

First detection of *Leishmania infantum* in *Phlebotomus kandelakii* using molecular methods in north-eastern Islamic Republic of Iran

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أول اكتشاف للآشمانيات الطفلية في الفاصدة الكانديلاكية باستخدام الطرائق الجزيئية في شمال شرق جمهورية إيران الإسلامية
ياور راثي، محمد رضا عبائي، محمد علي عشاق، عزت الدين جواديان، علي رضا صانعي، سينا رفيع زاده، كورورش أرزماني

الخلاصة: أصبحت مقاطعة شيرفان في شمال شرق جمهورية إيران الإسلامية بؤرة جديدة لداء اللاشمانيات الحشوي. وتهدف هذه الدراسة إلى التعرف على العوامل الناقلة للمرض، والطفيلي وأنواع الفواصد الناقلة في المنطقة خلال المدة ما بين تموز/ يوليو وأيلول/ سبتمبر من عام 2009 باستخدام تقنيات التفاعل التسلسلي للبوليمراز. وقد جمع الباحثون ألفين وثمانياً وثماناً فاصدة من سبع قرى، وتعرفوا على تسعة أنواع منها وهي الفاصدة الكانديلاكية (شرنيكوفا)، والفاصدة الكبيرة (أناندال)، والفاصدة الحليبية (الثيودورية)، والفاصدة الباباتاسية (سكوبولي)، والفاصدة السرجانية (باروت)، والفاصدة السرجانية النغمية (برنجل) بيرفيليف، والفاصدة الطويلة الخطوط (باروت) الفاصدة القوقازية (مارزونفسكي)، والسرجنتية الستونية (برينغل) والسرجنتية الغائصة (بيرفيليف). وقد كانت الفاصدة السرجنتية والفاصدة الكانديلاكية أكثر أنواع الفواصد شيوعاً، إذ يصل شيوع الأولى إلى 31.3% والثانية إلى 10%. ومن بين تسع وخمسين أنثى من نوع الفاصدة الكانديلاكية كانت هناك اثنان (3.4%) مصابتين بعدوى طبيعية بالآشمانيات الطفلية. وهذا هو الاكتشاف الأول لأنحاء الفاصدة الكانديلاكية عدوى طبيعية باللاشمانية الطفلية في هذا الإقليم مما يدل على أن الفاصدة الكانديلاكية قد تكون هي العامل الناقل للآشمانيات الطفلية في المنطقة، ولو أنها تحتل المرتبة الثانية من حيث أكثر أنواع الفواصد شيوعاً. ولا بد من استقصاء تفصيلي لبيولوجية هذا النوع، بما في ذلك الثوي ومقاومتها للمبيدات الحشرية.

ABSTRACT Shirvan district in north-eastern Islamic Republic of Iran is a new focus of visceral leishmaniasis. This study aimed to identify the vector(s), the parasite and the species composition of sand flies in the district during July–September 2009 using polymerase chain reaction techniques. In all, 2088 sand flies were collected from 3 villages. Nine sand fly species were identified: *Phlebotomus kandelakii* (Shchurenkova), *P. major* (Annandale), *P. halepensis* (Theodor), *P. papatasi* (Scopoli), *P. sergenti* (Parrot), *P. longidactus* (Parrot), *P. caucasicus* (Marzinovsky), *Sergentomyia sintoni* (Pringle) and *S. sumbarica* (Perfil'ev). *P. sergenti* and *P. kandelakii* were the most prevalent *Phlebotomus* species at 31.3% and 10.0% respectively. Of 59 female *P. kandelakii*, 2 (3.4%) were naturally infected with *L. infantum*. This is the first finding of natural infection of *P. kandelakii* by *L. infantum* in this region suggesting *P. kandelakii* may be the vector of *L. infantum* in the area although it is the second most prevalent phlebotomine species.

