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Sergentomyia species identification and their screening for possible infection to *Leishmania* spp. in Kaleybar, East-Azerbaijan province, Iran

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Abstract

Leishmaniasis is a protozoal and vector-borne disease. World health organization has considered the disease as a neglected tropical disease. *Phlebotomus* and *Lutzumyia* species (order: Diptera, family: Psychodidae) are human leishmaniasis vectors in new and old worlds. *Sergentomyia* spp. (Diptera, Psychodidae) are proven vectors of lizard leishmaniasis. Although some studies have identified human *Leishmania* parasites in *Sergentomyia*, their role in parasite circulation is unknown yet. Hence, the parasitological and molecular methods were used to study the possible *Leishmania* infection of *Sergentomyia* spp., in the human and canine visceral leishmaniasis endemic area in North West of Iran. Even though *Sergentomyia* specimens were caught in a dominant number compared to *Phlebotomus* spp., no *Leishmania* promastigote or DNA was detected in live-caught or sticky trap-caught specimens, respectively. *Sergentomyia* spp. are proven vectors of sauroleishmaniasis, and despite several global reports of *Leishmania* infection in *Sergentomyia* spp., such findings should be carefully interpreted to avoid false vector incriminations.

Introduction

Leishmaniasis is a vector-borne disease listed as one of the neglected tropical diseases by world health organization (WHO). Different species belonging to an obligatory intra-cellular parasite named *Leishmania* spp. are associated with different clinical forms of disease (visceral, cutaneous and mucocutaneous).[1] At least over 1.00 billion people are at risk of one kind of *Leishmania* infection. The visceral form is fatal; but, most cases are cutaneous and mainly occur in 10 countries including Iran.[1]

Leishmaniasis is caused by several *Leishmania* spp. that obligatory infect phagocytic host cells. It is the second most severe neglected tropical disease, behind malaria. Depending on the affected tissues, leishmaniasis can be cutaneous, visceral or mucocutaneous.[2,3] In Iran, *Leishmania major*, *L. tropica* and *L. infantum* are regarded as agents of the zoonotic cutaneous, anthroponotic cutaneous and visceral forms, respectively.[4,5] Estimated disability-adjusted life years (DALYs) in Iran due to leishmaniasis are 229714. For treatment, in addition to using the medicines such as meglumine anti-moniate and sodium stibogluconate, several studies have been performed on plant compounds.[6,7]

Leishmania is biologically transmitted by sand flies in a cyclo-propagative way, meaning that *Leishmania* parasites need Phlebotominae sand flies to propagate and complete their life cycle.[8] *Sergentomyia* species which belong to Phlebotominae subfamily, preferentially feed on reptiles which consequently are proven vectors of *Sauroleishmania.*[9,10]

Although several reports have indicated their blood-feeding on mammals including humans, rodents and dogs and human *Leishmania* parasites have been detected in *Sergentomyia dubia* and *S. schwetzi* in Senegal,[9,11-14] their role in human leishmaniasis circulation has remained under question. We survey the possible infection of *Sergentomyia* specimens in a visceral leishmaniasis (VL) focus in North West of Iran. Several Phlebotominae

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species, mainly belonging to *Larrossius* and *Adlerius* subgenus, are suspected of transmitting VL in main foci located in the North West, South and South West of the country, especially in nomadic places.[15-18]

Most of the present data in Iran are concentrated on the fanustic and phylogenetic studies of *Sergentomyia* specimens. As far as the authors know, there is just one report of isolation of *L. major* and *L. gerbilli* from *S. sintoni* in Iran[19] along with one report of virus isolation from *Sergentomyia* spp. in Golestan province, Iran.[20] The objectives of this study were (i) to identify *Sergentomyia* species from Kaleybar, East-Azerbaijan province, Iran, (ii) to search for promastigote forms in the mid-gut of live-caught *Sergentomyia* spp., and (iii) to detect *Leishmania* DNA in the sticky trap-caught *Sergentomyia* specimens using kDNA marker.

Materials and Methods

Ethics statement. The committee approved the project on the ethics of the Research Department of the School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran, under the permit number of IR.SBMU.MSP.REC.1397.483 (Grant No.: 14285).

