Research Article

# Monitoring of Vector-borne Diseases: Investigation of Feeding Preferences of the Sand Fly, *Phlebotomus perniciosus* (Diptera: Psychodidae) in a Focus of Cutaneous Leishmaniasis in Aichoun, North center of Morocco

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**ABSTRACT**

A survey was carried out to investigate the feeding habits of sand flies, with a focus on Cutaneous Leishmaniasis (CL) in Aichoun locality, North center of Morocco. The main objective of this study is to identify the blood meal of *Phlebotomus perniciosus*, vector of the leishmaniasis. CDC Light traps were selected as sampling methods and sand flies were collected during September and October 2013 in five stations and from 8:00PM to 6:00AM in outdoors habitats. In order to evaluate the role of phlebotomine sand fly in CL transmission, we identified the source of vertebrate blood by direct ELISA on nitro-cellulose membrane. The most prevalent species was *Phlebotomus perniciosus* (79.52%), followed by *Phlebotomus sergenti* (12.55%), *Phlebotomus papatasi* (7.47%), *Phlebotomus longicuspis* (0.29%) and *Phlebotomus ariasi* (0.14%). Among the 392 females collected, the source of blood was successfully identified with 67 of 151 (44.37%) sand flies and they were randomly selected and tested. Most females with which the blood source was identified belonged to *Phlebotomus perniciosus* (80.79%). Based on the results of blood meal analyses, the engorged females had been fed with a variety of vertebrate hosts, including humans, ruminants and chickens. The majority of analyzed blood reacted with anti-equine (34.61%) and anti-chicken (32.69%) sera. Forage ratios (FRs) indices gave different results for the large domestic animals. The FR for Sheep and Chicken was <1.0, indicating that *Phlebotomus perniciosus* had a preference on the other vertebrate hosts with FR > 1. This indicates that host choice was probably related to its availability rather than specific attractiveness. The results showed that the females of the engorged sand flies had been fed on a variety of vertebrate hosts, including humans, ruminants and chickens. This study will help to improve understanding the potential role of domestic animals in parasite transmission in CL endemic focus.

**Keywords:** Cutaneous Leishmaniasis, Phlebotomine Sand flies, direct ELISA, Blood meal identification, Forage ratio, *Phlebotomus perniciosus*, Aichoun, North center of Morocco.

## INTRODUCTION

According to World Health Organization (WHO), Leishmaniasis constitutes a serious public health risk.[1] On a worldwide scale, leishmaniasis infects between 1.5 and 2 million people each year (WHO, 2007).[2] In Morocco, leishmaniasis is an endemic disease posing a major threat to public health. 4319 cases of Cutaneous Leishmaniasis (CL) are reported and 107 cases of Visceral Leishmaniasis (VL).[3] From an epidemiological standpoint, 1242 cases of LC in the province of Sefrou were reported between 1997 and 2011 and he majority of cases are due to *Leishmania (L.) tropica* registered in the municipalities of El Menzel, Sefrou, Aghbalou, Tazouta Sidi Lahcen and Ain Chegag.[4]

The protozoan parasite is transmitted by sand flies, primarily by species of *Phlebotomus*. Zoonotic cutaneous leishmanisis is caused by *L. major* and transmitted by *Phlebotomus (Phlebotomus) papatasi.* [5,6,7] Anthroponotic CL (ACL) is caused by *L. tropica* is transmitted by *Ph. sergenti*.[5] There is also cutaneous and visceral leishmaniasis caused by *L. infantum* found in domestic dogs and humans and transmitted by *Phlebotomus (Ph.)* species of the subgenus *Larrossius*.[6]

Sand flies take blood meals from a wide variety of hosts, including human, livestock, dogs and chickens.[8] Transmission between vertebrate hosts is achieved by the blood-feeding habit of the Phlebotomine. Choice of a blood host is important for the parasite pathogen to complete its life cycle and to be transmitted to another host. The study of blood meal is important for an epidemiological survey. It allows orienting research which seeks to identify the probable reservoirs implicated in the epidemiological cycles of different forms of leishmaniasis.

