

IF: 0.925

Asian Pacific Journal of Tropical Medicine

journal homepage: www.apjtm.org



doi: 10.4103/1995-7645.225020

©2018 by the Asian Pacific Journal of Tropical Medicine. All rights reserved.

Phlebotomus (Adlerius) kabulensis (Diptera: Psychodidae) a new record sand fly species from Iran: Morphological and molecular aspects

Alireza Zahraei–Ramazani¹✉, Abedin Saghafipour², Yavar Rassi¹¹Department of Medical Entomology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran²Department of Public Health, Faculty of Health, Qom University of Medical Sciences, Qom, Iran

ARTICLE INFO

Article history:

Received 9 October 2017

Received in revised form 20 December 2017

Accepted 29 December 2017

Available online 2 February 2018

Keywords:

Phlebotomus (Adlerius) kabulensis

New geographical record

Morphometric

mtDNA cytochrome b gene

ABSTRACT

Objective: To represent a new geographical record, *Phlebotomus (Adlerius) kabulensis* (*P. kabulensis*), which is suspected to be a potential vector of visceral leishmaniasis. **Methods:** For the first time, *P. kabulensis* specimens were collected using the sticky paper traps method in outdoor places in mountainous areas with vegetation coverage of three provinces in Iran. Identification of males was based on ecological, morphological, morphometric and molecular (mtDNA cytochrome b gene sequences) criteria. Generally, males have two ascoids on the 8th antennal segment and one ascoid on segments 9th to 15th, aedeagus with normal obtuse-angled sub-terminal notch and coxite with 27–50 groups of hairs on the distal half of its body. **Results:** Morphometric measurement revealed that *P. kabulensis* specimens were the same as compared with seven other morphological characters in three provinces of the country but lengths of the coxite were significantly different. The PCR result of the cytochrome b (*Cyt b*)-mtDNA fragment shows 550-bp length, with its special nucleotide arrangement. The male and female of *P. kabulensis* were newly discovered members of the subgenus *Adlerius* from Iran. Initial DNA analysis indicated how distinct this species is. **Conclusions:** The results show that the *P. kabulensis* female will be identified by comparing with other *Adlerius* female groups regarding its morphometric characters and molecular sequencing.

1. Introduction

Phlebotomine sand fly's species recorded in Iran belong to the genera, *Phlebotomus* Rondani and Berté 1840, and *Sergentomyia* Franca and Parrot 1920[1]. The genus *Phlebotomus* has 12 subgenera[2]. Among them, the species of subgenus *Adlerius* Nitzulescu, in 1931 were reported to be the vectors of leishmaniasis and since then, a few studies have been carried out on this subgenus in Iran. Recently, eight species belonging to this subgenus: (i) *Phlebotomus (Adlerius) balcanicus* Theodor, 1958; (ii) *P. (Adl.) brevis* Theodor and Mesghali, 1964; (iii) *P. (Adl.) comatus* Artemiev 1978; (iv) *P. (Adl.) halepensis* Theodor, 1958;

(v) *P. (Adl.) kabulensis* Artemiev, 1978; (vi) *P. (Adl.) longiductus* Parrot, 1928; (vii) *P. (Adl.) turanicus* Artemiev, 1974 and (viii) *P. (Adl.) salangensis* Artemiev, 1978 have been reported in Iran[3-5]. *Phlebotomus chinensis* Newstead, 1916 is the type-species of this subgenus[6] (Artemiev, 1980), the vector of *Leishmania infantum* (*L. infantum*) in China[7]. The Phlebotomine sand flies of the subgenus *Adlerius* involved have seldom been identified, because despite the taxonomic efforts of Artemiev (1980) and others, the morphological characters have not clearly been distinguished between the females of about 20 *Adlerius* species[8]. In addition, identification of male *Adlerius* by morphological means replace was difficult by remains

This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-Share Alike 3.0 License, which allows others to remix, tweak and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

©2018 Asian Pacific Journal of Tropical Medicine Produced by Wolters Kluwer- Medknow

How to cite this article: Alireza Zahraei-Ramazani, Abedin Saghafipour, Yavar Rassi. *Phlebotomus (Adlerius) kabulensis* (Diptera: Psychodidae) a new record sand fly species from Iran: Morphological and molecular aspects. Asian Pac J Trop Med 2018; 11(2): 131-135.