Study area. During an inventory research on sand flies in Kaleybar and Khoda-Afarin counties (East-Azerbaijan province, Northwest Iran; Fig. 1), where VL cases had been recorded in recent years, sand flies were collected.[21]

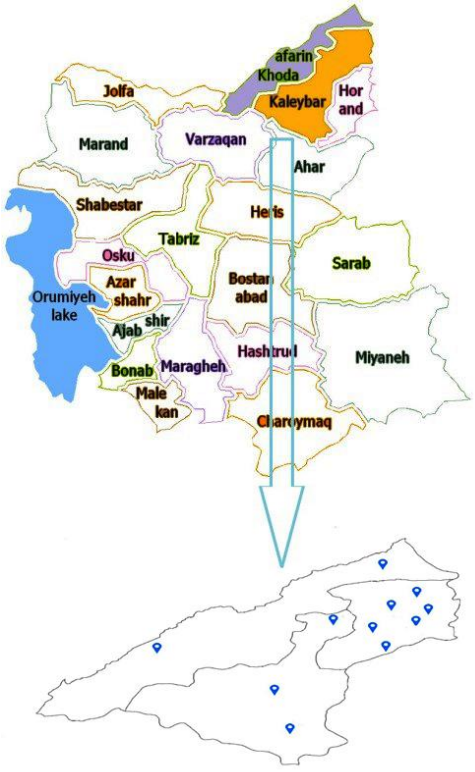

Fig. 1. Map of East-Azerbaijan province of Iran and details of the study areas and sand flies' collection sites.

The climate is moderate mountainous and the annual cumulative rainfall is 372 mm, with a mean average annual temperature and relative humidity of 13.60 ˚C and about 60.00%, respectively.[22] Sand fly specimens were collected in 11 villages, from different indoor locations (especially patient's house, if possible), domestic animal barns, ruins, dogs' shelters, grooves of walls and rocks and rodents nests. Sand flies were collected from July to late September 2018, using two methods including: 1) sticky traps to collect dead sand flies for molecular parasite detection being set before sunset and retrieved the following day, early in the morning; due to funding constraints and, more crucially, the confluence of the COVID-19 epidemic with the subsequent seasonal activity of sand flies, it was impossible to work in the field again in 2019 and the sand flies were carefully removed from traps using a fine paintbrush wetted with acetone and preserved in 96.00% ethanol, and 2) aspirator for collecting live sand flies to detect *Leishmania* promastigotes carried out between 7:00 to 10:00 AM.

Species identification and preparation. A common species identification method was used based on the morphology of female spermathecae and pharyngeal armature.[23] The specimens being narrow, lanceolate and symmetrical throughout the length having cibarium with one or more rows of teeth and pigment patch were considered to belong to the *Sergentomyia* genus.[23] They were then processed for species identification, promastigote detection (for live-caught specimens) and molecular detection of *Leishmania* spp. (for dead-caught specimens).

Identification at the species level. The head and final few abdominal segments were sliced using tiny, sterile needles, mounted between slide and cover slide with Puri's medium and then, coded identically to the body. The slides were carefully observed using a stereozoom microscope (CZM4; Labomed, Fremont, USA) at 10 and 40× magnifications and identified at species level using reliable identification keys.[24,25]

Promastigote detection. Female *Sergentomyia* spp. caught alive by aspirator were immobilized by putting in the fridge. Then, they were immediately dissected using sterile syringes in a drop of sterile saline (0.90%), and their mid-gut was microscopically examined for the presence of *Leishmania* promastigotes.

DNA extraction and polymerase chain reaction (PCR). Genomic DNA was extracted using the phenol/ chloroform method. According to Noyes *et al*. *Leishmania* kDNA was amplified by nested PCR.[26] The pooling method was used to amplify the specimen's genome to save time and budget. External primers for the first run were CSB2XF (CGAGTAGCAGAAACTCCCGTTCA) and CSB1XR (ATTTTTCGCGATTTTCGCAGAACG).[27] In the second run, internal primers, 13Z (ACTGGGGGTTGGTGTAAAATAG), LiR (TCGCAGAACGCCCCT) and 0.50 µL of the first round of

