کارگاه‌های آموزشی مرکز اطلاعات علمی

مقاله نویسی علوم انسانی

اصول تنظیم قراردادها

آموزش مهارت های کاربردی در تدوین و چاپ مقاله
Original Article
Sand Flies of the Subgenus Adlerius (Diptera: Psychodidae) in an Endemic Focus of Visceral Leishmaniasis and Introduction of Phlebotomus (Adlerius) comatus as a New Record for Iran

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(Received 12 Dec 2012; accepted 22 Jan 2013)

Abstract
Background: Sand flies of subgenus Adlerius has a wide geographical distribution in Iran and are mostly found in wild form in mountainous areas. They are always considered as probable vectors of visceral leishmaniasis. The objective of this study was to determine the Adlerius species and its composition in an endemic focus of zoonotic visceral leishmaniasis in northwest of the country.

Methods: Sand flies were collected from 6 different areas of Azarbaijan-e-Sharqi Province using sticky paper traps from August to September which is active season for sand flies in this area, in 2009. The flies were mounted and identified. The length of third antennal segments, ascoid, labrum, coxite, surstyle, style, aedeagus, genital filament, genital pump, width of style, and the end of aedeagus were measured and the number of costal hairs group was also counted as the morphological characters.

Results: A total of 30 adult sand flies, (26 males and 4 females) including Phlebotomus halepensis (46.8%), P. longiductus (13.3%), P. balcanicus (23.3%), P. comatus (3.3%), and Adlerius spp. (13.3%) belong to subgenus Adlerius were identified respectively in 6 counties. One P. comatus male was captured in front of a cave located in the hillside of a mountain covered with the vegetation in Varzeqan area.

Conclusion: The presence of at least 5 species of the subgenus Adlerius in Azarbaijan-e-Sharqi Province, an endemic focus of zoonotic visceral leishmaniasis in Iran, shows that the risk of parasite transmission among man and reservoir animals is high during the active season of sand flies. P. comatus is a new record for Iran and needs to be added to the list of Iranian phlebotomines of subgenus Adlerius.

Keywords: Phlebotomus (Adlerius) comatus, Phlebotomine sand flies, Visceral leishmaniasis, New species, Iran

Introduction

Visceral Leishmaniasis (VL) is a fatal protozoan disease caused by Leishmania donovani complex (Kinetoplastidae: Trypanosomatidae) transmitted by the bites of phlebotomine sand flies (Diptera: Psychodidae) of the genus Phlebotomus in The Old World and Lutzomyia in The New World. It is a serious illness that gives rise to epidemics and causes high mortality if left untreated. Visceral leishmaniasis appears as an opportunistic disease associated with the HIV infection and in other immune suppressed patients (Desjeux and Alvar 2003).
Leishmania infantum Nicolle, 1908 can also cause cutaneous lesions (WHO 2010). There are at least 7 endemic foci of Zoonotic Visceral Leishmaniasis (ZVL) in Iran as follows:

In Iran, some parts of Fars Province in the south and Ardabil Province in the northwest are indicated as the highly endemic areas while some parts of Azarbaijan-e-Sharqi Province in the northwest, Saveh and Qom in the central, Khorramabad area of Lorestan Province in the west, some parts of Khuzestan Province in the southwest and Khorassan-e-Shomali area in the northeast are known as the low endemic areas. In all foci, kala-azar is caused by L. infantum. Dogs, foxes and Jackals are main reservoir hosts (Yaghoobi-Ershadi 2012). Kala-azar is endemic in four counties of Azarbaijan-e-Sharqi province including Ahar, Sarab, Kaleybar and Azarshahr.

Five species of sand flies including P. kandekaki, P. perfiliewi, P. keshishiani, P. neglectus and P. alexandri are considered as probable vector species of ZVL in Iran (Yaghoobi-Ershadi 2012).

The subgenus Adlerius Nitzulescu includes about 20 species which some are suspected or proven vectors of ZVL in the old world, as follows:

Phlebotomus balcanicus in Armenia and Georgia, P. longiductus in China, Kazakhstan, Kyrgyzstan, Ukraine and Uzbekistan, P. halepensis in Syrian Arabie Republic and Georgia (WHO 2010).