✉First and corresponding author: Alireza Zahraei-Ramazani, Department of Medical Entomology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
Tel: +98 9133150287
Fax: +98 2188951393
E-mail: azahraei@tums.ac.ir
Foundation project: This study was sponsored by the Deputy of Research Affairs, Tehran University of Medical Sciences with project number: 5146-27-01-86.

a difficult task. They differed slightly because some morphological characters were closely related, for instance the location and number of hairs on coxite are the most important morphological characteristic features in identifying and distinguishing *Adlerius* species from others. At times, it is important to identify the number and location of hairs on the body of different species of sand flies to check how similar or different they are. According to morphological keys, total number of coxite hairs in *P. kabulensis* are between 27–50 and in *P. longiductus* the hairs on coxite are 50–85; therefore, when a specimen collected has 50 hairs on its coxite, it is difficult to say which of the two species it belongs to. Also, the antennae of sand fly is the first morphological character; in preparing morphological slides careful handling and care are needed during collection and mounting because of their minute and delicate body parts. The antennae usually break and disappear. Without the antennae, species identification becomes difficult or impossible. Also, the females of the subgenus *Adlerius* do not have morphological keys for species identification and the inclusion of its species can be justified on the basis of characters of the males' morphology[9,10]. Because morphological characters do not distinguish between species of the *Adlerius* subgenus, it is important to thoroughly revise the identification key based on the morphological characters. Additionally, access to modern molecular techniques and genetic information regarding these vectors and detailed classification of their systematic positions, it seems far too ambitious to state that genetic information will help to elucidate the biological, morphological, ecological and effective leishmaniasis control methods. In this study, we compared the morphological and morphometric characteristics of *P. kabulensis* male specimens and also extracted the sequence of cytochrome b (*Cyt b*) [Mitochondrial DNA (mtDNA)] gene of *P. kabulensis* sand fly in order to identify and distinguish females of this species in subsequent studies. Also, we compared the 68 sequences with that of species in the GenBank. This gene is normally 69 inherited maternally and had been used to relate geographical populations of many species of 70 *Larrousius* in the Mediterranean sub-region[11,12]. The aim of this study was to detect *P. kabulensis* from other *Adlerius* species regarding its morphometric characters and molecular sequencing.

2. Materials and methods

2.1. Study area

Sticky paper traps were used for collecting *P. kabulensis* specimens in mountainous areas with vegetation coverage (e.g., gardens, mountainous caves, reservoir hosts, trees, river side and stones) in outdoor places of Ilam province of Iran between 5 p.m. and 7 a.m. (33°38'14" N, 46°25'21" E and altitude: 1 381 m above sea level),

Lorestan (33.4871° N, 48.3538° E and altitude: 4 050 m above sea level) and Esfahan (32°39'25" N, 51°40'39" E and altitude: 1 571 m above sea level) provinces. In Esfahan province, sand flies were collected in August 2016. In Lorestan and Ilam provinces, July 2016 was the time of collection.

2.2. Monitoring sand flies

Sand fly specimens were preserved in glass containers with 96% ethanol. Sand flies were washed twice with distilled water. The head as well as the 4th and the 5th ends alongside the genitalia of the male sand fly were mounted separately on every slide and two drops of puri's medium were added and the cover slip was gently put on the specimen[10]. These slides of male sand flies were prepared for morphological identification and the morphometric measurements using standard keys[10,13-15]. Eight characters were measured on the 13 *P. kabulensis* specimens in order to know if there was any difference between these specimens in the three provinces. The morphometric measurements were done using ocular micrometer and Olympus Microscope (ch-2).

2.3. Statistical analysis

Begin sentence with difference of morphological measurements were carried out with ANOVA and Kruskal–Wallis test with a priori level of significance set at $P < 0.05$. Given that statistical tests were significantly different, post Hoc-Bonferroni test was used[16]. For the molecular description and identification of *P. kabulensis* females by pair-wise alignment comparison in subsequent studies, the authors used DNA sequence of the males for the *cytb*-mtDNA gene. The remaining sections of the abdomen, wings and legs of the sand fly were preserved in 96% ethanol in 1.5 mL sterile micro tubes for DNA extraction. The sequences were compared with sequences available in GenBank using BLAST available on <http://blast.ncbi.nlm.nih.gov>.