Première détection de *Leishmania infantum* dans *Phlebotomus kandelakii* à l'aide de méthodes moléculaires dans le nord-est de la République islamique d'Iran

RÉSUMÉ Le district de Shirvan dans le nord-est de la République islamique d'Iran est un nouveau foyer de leishmaniose viscérale. La présente étude visait à identifier le(s) vecteur(s), le parasite et la composition des espèces des phlébotomes dans le district, de juillet à septembre 2009, à l'aide de techniques d'amplification en chaîne par polymérase. Au total, 2088 phlébotomes ont été prélevés dans trois villages. Neuf espèces de phlébotomes ont été identifiées : *Phlebotomus kandelakii* (Shchurenkova), *P. major* (Annandale), *P. halepensis* (Theodor), *P. papatasi* (Scopoli), *P. sergenti* (Parrot), *P. longidactus* (Parrot), *P. caucasicus* (Marzinovsky), *Sergentomyia sintoni* (Pringle) et *S. sumbarica* (Perfil'ev). *P. sergenti* et *P. kandelakii* étaient les espèces de phlébotomes les plus répandues (31,3 % et 10 % de l'échantillon respectivement). Sur 59 *P. kandelakii* femelles, deux (3,4 %) étaient naturellement infectées par *L. infantum*. Il s'agit de la première détection d'une infection naturelle de *P. kandelakii* par *L. infantum* dans la région, suggérant que *P. kandelakii* pourrait être le vecteur de *L. infantum* dans la zone, même si elle n'est que la deuxième espèce la plus répandue de phlébotomes.

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Introduction

Leishmaniasis are a broad spectrum of diseases, ranging from self-limiting localized cutaneous lesions to visceral leishmaniasis (VL) with fatal spontaneous evolution [1]. The visceral form of the disease is the most severe and is nearly always fatal if left untreated [2].

More than 90% of VL cases in the world are reported from Bangladesh, Brazil, India and Sudan [3]. VL caused by *Leishmania infantum* affects approximately half a million new patients each year in the Mediterranean region and Latin America [4]. In the Mediterranean basin, domestic dogs (*Canis familiaris*) are the principal reservoir host, and some species of sand flies belonging to the subgenus *Larrousius* are the primary vectors [3].

VL occurs sporadically throughout the Islamic Republic of Iran. However, there are 4 important endemic foci in the country: Ardebil and East Azerbaijan provinces in the north-west of the country and Fars and Bushehr provinces in the south [5–8]. Two sand fly species, *Phlebotomus (Lar.) perfiliewi* (Parrot) and *P. (Lar.) kandelakii* (Shchurenkova) have been shown to be vectors of *L. infantum* in north-western Islamic Republic of Iran [9–11]. The species of *P. (Paraphlebotomus) alexandri* (Sinton) and *P. (Lar.) major* (Annandale) have been found naturally infected with *L. infantum* and are the VL vectors in the south part of the country [12–14]. *P. (Lar.) keshishiani* (Shchurenkova) and *P. (Para.) caucasicus* have been reported infected with promastigotes in southern Islamic Republic of Iran but no parasite identification was carried out [15,16].

This study was carried out to identify the vector(s) and the parasite, as well as the species composition of sand flies in a new VL focus, Shirvan district, in north-eastern Islamic Republic of Iran.

Methods

Study area

The study was conducted in Shirvan district, North Khorassan province, north-eastern Islamic Republic of Iran. This region is 1350 meters above sea level. The total population of Shirvan district was around 164 000 in 2006. The northern part of Shirvan is mountainous with cold weather and the southern part has a temperate climate due to the flow of the Atrak River. The warm season is short (mid-May to mid-September) in the mountainous areas. The main occupations of the population are farming and raising animal.

Sand fly collection

Based on previous mass screening of dogs using the DOT test and confirmation that canine species as a reservoir of VL in Shirvan district (Mohebbali et al., unpublished data, 2008), 3 villages, Gholban, Starkhi and Hosseinabad, were selected. Sand flies were collected biweekly from indoors (e.g. bedroom, guest bedroom, toilet, and stable) as well as outdoors (wall cracks and crevices and animal burrows) by using sticky paper (60 papers per village) during July–September 2009. All traps were installed at sunset and collected near sunrise. The sand fly specimens were washed in 96% ethanol alcohol to get rid of the sticky materials and to preserve them. Dissection of preserved sand flies was done in phosphate buffered saline (PBS) solution. The terminal segments of the abdomen containing the spermatheca and the heads of females were removed and mounted in a drop of Puri medium and identified to species level using keys of Theodor and Mesghali [17]. The remains of the bodies of the sand flies were kept individually in 96% alcohol and stored at –20 °C for molecular analysis.

