *Phlebotomus papatasi* as the primary vector of parasite to human in the studied regions. Infection of *P. caucasicus* to leishmanial parasite was the other finding of this study. *Leishmania major* was isolated and identified from this species. This is the third report, on isolation and identification of *L. major* in *P.caucasicus* in Iran. The first and second reports is related to Isfahan and Yazd provinces of Iran (Yaghoobi-Ershadi *et al*., 2001, 1994).

Because the low infection rate (4.2%) and absence of *P. caucasicus* in indoors (room, -toilet, stable), it seems, this species can only help for maintenance of disease in rodents burrow and play second role as the vector of disease.

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