The main key to species identification of subgenus Adlerius of Iran was prepared by Seyedi-Rashiti and Nadim (1992). They believed that females of the subgenus are indistinguishable. So the key is based on male morphology as follows:

1- Coxite with less than 29 hairs. Subterminal tubercle of aedeagus 19–28 mm from tip. Aedeagus short and thick. P. brevis
2- Coxite with more than 30 hairs. Aedeagus long. P. neglectus
3- Aedeagus with rectangular subterminal notch, coxite with 50 hairs or less (39–40). P. halepensis
- Subterminal tubercle of aedeagus not rectangular. Coxite with more than 50 hairs.
3- Coxite with 50–80 hairs. Genital filaments very long 8–15 times length of pump. P. longiductus
- Coxite with 69–105 hairs. Genital filament 8 times length of pump. P. balcanicus

In the last 3 decades ZVL has become an endemic disease in Azarbaijan-e-Sharqi Province, northwest of the country.

The objective of this study was to determine the Adlerius species and their population size in an endemic focus of ZVL in northwest of the country.

Materials and Methods

Study area

The Azarbaijan-e-Sharqi Province is located in the northwest of Iran (38.0766° N, 46.2800° E) bordering Armenia and the Republic of Azerbaijan countries and the provinces of Ardabil, Western Azarbaijan, and Zanjan (Fig. 1). It covers an area of approximately 47830 km², with altitude range of 1310 m in plain locale to 3722 m in highland locale. It has a cool and dry climate being in the main a mountainous region. Temperatures run between -17 °C in the winter and up to 40.6 °C in the summer. The annual precipitation is about 255 mm. It has a population of 3691270 people (SCI, 2006).

Sand flies collection

Sand flies were collected monthly from 6 different areas including Tabriz, Ahar, Sharabian-Esmailabad, Varzeqan, Kaleybar and Marand in Azarbaijan-e-Sharqi Province using 50 sticky paper traps (castor oil coated white papers 15x21 cm) from the beginning (August) to the end (September) of the active season in 2009. Traps were installed before sunset and collected before sunrise. Collected sand flies...
were removed from sticky papers using needle dipped and stored in 75% ethanol. Females of sand flies belong to the subgenus Adlerius are more or less morphologically identical and there is no key for identification so only male specimens were selected for morphological study (Seyedi-Rasht and Nadim 1992).

Mounting
The head and genitalia of individual male sand flies were cut off within a fresh drop of sterile saline on a clean slide then they were transferred to a drop of puri’s media between a slide and cover slip (Smart et al. 1965). Identification of specimens was carried out after 24 h using the keys of Theodor and Mesghali 1964, Artemiev 1973, Lewis 1982, Seyedi-Rasht and Nadim 1992.

Morphometric measurements
Morphometric measurements were done by Olympus microscope (CH-2) and photographs were taken by camera of Leica microscope (Model: DM 2000).

The specimen characters on the length of third antennal segments, ascod, labrum, coxite, surstyle, style, aedeagus, genital filament, genital pump, and also the width of style were measured with different magnifications. In addition, for the P. comatus male, the end of aedeagus was measured. Number of costal hairs group, a very important morphological character, for all Adlerius specimens were counted. The entire given measurements are in micrometer (μm).

Results
Adult sand flies belong to genus Phlebotomus were collected and identified. Males were significantly larger numbers than females from the area sampled (30:4). Thirty four Phlebotomus sand flies belong to the subgenus Adlerius (30 males and 4 females) were identified. Phlebotomus halepensis (46.8%), P. longiductus (13.3%), P. balcanicus (23.3%), P. comatus (3.3%), and females of Adlerius spp. (13.3%) have been captured during this study in 6 areas of the Azarbaijan-e-Sharqi Province (Table 1).

Phlebotomus halepensis was present in all areas. The relative abundance of this species was 46.8%, higher than of the other species in comparison. The most captured Adlerius specimens, including P. halepensis, P. longiductus and P. balcanicus, were related to Varzeqan area.