The objective of this study is to identify the blood meal of the most abundant sand fly in Aichoun locality North center of Morocco; it will help improving the understanding of the potential role of domestic animals in parasite transmission in CL endemic focus.

## MATERIALS AND METHODS

### *Study area*

The study was conducted at different sites in Aichoun (33° 39'N, 04° 38'W) situated in northwest of Atlas Mountain

in Sefrou Province, North center of Morocco. The area is rural, lies at altitudes of 700-800m above sea level and has a semiarid climate with mean annual rainfall about 450mm. The majority of houses are built with clay and straw.

**Sand fly collection**

Collections were carried out in September and October, on five stations distributed to cover all supervised area. Sand fly collections were performed bi-weekly by CDC Light traps (Communicable Disease Centers of Atlanta, USA), a total of ten CDC Light traps were set at each trapping campaign and they were always positioned at a height of 60 cm above the ground. The traps were placed overnight from 6 pm to 8 am.

**Identification of sand fly species**

The collected sand flies were placed in glass vials containing 96% of ethanol and identified to species, by examination of the morphology of the pharyngeal armatures, spermathecae for the females and external genitalia for the males, using morphological key and the Activity guide of sand flies.[9,4] Morphological differentiation of the two sympatric species: *Ph. longicuspis* and *Ph. perniciosus* was made according to a description by Berchi et al. (2007).[10]

After recording the sampling data and locations, sand fly specimens were washed twice in sterile distilled water. Each specimen was then dissected in fresh drop of normal sterile saline by cutting off the head and abdominal terminalia with sterilized forceps and the single used mounted needles. The remainder of the body was stored in 96% ethanol. Of the many samples collected in the region, the source of blood was successfully identified in 67 of 151 sand flies, randomly selected and tested. Undetermined blood meals were probably due to the sand fly taking only a small amount of fresh blood or having time to digest most of the meal before capture.

**Production of Polyclonal Antibodies against animal species Immunoglobulin's**

Blood samples of various animals' species existing in the study area are collected. After centrifugation of 4000 rpm / min for 15 min, the sera were retained and stored in -20°C until it was used. The serums separated were used to Immunoglobulin isolation using ammonium sulfate precipitation. Add 210 mg of ammonium sulfate in a volume of 1 ml of each serum, let 3 hours at 4°C, centrifuge at 9000 rpm for 20 min and then retrieve the pellet in 2 ml of PBS (pH = 7. 4).

To study the host-blood preferences of sand fly, rabbits were immunized against animal immunoglobulins during 4 weeks using Freud adjuvant.

After one month, animals were sacrificed and blood samples were collected in dry tubes. After centrifugation for 15 minutes at 4000 rpm at 4ºC, sodium azide 0.02% was added to the sera and then frozen at - 20ºC until use.

**Testing of blood meals by a direct ELISA on nitrocellulose**

The blood origin was determined by a direct ELISA on nitro-cellulose membrane. Each female was homogenized in 50 µl of the buffered saline phosphate pH 8.4 (BBS). Then 1µl was coated onto nitro-cellulose membrane and left to dry at a temperature room for 15min. For blocking the remaining binding sites, membranes were incubated with skimmed milk for 1 h on a plate shaker. Membrane was then washed three times with BBS containing 0.1% Tween 20 (BBS-Tw20).[11]

The blood origin was detected by the addition of the anti-animal prepared sera (anti-chicken, anti-sheep, anti-dog, anti-cow, anti-cat, anti-horse, anti-mouse, anti-rat, anti-donkey, anti-human, and anti-goat). The conjugate preparations were incubated on the nitrocellulose membranes for 1 h on the plate shaker. After the washings, the Conjugate (anti-rabbit IgG peroxidase labeled) was added and nitrocellulose membranes incubated for 1 h on the plate shaker. Immune complex were then revealed by the addition of DAB (3.3'-diaminobenzidine) solution 0.1% and incubation of membranes for 30 min in the dark. The enzyme reaction was stopped by washing with tap water. Samples were considered positive if dark brown spots appeared.