DNA extraction

DNA of the specimens was extracted using the Bioneer Genomic DNA

Extraction Kit (North Korea), according to the manufacturer's instructions. Extraction was carried out on the remaining body of the individual sand fly and stored at 4 °C. Double distilled water and DNA from *L. major*, *L. tropica*, and *L. infantum*, provided to the Iranian Institute of Pasteur by the World Health Organization, were used as negative and positive controls.

DNA amplification and PCR-RFLPs

Primary examination for infection of sand flies with *Leishmania* species was performed using nested-polymerase chain reaction (PCR) against the minicircle kinetoplast (k)-DNA using the following primers [18]: CS-B2XF (forward): 5'-C/GA/GTA/GCAGAAAC/TCCCGTTCA-3' (20 bp); CSB1XR (reverse): 5'-ATTTTTCG/CGA/TTT/CGCAGAACG-3' (20 bp); 13Z (forward): 5'-ACTGGGGGTG-GTGTAATAAG-3' (22 bp); LIR (reverse): 5'-TCGCAGAACGCC-CCT-3' (15 bp).

Restriction fragment length polymorphism (RFLP) PCR was done. Positive samples against kDNA were tested against the ribosomal internal transcribed spacer 1 (ITS1) region using the primers LITSR (5'-CTG-GATCATTTCCGATG-3') and L5.8S (5'-TGATACCACTTATCG-CACTT-3') followed by digestion by *HaeIII* [19].

The PCR products were run along with a 100 bp ladder on 1.2% agarose gel containing ethidium bromide for 1 h at 80 V. The gel was observed on a ultraviolet (UV) transilluminator and then digital photographs were prepared. Parasites were identified by comparison with positive controls of *L. infantum*, *L. major* and *L. tropica* and molecular weight markers.

We added 2 µL *HaeIII* to the ITS1 PCR products (20 µL) at 37 °C for 12 h with conditions recommended by the supplier (Fermentas, Germany). The restriction fragments were subjected

to electrophoresis in 3% agarose gel containing ethidium bromide (0.5 µg/mL) for 3 h at 65 V and observed on a UV transilluminator [20,21].

Cathepsin B-like cysteine protease E/F PCR

Cathepsin B-like cysteine protease (cpb)E/F PCR was done. This PCR is species-specific for *L. donovani* complex and was developed by Hide and Bañuls [21]. cpbE/F amplification was done in 30 µL with 6 pmol of each primer (forward: 50-CGTGACGCCGGT-GAAGAAT-30; reverse: 50-CGTG-CACTCGGCCGTCTT-30), 4.5 nmol dNTPs, 1 U *Taq* polymerase, 3 µL buffer 10x and 1 µL of DNA extracted from individual sand flies. Thirty cycles were necessary for amplification (denaturation 30 s at 94 °C, annealing 1 min at 62 °C and elongation 1 min at 72 °C), followed by 10 min at 72 °C. All of the amplification reactions were analysed by 1%–1.5% agarose gel electrophoresis, followed by ethidium bromide staining and visualization under UV light. Standard DNA fragments (100 bp ladder, Fermentas) were used to permit sizing.

Restriction of cpb PCR fragments

This assay was developed by Oshaghi et al. [22] for discrimination of *L. infantum* from *L. donovani* using restriction of cpb PCR products by *Dra*III enzyme. Digestion was carried out in a total volume

of 20 µL, with approximately 10 ± 5 ng of DNA (10–15 µL PCR products) and 5 U of restriction enzyme in the recommended buffer, overnight at the recommended temperature. Restriction fragments were separated at 120 V for 1 h in 2% agarose gel and ethidium bromide staining.