3. Results

In total of 46 specimens of *P. kabulensis* were collected, of which 14 specimens were from Esfahan, 15 from Lorestan and 17 were from Ilam provinces. The main morphological characters of subgenus *Adlerius* were: the coxite has no lobe, the style bears five spines, and the paramere was not truncated and carries no ventral process. The most aedeagus form has a sub-terminal minute fin-like barb. The female's spermatheca was incompletely segmented. In addition, the morphological description of male *P. kabulensis* was; presence of one ascoid on antennal segments 9–15, antenna 8 with two ascoids, aedeagus with normal obtuse-angled sub-

terminal notch, a group of 27–50 hairs on coxite with dark body, sub terminal tooth in aedeagus is 12 μm , coxite is moderately wide, distal border of the hairy spot is about 0.50% of the coxite and genital filament/genital pump was seven (Figures 1 and 2). Morphometric measurements among the specimens of *P. kabulensis* in the three provinces showed there was no significant difference between (i) the length of A3, (ii) the length of epipharynx, the length of surstyles, (iv) the length of styles, (v) the width of styles, (vi) the length of aedeagus, and (vii) the number of hairs on coxite (Table 1). Therefore, this indicates the *P. kabulensis* specimens in the three provinces were within the same population or there was adequate gene flow to keep the groups similar. But there was a significant difference between the lengths of the coxite of the specimens from the three provinces ($P = 0.045$). Post Hoc-Bonferroni test shows that the length of coxite of *P. kabulensis* specimens in Lorestan province was significantly more than that from Ilam province (Table 2). This showed that they were not morphologically similar judging from the length of the coxite. In the conduct of the molecular experiment, DNA was extracted from 12 specimens of *P. kabulensis* sand fly from which five were used for mtDNA-PCR. PCR product of a male specimen was sent to the Department of Genetics, Faculty of Health Tehran University of Medical Sciences (TUMS) for sequencing. In this study, PCR experiments for *Cyt b* gene, primers CB3-PDR and N1N-PDR were used. These primers amplified a fragment of 550 bp of the mitochondrial genome of Males specimens which were captured from mountainous areas with vegetation within Poldokhtar Township in Lorestan province. This was the first time the sequenced result of *P. kabulensis* has been submitted to the GenBank from Iran (ACCESSION NO: JX885994). The comparison alignment of the 528 nucleotides with the specimens in the GenBank using NCBI-BLAST software showed that was 87% similarity with *P. (Adl.) chinensis* with the accession number: HM747243.1 and E-value $2e-174$ (Figure 3). The *P. kabulensis* specimens have been deposited in the corresponding author's medical entomology laboratory in Tehran University of Medical Sciences, Iran.

Table 1

Samples used in morphometric study of *P. (Adl.) kabulensis* specimens in Iran.

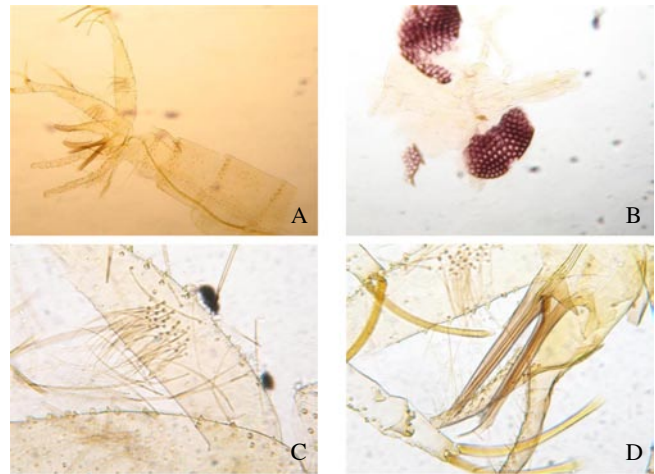
Molecular no.	Length (μm)						Width of style (μm)	Number of setae
	A3	Labrum	Coxite	Surstyle	Style	Aedeagus		
EN87	36	26	36	37	18	17	3	39
EN72	25	30	35	44	19	17	4	36
EN78	30	24	30	35	15	17	3	31
LP7	32	24	32	38	16	16	3	32
LPM5	-	-	39	40	19	17	3	36
LP8	-	-	39	40	18	19	3	40
LPM10	-	27	35	35	-	17	3	40
Em6 5	31	25	32	36	16	16	3	29
Em6 2	36	27	32	35	16	17	3	38
Em3' 11	35	26	32	36	16	16	3	30
Em3' 12	30	25	32	36	16	16	3	26
Em6 16	37	27	32	35	16	16	3	29
Em6 11	30	25	30	35	15	16	3	29

Table 2

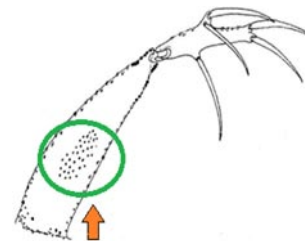
Post Hoc-Bonferroni test results showing comparison of the differences of the length of coxite of *P. kabulensis* specimens.