**Data analysis**

The present paper applies the forage ratio technique to answer these questions.[12,13] The technique was used to study the food habits of sand flies and to determine the preferences of sand flies for the different vertebrate hosts existing at the collecting sites. The procedure simply compares the percent of use with the percent of abundance. Applied to blood-sucking mosquitoes, this is the percent of engorged mosquitoes which have fed upon a given vertebrate host divided by the percent which it comprises of the total population of hosts available in the mosquito's habitat. According to the method described by these authors, a forage ratio of one indicates neither preference nor avoidance of the indicated host animal; forage ratio significantly greater than one (>1) indicate selective preference; and values less than one (<1) indicate avoidance in favor of other hosts.

## RESULTS

***Sand fly fauna composition***

The numbers of sand fly specimens collected and identified at each site are shown in Table 1. A total of 669 sand flies were obtained during these months, with 41.40% males. Four species of *Phlebotomus* were identified. *Ph. perniciosus* (79.52%) was the most abundant especially in stable henhouse (station 5), while *Ph. sergenti* (12.55%) was recorded at all sites, mainly in stable henhouse. *Ph. papatasi* (7.47%) was present in three out of four sites monitored predominating in stable henhouse followed by *Ph. longicuspis* (0.29%) and *Ph. ariasi* (0.14%) were present successively in stable henhouse and multi-species pen. For engorged females,

151 blood-fed females were collected; *Ph. perniciosus* was the prevalent species (80.79%) followed by *Ph. papatasi* (9.93%) and *Ph. sergenti* (7.94%); *Ph. longicuspis* was represented by only two specimens.

**Table 1:** Abundance of sand fly species in five collecting sites in Aichoun locality, Sefrou province, 2013 during 2 month of entomological survey (September and October)

| Station | Collection site | Habitat composition | *Sandflies collected with relative abundance (%)* | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | ***Ph.sergenti (12, 55%)*** | | | ***Ph.perniciosus (79, 52%)*** | | | ***Ph.papatasi (7, 47%)*** | | | ***Ph.longicuspis (0, 29%)*** | | | ***Ph.ariasi (0, 14%)*** | | |
| | | | F | | M | F | | M | F | | M | F | | M | F | | M |
| | | | G | N | | G | N | | G | N | | G | N | | G | N | |
| **1** | multi-species pen | Cow, Dog, Horse, Sheep, Chicken, Cat, Human | 0 | 0 | 7 | 42 | 32 | 92 | 1 | 0 | 2 | 0 | 0 | 0 | 0 | 1 | 0 |
| **2** | sheep pen | Sheep, Dog, Cat, Chicken, Human | 0 | 0 | 0 | 3 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **3** | the winery | Goat, Donkey, Dog, Cat, Chicken, Human | 2 | 0 | 1 | 5 | 4 | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **4** | sheep pen | Sheep, Dog, Goat, Human | 2 | 3 | 2 | 1 | 5 | 5 | 0 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 0 |
| **5** | stable henhouse | Cow, Dog, Donkey, Cat, Chicken, Horse, Sheep, Human | 8 | 0 | 59 | 71 | 70 | 191 | 13 | 9 | 18 | 2 | 0 | 0 | 0 | 0 | 0 |

F: Female; M: Male; G: Engorged female; N: Non engorged female

**Host feeding preferences**

For the blood meal identification, the source of blood was successfully identified in 67 of 151 of the tested sand flies (44.37%). We have chosen the station 5 for two reason; the majority of sand fly and the existing different domestic animals in as different station are present at station S5. The females engorged had fed from a variety of vertebrate hosts, including humans, ruminants and chickens. Most females for whom the blood source was identified belonged to *Ph. perniciosus* (52/67). The majority of the sand fly specimens collected inside the stable hen house (S5) was found to have fed on the animals occupying this shelter. Some of these flies are depicted where it can be clearly seen by the spots marked on nitrocellulose membrane. The identification of blood meals demonstrated that the majority of *Ph. perniciosus* reacted with anti-equine (34.61%) and anti-chicken (32.69%) sera. The remaining females were positive for anti-body sera are shown in Table 2.