Results

Altogether, 2088 sand flies were collected from the above-mentioned villages (3 locations per village) and identified. *P. sergenti* (Parrot) and *P. kandelakii* (Shchurenkova) were the most prevalent *Phlebotomus* species found at 31.3% and 10% respectively. Other species included *P. papatasi* (Scopoli) (6.3%), *P. caucasicus* (Marzinovsky) (5.3%), *P. major* (Anandale) (5.2%), *P. halepensis* (Theodor) (0.9%), *P. longidactus* (Parrot) (0.8%), *Sergentomyia sumbarica* (Perfil'ev) (27.5%) and *S. sintoni* (Pringle) (12.7%). Among the sand flies collected 31% were female and the rest were male. Abdominal examination of female specimens showed 70% were unfed, 15% semigravid, 10% gravid and 5% blood fed (Table 1). In total 260 females (40% of all collected females) were selected for PCR examination and parasite detection.

Of 260 female specimens tested for promastigote infection, only 2 out of 59

(3.4%) *P. kandelakii* were found naturally infected with *L. infantum/L. donovani*. This was observed in the kDNA nested-PCR amplification assays where a 680 bp PCR band was produced. This length of PCR in the system is assigned to *L. infantum/L. donovani*. This was then confirmed by ITS1 PCR-RFLP using *Hae*III enzyme. The diagnostic fragments are 220 and 140 bp for *L. major*, 200, 80 and 60 bp for *L. infantum/L. donovani*, and 2 fragments of 200 and 60 bp are for *L. tropica*. The result of PCR-RFLP revealed 200, 80 and 60 bp bands, which is indicative of *L. infantum/L. donovani* (Figure 1).

To discriminate between *L. infantum* and *L. donovani*, cpb PCR was performed and a 702 bp was produced for both specimens. The restriction digestion of the cpb PCR products with the enzyme *Dra*III gave intact PCR products (702 bp) that were associated with the presence of *L. infantum* (Figure 2).

Of 59 female *P. kandelakii*, 2 (3.4%) were found naturally infected with *L. infantum*. One infected specimen was caught from a living room and the other from a yard in Starkhi village.

This is the first report of *P. kandelakii* naturally infected with *L. infantum* in Shirvan district, northern Khorassan province, north-eastern Islamic Republic of Iran.

Table 1 Species composition of sand flies in Shirvan district, north-eastern Islamic Republic of Iran, 2010

Species	Male	Female	Unfed	Fed	Semi-gravid	Gravid	Total	
	No.	No.	No.	No.	No.	No.	No.	%
<i>Phlebotomus kandelakii</i>	150	59	40	3	6	10	209	10
<i>P. major</i>	99	10	10	0	0	0	109	5.2
<i>P. halepensis</i>	19	0	0	0	0	0	19	0.9
<i>P. papatasi</i>	81	50	34	4	5	7	131	6.3
<i>P. sergenti</i>	451	203	140	10	30	23	654	31.3
<i>P. longidactus</i>	17	0	0	0	0	0	17	0.8
<i>P. caucasicus</i>	110	0	0	0	0	0	110	5.3
<i>Sergentomyia sintoni</i>	125	140	100	7	21	12	265	12.7
<i>S. sumbarica</i>	388	186	130	8	35	13	574	27.5
Total	1440 (69%)	648 (31%)	454 (70%)	32 (5%)	97 (15%)	65 (10%)	2088	100