PCR products were used. The first stage of PCR was performed as follows: 5-min initial denaturation phase at 94.00 ˚C, 30 cycles of 1 min steps at 94.00 ˚C, 1 min steps at 55.00 ˚C as an annealing step, 1 min steps at 72.00 ˚C and 5 min extension at 72.00 ˚C at the end. It is worth mentioning that the amplification was conducted with a final volume of 10.00 µL, consisting of 4.50 µL of distilled water, 1.00 µL of each primer (10.00 pmol of each primer), 7.50 µL of 2X Master Mix (Ampliqon, Odense, Denmark), and 2.00 µL (5.00 ng) of diluted extracted DNA. The second step was exactly the same as the first step; only the annealing temperature was carried out at 57.00 ˚C, and the DNA was replaced by the product of the first step.[28] The amplification products were analyzed on 1.50% agarose gels and negative and positive controls were also monitored. The following reference strains were obtained from Department of Medical Parasitology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran and used as positive controls: *L. infantum*: MCAN/IR/07/Moheb.gh, *L. tropica*: MHOM/IR/02/Mash10 and *L. major*: MRHO/IR/75/ER. The expected amplified bands for *L. infantum*, *L. major* and *L. tropica* were 720, 570 and 750 bp, respectively.

Results

Sand flies' identification and species composition. Through the field works, 617 female sand fly specimens were captured by the sticky traps (577) and aspirator (40) methods. In the present research, only *Sergentomyia* specimens were subjected to further inquiry, including species-level identification and DNA analysis. Among 577 sand flies collected by sticky traps, microscopic identification at the genus level revealed that specimens belonging to the *Sergentomyia* genus (300 specimens; 52.00%) were more prevalent than those belonging to the *Phlebotomus* genus (277 specimens; 48.00%). Two species were identified among *Sergentomyia* specimens; the dominant one was *S. dentata* (80.60%) based on the following morphological characteristics: Median teeth of cibarium were shorter than the lateral ones (Fig. 2A) and buccal teeth ranged about 18-20 (Fig. 2B), and the other one was *S. sintoni* (19.40%), with uniform size cibarium teeth and less than 20 buccal teeth (Figs. 3A and 3B).

Dissection of *Sergentomyia* specimens for promastigote detection. A total of 40 *Sergentomyia* specimens were captured alive by the aspirator and processed as detailed above. All these specimens were identified as *S. dentata*. None of 40 female *S. dentata* was found to contain promastigotes.

Molecular detection of *Leishmania* kDNA. In total, 29 pools (6 for *S. sintoni* and 23 for *S. dentata*) were generated using female specimens. They were unfed and pooled according to the collection site and species. No kDNA-*Leishmania* was detected among the samples (Fig. 4).

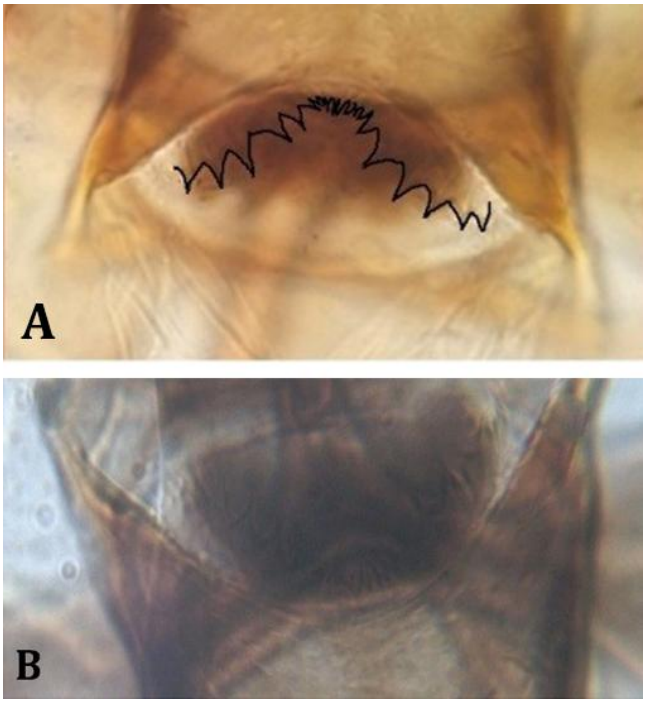

Fig. 2. Morphological characteristics of *Sergentomyia dentate*. **A)** Buccal teeth range about 18 - 20; **B)** Median teeth of cibarium are shorter than the lateral ones (40×).