The altitudinal distribution and abundance of all Adlerius species collected from the study areas are shown in Table 1. Phlebotomus halepensis was found at an altitude range of 1200–1670 m. P. longiductus was captured from two different altitudes at 1200 and 1670 m, while P. balcanicus and P. comatus were only trapped at 1670 m. As the table shows different species of subgenus Adlerius have been collected at altitude of 1670 m in Varzeqan area. It seems that the highest altitudes are preferred for breeding places of these phlebotomine sand flies.

We found only one Phlebotomus comatus male from Varzeqan area, which was a new record for Iran. It was collected in front of a cave located in the hillside of a mountain covered with the vegetation in Varzeqan area. The humidity was measured as normal (60%). Table 2 shows the measurements of the species. In this specimen, the coxite is wide with dense group of about 176 hairs. The hairy spots are located almost the middle of the coxite. The paramere is pigmented at the end. Aedeagus with subterminal tooth at 23 μm from the end. The length of genital filament is 931 μm and F/P=6.5. Figure 2 shows photos of some important characters on the head eg, antennal segment 3, clypeus, epipharynx and palp and also the genitalian parts at the end of the abdomen eg, genital filament, genital pump, length and width of aedeagus, dense group of 176 hairs on coxite and pigmented paramere.
Table 1. Sand flies of subgenus *Adlerius* and their relative abundance (%) in 6 areas of Azarbaijan-e-Sharqi, an endemic province of visceral leishmaniasis in northwest of Iran

| Row | Area        | Altitude* | \( P. halepensis \) | \( P. longiductus \) | \( P. balcanicus \) | \( P. comatus \) | Adlerius sp. (Female) | Total |
|-----|-------------|-----------|---------------------|---------------------|---------------------|---------------------|-----------------------|-------|
| 1   | Tabriz      | 1200 m    | 1                   | 25                  | 3                   | 75                  | 0                     | 4     |
| 2   | Ahar        | 1367 m    | 3                   | 100                 | 0                   | 0                   | 0                     | 3     |
| 3   | Mianeh      | 1300 m    | 1                   | 25                  | 0                   | 0                   | 0                     | 3     |
| 4   | Varzeqan    | 1670 m    | 6                   | 40                  | 1                   | 6.7                 | 6                     | 15    |
| 5   | Kaleibar    | 1240 m    | 2                   | 100                 | 0                   | 0                   | 0                     | 2     |
| 6   | Marand      | 1334 m    | 1                   | 50                  | 0                   | 1                   | 50                    | 2     |
|     | Total       |           |                     |                     |                     |                     |                       | 4     |

*(Statistical Center of Iran, 2006)*

Fig. 1. Map of Azarbaijan-e-Sharqi province, showing sand fly sampling areas
Table 2. The comparison of morphometric measurements (μm) on males of *Phlebotomus (Adlerius) comatus* from Iran and Afghanistan

| Country | Iran Present study | Afghanistan (Seyedi-Rashti and Nadim) * |
|---------|-------------------|----------------------------------------|
| Number of Coxal setae | 176 | - |
| Ascoid 3 Length | 49 | - |
| Labrum Length | 270 | 270–300 |
| Clypeous Length | 180 | - |
| Cibarium teeth | Unarmed | Unarmed |
| Palp1 Length | 60 | - |
| Palp2 Length | 175 | - |
| Palp3 Length | 190 | - |
| Coxite Length | 450 | 420 |
| Surstyle Length | 450 | - |
| Style Length | 220 | 200 |
| Style width | 50 | - |
| Aedeagus Length | 170 | - |
| Aedeagus End | Subterminal tooth | - |
| A3 Length | 399 | 310–370 |
| Genital Filament | 931 | - |
| Genital Pump | 144 | - |

*(Seyedi-Rashti and Nadim 1992)

**Fig. 2.** *Phlebotomus (Adlerius) comatus* Artemiev male: 1A, Antennal 3(×400), 1B, Pharynx and pharyngeal armature (×400), 1C, Epipharynx (labellum, maxilla) and palp (×400), 1D, Genital filament, genital pump, Surstyle (×100), 1E, dense group of 176 hairs on coxite (×1000), 1F, Length and width of aedeagus, paramere (×630), 1G, Style and the seta(×400), 1H, Pigmented paramere (×400), 1I, Clypeus(×400)
Discussion

Most species of the subgenus Adlerius were considered as subspecies of Phlebotomus chinensis (Lewis 1978). Theodor and Mesghali (1964) supposed that some of the subspecies may have to be made into species. After the examination of numerous specimens from Afghanistan, central Asia and Caucasus it was concluded that the supposition of these scientists was right (Artemiev 1973).