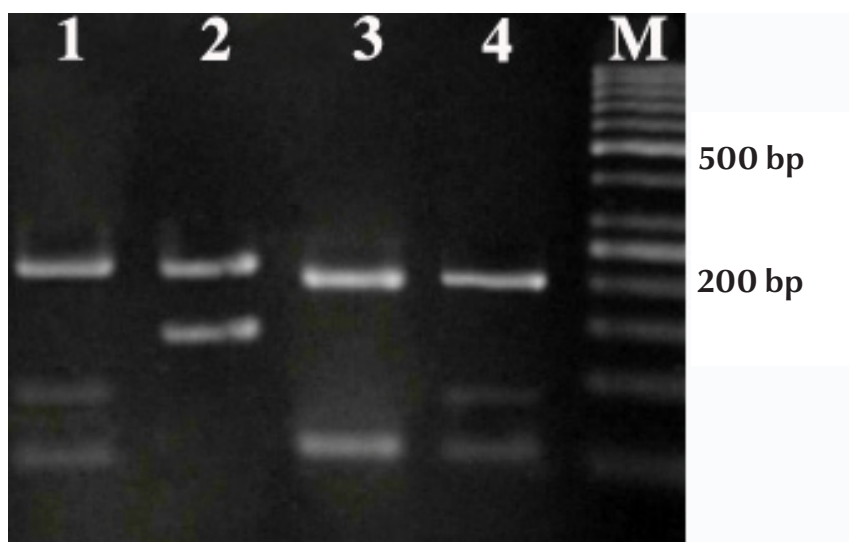


Figure 1 ITS1-rDNA PCR-RFLP analysis of selected strains of *Leishmania* species using *HaeIII* restriction enzyme. Lanes: M, 100 bp ladder (Fermentas); 1, infected *Phlebotomus kandelakii* to *L. infantum*; 2, *L. major* (MHOM/IR/75/ER: serial code of the standard *L. major* species at the reference laboratory of the Parasitology Department, Tehran University of Medical Sciences); 3, *L. tropica* (MHOM/IR/03/Mash-878); 4, *L. infantum* (positive control)

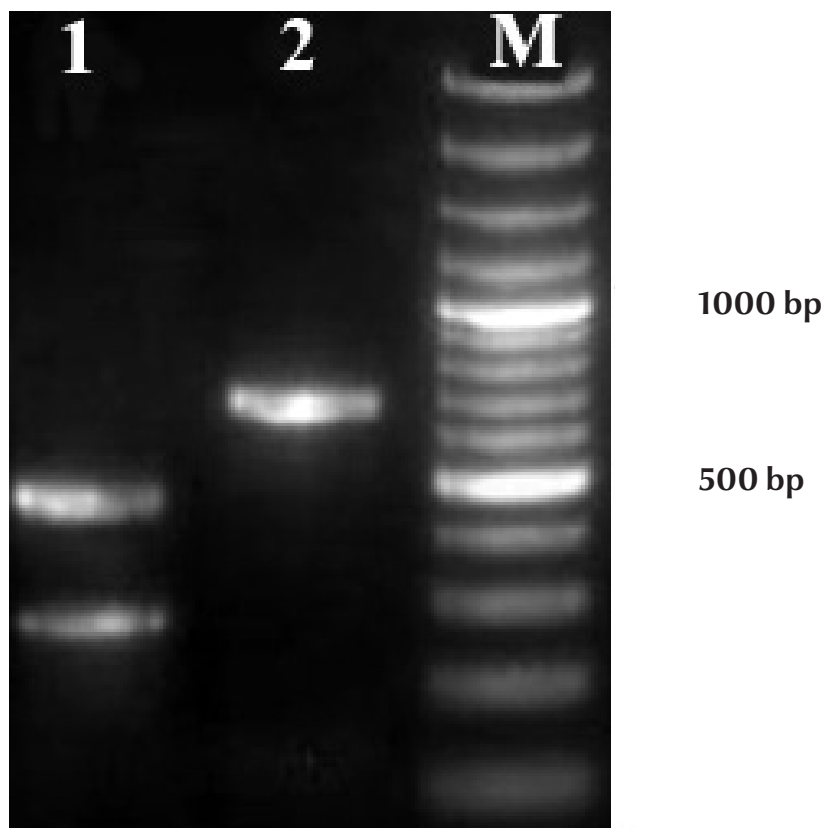


Figure 2 cpbE/F gene PCR-RFLP analysis of selected strains of *Leishmania donovani* complex with the use of the restriction enzyme *DraIII*. Lanes: M, 100 bp ladder (Fermentas); 1, *L. donovani*: cpbF/cpbE (MHOM/DZ/82/LIPA59); 2, *L. infantum* from infected *Phlebotomus kandelakii*

Discussion

The ecology and epidemiology of leishmaniasis are important measures for management and planning of disease control. Entomological surveys accompanied by epidemiological data provide essential information for the design of control programmes of the disease.