Province	Length of coxite ^A	95% Confidence interval
Esfahan	2.000 00±1.713 91 ^a	-7.896 5–2.729 9
Lorestan	2.583 33±1.851 24 ^{ab}	-2.919 1–6.919 1
Ilam	2.000 00±1.713 91 ^{ac}	0.092 9–9.073 8

^AData are expressed as Mean±SEM. Data with the same small letter (superscript) are not statistically different, while those with different letters are statistically different ($P < 0.05$).

**Figure 1.** Male *P. (Adl.) kabulensis* Artemiev, 1978.

(1A) Genital filament, genital pump and surstyle; (1B) Clypeus, epipharynx (labellum, maxilla) and palp; (1C) Dense group of 33 hairs on coxite; (1D) Length and width of aedeagus and paramere.

**Figure 2.** Drawing of coxite and dense group of hairs in *P. (Adl.) kabulensis* Artemiev, 1978.

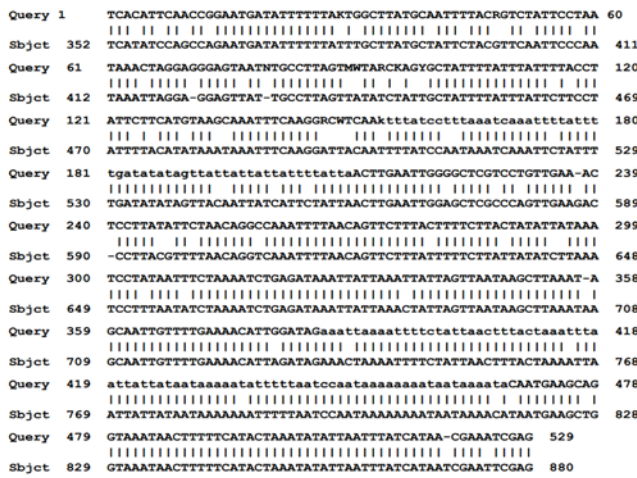


Figure 3. Sequence comparison of cytochrome b (*Cyt b*)-mitochondrial DNA between *P. (Adl.) kabulensis* specimen (Query, with a molecular number of LPM5) and *Phlebotomus chinensis* haplotype CYTB-10 (Sbjct, accession no. HM747243.1; partial cds; mitochondrial) in the GenBank.

The score, expect value, identities, gaps and strand are 621 bits(336), 2e-174, 465/532(87%), 6/532(1%), and Plus/Plus, respectively.

4. Discussion

The *Phlebotomus* genus entails all known medically important species in the Old World, including incriminated vectors of mammalian *Leishmania* species and of sand fly fever serogroup of arboviruses[17,18]. This genus included 12 subgenera and *Adlerius* Nitzulescu (1931) is one of the important subgenus in the Old World as it contains the species of leishmaniasis vectors[7]. It is a Central Asian species and it is possible that a number of Central Asiatic species have entered Afghanistan and Iran from central Asia. Artemiev (1978) revised the subgenus *Adlerius* specimens of Afghanistan. He captured *P. (Adl.) kabulensis* Artemiev (1978) which is found in dwellings and is rather thermophilic and hydrophilic. Since this species belongs to the subgenus *Adlerius* groups; he said, perhaps it can transfer visceral leishmaniasis agents like other species of this subgenus. But so far this has not been reported. Generally, there are five factors that all must be satisfied for vector incrimination: finding parasite in wild specimens, transmission from host to vector (in the laboratory), transmission from vector to vertebrate (Lab.), correlation of vector presence with disease outbreak, and laboratory confirmation that life cycle of parasite can be maintained in the purported vector. Although finding the parasite in collected specimens from the wild does not necessarily indicate that the species is a vector. Before the present study, known sand fly species of the subgenus *Adlerius* fauna from Iran were four species and this has been confirmed by senior sand fly specialists[1,10]. Currently, there are eight species of the subgenus *Adlerius* in Iran. So, it can be mentioned that through further field studies, one can discover a greater number of new records or even newer species due to the large area of the country. During the conduct of this study across