Males of all described Adlerius species from Iran can be easily identified according to antennal formula, position of the coxal tuft and its number of setae and shape of aedeagus, but females are much more uniform. Prior to this study six species including P. halepensis, P. brevis, P. longiductus, P. balcanicus, P. turanicus and P. salangensis were recorded from Iran (Seyedi-Rashhti and Javadian 2008, Akhundi et al. 2012). Whether P. kabulensis has also been recorded in the past (Kasiri et al. 2000) but it has not been confirmed by senior entomologists in the country yet. Some morphometric measurements have been processed to characterize another new species for the list of phlebotomine sand flies of Iran (Table 3). Artemiev revised the Adlerius of Afghanistan in 1978. He caught and identified 44 males of P. comatus from Northern and Central Afghanistan including Kala-i-Naw (Badghis), Khulm, Bamyan and Gorband valley. He mentioned that it is a species of rocky mountain (1000–2600 m) and it seems to be moderately therophilic and hydrophilic. He described that male of P. comatus has antennal formula 2/3–5, 1/6–15, coxite very wide, with dense group of 165(126–203) hairs, distal border of the hairy spot at 0.47 (0.41–0.51) of coxite, paramere with wide pigmented end, aedeagus rather short, with subterminal tooth a 20 (16–24) .m from the end, Genital filaments 1040 (800–1280) .m long, F/P=8.2 (6.9-9.4).

Seyedi-Rashhti and Nadim (1992) mentioned that in Afghan specimens of P. comatus, the measurements are as follows: the length of antennal segment 3, 310–370 .m, labrum 270–300 .m, coxite 420 .m and style 200 .m (Table 3).

The Iranian male specimen of P. comatus has 176 numbers of coxal hairs. It is in the range record numbers of the Afghan specimen by Artemiev in 1978. Antenna 3 observed to be longer than flies recorded by Seyedi-Rashhti and Nadim (1992) but labrum is in the range record of the latter authors. The length of coxite is larger and style length is also bigger than which was described by Seyedi-Rashhti and Nadim in 1992.

According to the literature P. halepensis was recorded by Theodor and Mesghali from Tabriz county, Azarbaijan-e-Sharqi Province in 1964 and from Sarab county by Mesghali in 1961. During an entomological study of visceral leishmaniasis P. halepensis, P. brevis, P. longiductus and P. balcanicus were recorded in Kaleybar county at the same province in 2000 (Rassi et al. 2000). Our captures and identifications showed that P. halepensis, P. longiductus and P. balcanicus are common species in this province.

Phlebotomus brevis which had recorded from the area previously was not found in this study. Phlebotomus halepensis and P. brevis have wide distribution in Iran at altitudes between 800 and 1700 m above sea level mostly in mountainous regions. Phlebotomus longiductus and P. balcanicus have limited distribution in comparison of P. halepensis and P. brevis in the country. These four species are present in sympathy in the northwest of Iran (Ardehali et al. 1995).

We caught the Iranian specimen of P. comatus from Varzeqan area in elevation of 1670 m. Its coxite is also wide, paramere with wide pigmented ends. In comparison with other species aedeagus is short with subterminal tooth at 23 .m from the end and it is in the range record numbers of Artemiv’s
specimens. The ratio of F/P in Iranian specimens is also in the range of his record from Afghanistan.

It should be mentioned that Varzaqan is the mining center for base metal mining as well as gold and farming is the predominant occupation in the rural areas. Recently Spruce trees have also been planted as the start of a forestry industry at the same place.

More investigations are needed to reveal the distribution of *P. comatus* in Iran and its role in the transmission of *L. infantum* in the study area remains to be specified.

Acknowledgments

Authors thank the leishmaniasis staff of Esfahan, Meshkinshahr and Yazd health research stations, National Institute of Health Research for their contribution in sand fly collections. The study was funded by Deputy of Research of Tehran University of Medical Sciences through project number: 5146-27-01-86. The authors declare that there is no conflict of interest.

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