Several epidemiological and entomological findings, including anthropophily, common infection of the sand flies with the same *Leishmania* parasite found in the patients in the same places, suggest the capacity of the sand fly to be a vector [17]. In the current study, infection of *P. kandelakii* with *L. infantum* was confirmed using sensitive PCR technique.

Isoenzyme detection methods provide the gold-standard for identifying species and reference strains of *Leishmania*, but this method has disadvantage as it requires the culture of a large number of parasites and primary isolates can easily become contaminated, or a mixed infection can yield only the strain that grows fast in laboratory conditions [23]. The highly sensitive technique of PCR has been used before to detect *Leishmania* in New and Old World sand flies [24,25]. Some species of sand flies belong to subgenus *Larroussius* and are potential vectors of VL in the Mediterranean basin. Aransay et al. in 2000 used this method in Greece for detection of *Leishmania* infection in *P. (Lar.) neglectus*, *P. (Lar.) tobbi*, *P. (Lar.) simici*, and *P. (Para.) alexandri* using a semi-nested PCR technique [9]. Based on PCR detection and sequencing of the parasite cpb, Oshghi et al. in 2009 confirmed *P. perfiliewi transcaucasicus* was circulating both *L. donovani* and *L. infantum* in north-western Islamic Republic of Iran [11]. In our study species-specific PCR of the cpbE/F gene against the 2 positive ITS rDNA specimens of *P. kandelakii* revealed the presence of *L. infantum* in the sand fly. This method is able to separate infection of sand flies with *L. donovani*

complex, i.e. to distinguish *L. donovani* from *L. infantum* [22].

P. kandelakii has been reported as a primary and proven vector of VL in an important focus of disease, i.e. north-western Islamic Republic of Iran [9]. This species has been reported in central Asia, Afghanistan, Islamic Republic of Iran, Lebanon, Turkey and the former USSR [23]. *P. kandelakii* is very hydrophilic, moderately thermophilic and bites man and large animals easily in Afghanistan [26,27]; it is also considered to be a vector of VL in the South Caucasus region [27].

The results of blood meal analyses indicate the *P. kandelakii* collected in north-western Islamic Republic of Iran are strongly anthropophilic with 32.8% containing human blood and 21.2% canine blood. *P. kandelakii* may also

therefore play an important role in the transmission of VL to dogs, which are the main domestic reservoir of disease [28]. Among the female sand flies examined, only 2 specimens of *P. kandelakii* were found naturally infected by *L. infantum* parasites. Both infected sand flies were empty and it seems the females' longevity was enough to complete the parasite's cycle in its body.

Based on its natural infection with *L. infantum* and the fact that it was the only species found infected with *L. infantum*, we conclude that *P. kandelakii* can be incriminated as the vector of VL in north-eastern Islamic Republic of Iran. This is the first detection of *L. infantum* in *P. kandelakii* from Shirvan district, north-eastern Islamic Republic of Iran. It is recommended that the detailed biology of *P. kandelakii*,

particularly its host preference, should be investigated using the available molecular method. This, in combination with other ecological data, could be used in vector control measures of VL in the region [29].

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Handbook for integrated vector management

Integrated vector management (IVM) is a rational decision-making process for optimal use of resources for vector control. The aim of the IVM approach is to contribute to achievement of the global targets set for vector-borne disease control, by making vector control more efficient, cost-effective, ecologically sound and sustainable. The *handbook for integrated vector management* presents an operational framework to guide managers and those implementing vector-borne disease control programmes in designing more efficient, cost-effective systems. The handbook discusses the policy and institutional framework for IVM; planning and implementation of IVM; capacity-building, including human resource development; the core functions and essential competence required for IVM at central and local levels; the elements and processes of IVM; and a comprehensive framework for monitoring and evaluation of IVM, including indicators and methods for measuring process, outcomes and impact.

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