three provinces (Esfahan, Lorestan and Ilam) in Iran, *P. kabulensis* was collected in outdoor places situated far from human dwellings. These three provinces are located on the visceral leishmaniasis areas of the foothills to high altitude of the Zagros mountain range in the country and this geographical climate is similar to the dwellings of this species in Afghanistan. However, previous field surveys have given evidence of the subgenus's anthropophilic nature which is usual in human houses, cattle sheds and gardens[14], but the researchers collected all of them outside human dwellings. The collections were made by sticky paper traps but testing by other methods like CDC light traps may be more efficiency for this species. The *P. kabulensis* has not been naturally and/or experimentally proven as vector of leishmaniasis; however, it is important to note that its occurrence alongside known and proven vectors in *L. infantum* endemic regions enhances the risk of transmission. Nevertheless, the *P. kabulensis* of the subgenera *Adlerius*, which includes the potential vectors of Mediterranean kala-azar, suggests that the role of this species should be given serious attention in Iran. In this country, *P. kabulensis* is very uncommon and even the few that are present show little considerable variation in morphological characters. In this study, *P. kabulensis* species of sand flies were collected at low densities and in small numbers together with other local vectors; therefore, presently, there is little information available regarding its biology and ecology. Ecologically, the successful completion of the life cycle of *L. infantum* depends entirely on the number of efficient vector specimens. Based on the observed densities in nature, *P. kabulensis* cannot restrict *L. infantum* life cycle to itself. Notwithstanding, we cannot rule out the possibility that *P. kabulensis* plays a role in the transmission of *L. infantum*, as in cases of canine leishmaniasis. Sand fly taxonomy in most cases is based on morphometric means and is either measurable or countable characters. In this present study, length of the 3rd segment of antennae, length of style, length of epipharynx, length of coxite, width of style, length of substyle, length of aedeagus and number of hairs on coxite, eight taxonomic characters in all were analyzed. Aside these characteristic features, other measurable characters were illustrated in order to describe the morphology of the *P. kabulensis*. Some taxonomic characters of *P. kabulensis* from Iran have shown considerable morphological similarities such as the sub-terminal tooth in aedeagus, Filament/Pump and the hairs on coxite, having been compared with the published data of this species from Afghanistan[14]. The mean and standard deviation of eight morphometric characters of the species aided the researchers in the field of ecological study and control of the vectors. DNA sequences alone can be used for species identification. Previous studies have been showed that the PCR technique is an appropriate tool for detecting sand flies species from others[19-21]. *P. kabulensis* was studied and identified by morphological, morphometric and molecular characterization. The present study suggests a sand fly survey in Iran. Initial DNA analysis has shown how distinct this species is. More entomological intensive studies are needed in order to discover its distribution and abundance as well as further molecular comparisons to effectively control this species in Iran. This is because of its similarity with the female

specimen of *Adlerius* group sand flies in morphological characters and the morphological identification of the females of *P. kabulensis* sand fly is impossible too. So, by extracting and sequencing the DNA of the males of *P. kabulensis* sand fly, we can identify the female of this species by comparing their sequences in the future studies. It is feasible that the populations share vector competence. The natural or experimental infection of the *P. kabulensis* with *Leishmania* parasites thus seems attractive for future testing.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgment

Authors express their sincerest appreciation to the Mr. Amrolah Azarm for drawing the tables and set-out figures for publishing. This study was sponsored by the Deputy of Research Affairs, Tehran University of Medical Sciences with project number: 5146-27-01-86.