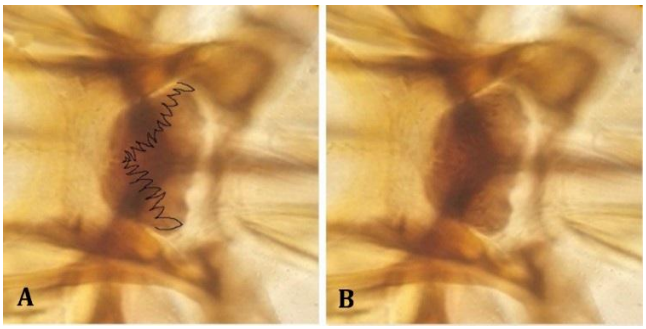

Fig. 3. Morphological characteristics of *Sergentomyia sintoni*. **A)** Buccal teeth are less than 20; **B)** Cibarium teeth have uniform size (40×).

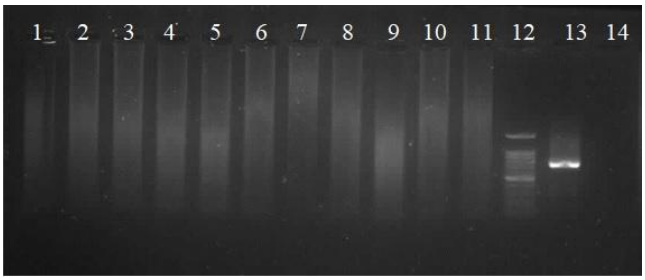

Fig. 4. Electrophoresis of *Leishmania* kDNA in *Sergentomyia* specimens collected from Kaleybar, East-Azerbaijan province, Iran. Lanes 1 to 11: Current study samples; Lane 12: Ladder marker 100 bp (Sinaclon, Tehran, Iran); Lane13: Standard strain of *Leishmania infantum* (MCAN/IR/07/Moheb.gh); Lane 14: Negative control.

Discussion

Sergentomyia specimens were the most numerous sand flies obtained in this investigation, consistent with previous reports of their supremacy over other sand flies.[14,29,30] In addition to being plentiful, the transmission function of a suspected species would be more plausible if metacyclic promastigotes of *Leishmania* spp were discovered in wild females, particularly those that had not been fed. Natural *Leishmania* infection was reported in *S. schwetzi* and *S. dubia.* Motile metacyclic form of *L. infantum* was found in the anterior part of the mid-gut in 0.40% of *S. dubia* and 0.79% of *S. schwetzi*. Moreover, more than 2.00% of these positive females were unfed, showing that the parasite could survive during blood-meal digestion or probably egg lying.[8] Senghor *et al*., have put this dogma "Leishmaniasis in the old world exclusively transmits by *Phlebotomus* species" under a challenge in their article.[14] In the current study, no promastigote was detected in live-caught *S. dentata*; this could be in terms of small sample size or probably in terms of the presence of *Phlebotomus* species as a proven and more efficient vector of leishmaniasis.

Promastigote detection in the mid-gut is valuable and highly recommended; but it is tedious and needs to be done by a well-trained and expert person. Furthermore, molecular techniques allowed the detection of *Leishmania* DNA within the sand flies. Here, we used a k-DNA marker to trace the *Leishmania* genome in all sticky traps-caught *S. dentata* and *S. sintoni*. *Leishmania* parasite has a single mitochondrion called kinetoplast containing a large number of kinetoplast DNA copies, making it suitable to detect the parasites, especially in the specimens that naturally contain a small number of parasites such as sand flies;[31] hence, we could not find any *Leishmania* DNA in *Sergentomyia* specimens. Final step of designating a vector to a particular sand fly, experimental transmitting of *Leishmania* to a susceptible host, is crucial;[8,10,11] due to challenges regarding sand fly colonization in laboratories, it is too difficult to test this criterion.

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Conflict of interest

There is no conflict of interest in this study.

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