References

- [1] Yaghoobi-Ershadi MR. Phlebotomine sand flies (Diptera: Psychodidae) in Iran and their role on *Leishmania* transmission. *J Arthropod Borne Dis* 2012; **6**(1): 1-17.
- [2] Alten B. Speciation and dispersion hypotheses of Phlebotomine sand flies of the subgenus *Paraphlebotomus* (Diptera:Psychodidae): The case in Turkey. *Hacetatepe J Biol Chem* 2010; **38**(3): 229-246.
- [3] Akhundi M, Parvizi P, Baghaei A, Depaquit J. The subgenus *Adlerius* Nitzulescu (Diptera: Psychodidae, *Phlebotomus*) in Iran. *Acta Tropica* 2012; **122**(1): 7-15.
- [4] Zahraei-Ramazani AR, Kumar D, Yaghoobi-Ershadi MR, Naghian A, Jafari R, Shirzadi MR, et al. 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. *J of Arthropod-Borne Dis* 2013; **7**(1): 1-7.
- [5] Zahraei-Ramazani AR, Kumar D, Mirhendi H, Sundar S, Mishra R, Moin-Vaziri V, et al. A study of morphological and genotypic variations in the population of the subgenus *Adlerius* (Diptera: Psychodidae: Phlebotominae) in Iran. *J Arthropod-Borne Dis* 2015; **9**(1): 84-97.
- [6] Artemiev, MM. A revision of sand flies of the subgenus *Adlerius* (Diptera, Phlebotominae, *Phlebotomus*). *Zoologicheskii Zhurnal* 1980; **59**: 1177-1193.
- [7] World Health Organization. *Control of leishmaniasis. Technical Report Series 2010*. Geneva: WHO Export Committee; 2010: p. 949.
- [8] Seccombe AK, Ready PD, Huddleston LM. A catalogue of Old World Phlebotomine sand flies (Diptera: Psychodidae, Phlebotominae). *Occas Pap Syst Entomol* 1993; **8**: 1-57.
- [9] Seyedi-Rashti MA, Nadim A. The genus *Phlebotomus* (Diptera: Psychodidae: Phlebotominae) of the countries of the Eastern Mediterranean Region. *Iran J Public Health* 1992; **21**: 11-50.
- [10]Zahraei-Ramazani A, Leshan WD. Phlebotominae sand flies- Morphological and molecular approaches. Germany: Laplam-bert Publishing; 2016, p. 27-31.
- [11]Essegheir S, Ready PD, Killick-Kendrick R, Ben-Ismail R. Mitochondrial haplotypes and phylogeography of *Phlebotomus* vectors of *Leishmania major*. *Insect Mol Biol* 1997; **6**: 211-225.
- [12]Essegheir S, Ready PD, Ben-Ismail R. Speciation of *Phlebotomus* sand flies of the subgenus *Larrousius* coincided with the late Miocene-Pliocene aridification of the Mediterranean sub region. *Biol J Linn Soc* 2000; **70**: 189-219.
- [13]Theodor O, Mesghali A. On the Phlebotominae of Iran. *J Med Entomol* 1964; **1**: 285-300.
- [14]Artemiev MM. Sandflies (Diptera, Psychodidae, Phlebotominae) of Afghanistan Kabul. Ministry of Public Health; 1978, p. 4-87
- [15]Lewis DJ. A taxonomic review of the genus *Phlebotomus* (Diptera: Phlebotominae). *Bull Brit Mus Nat Hist (Ent)* 1982; **45**: 121-209.
- [16]Norusis, Marija J. PASW statistics 18 guide to data analysis. Prentice Upper Saddle River, New Jersey: Hall Press; 2010, p. 361-391.
- [17]Tesh RB. *Phlebotomus* fevers. In: Monath TP, editor. *The arboviruses: Epidemiology and ecology*. Boca Raton: CRC Press; 1988, p. 15-27.
- [18]Verani P, Ciufolini MG, Caciolli S, Renzi A, Nicoletti L, Sabatinelli G, et al. Ecology of viruses isolated from sand flies in Italy and characterized of a new *Phlebovirus* (Arbia virus). *Am J Trop Med Hyg* 1988; **8**: 433-439.
- [19]Tiwary P, Kumar D, Rai M, Sundar S. PCR-RFLP based method for molecular differentiation of sand fly species *Phlebotomus argentipes*, *Phlebotomus papatasi* and *Sergentomyia babu* found in India. *J Med Entomol* 2012; **49**: 1515-1518.
- [20]Gajapathy K, Peiris LB, Goodacre SL, Silva A, Jude PJ, Surendran SN. Molecular identification of potential leishmaniasis vector species within the *Phlebotomus (Euphlebotomus) argentipes* species complex in Sri Lanka. *Parasit Vectors* 2013; **6**: 302.
- [21]Khalid N, Elnaiem D, Aboud M, Al Rabba F, Triplet F. Morphometric and molecular differentiation of *Phlebotomus (Phlebotomus)* sandflies. *Med Vet Entomol* 2010; **24**(4): 352-360.