**Table 2:** Percent of blood-meal identification of the female of *Ph. perniciosus* captured in stable henhouse, in Aichoun locality, Sefrou province, September to October 2013, by direct ELISA on nitro-cellulose membrane

| Habitat composition in Station 5 (Stable henhouse) | Species of sandflies (number of female engorged) | Vertebrate used as source of blood-meal | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Cow | Dog | Horse | Cat | Human | Chicken | Donkey | Sheep | Goat |
| Cow, Dog, Donkey, Cat, Chicken,Horse, Sheep, Human | *Ph. perniciosus* (n=52) | 16.41 | 17.91 | 26.86 | 16.41 | 17.91 | 25.37 | 19.4 | 14.92 | 17.91 |
| | *Ph. sergenti* (n = 6) | 7.46 | 4.47 | 2.98 | 5.97 | 1.49 | 2.98 | 0 | 0 | 1.67 |
| | *Ph. longicuspis* (n=2) | 1.49 | 1.49 | 1.49 | 1.49 | 1.49 | 2.98 | 1.49 | 1.49 | 2.98 |
| | *Ph. papatasi* (n=7) | 0 | 0 | 0 | 0 | 1.49 | 0 | 0 | 0 | 1.49 |

If we looked the results of ELISA on nitrocellulose for *Ph. perniciosus* collected in Aichoun, we might say that the different animals anti-sera showed positively for engorged females species and the highest positivity observed in blood of horse (26.86%%) and chicken (25.37%) followed by blood of Donkey (19.4%), Dog (17.91%), Human and Goat with 17.91%, Cow and Cat with 16.41% and blood of sheep has also been identified with 14.92%. All hosts tested were present at the same time, *Ph. perniciosus* was found to have fed on almost all of them.

The FR for Sheep was < 1.0, indicating that *Ph. perniciosus* had a preference for other vertebrate hosts and apparently fed upon Sheep only as a “last resort” because of the unavailability of other host animals. Forage ratios were calculated for different vertebrate hosts; and those with FRs > 1.0 are shown in table 3

indicating that *Ph. perniciosus* had a preference for various animals.

**Table 3:** Percent of *Ph. perniciosus* reacting with each anti-species reagent and the corresponding forage ratio (FR) in stable henhouse, Aichoun locality, Sefrou province, 2013

| Species of *Ph. perniciosus* | | | |
|---|---|---|---|
| **Species of host** | **% Blood Meals (A)** | **% Host Population (B)** | **Forage Ratio (A/B)** |
| Cow | 16,41 | 3,24 | 5,06 |
| Dog | 17,91 | 3,82 | 4,68 |
| Horse | 26,86 | 1,09 | 24,64 |
| Cat | 16,41 | 2,73 | 6,01 |
| Human | 17,91 | 13,66 | 1,31 |
| Donkey | 19,4 | 2,73 | 7,1 |
| Sheep | 14,92 | 53,55 | 0,27 |
| Goat | 17,91 | 2,18 | 8,21 |
| Chicken | 25,37 | 16,93 | 1,49 |

Similarly to what has been observed by others.[12,15] The blood meal analysis of the engorged *Ph. perniciosus* revealed that this species fed on a broad variety of vertebrates hosts (Horses, Cat, Dog, Goat, Human, Donkey and Chickens) highlighting its opportunistic feeding behavior.

## DISCUSSION

Knowledge on the host preferences of phlebotomine sand flies is essential to have an idea of their vectorial capacity for different leishmaniasis foci in Aichoun, where sporadic human cases of CL continue to occur every year. Among 23 species described in Morocco, five sand fly species (22%) were identified in this area. *Ph. perniciosus* was clearly the dominant species, exceeding the cumulative abundance of the other species identified (79.52%); this species are proven vectors of the protozoan parasite of *L. infantum*.[14] The second predominant species identified was *Ph. sergenti* (12.55%), the confirmed vector of *L. tropica* throughout North Africa, the Middle East and Central Asia.[15, 16] In general, our survey confirms what previously observed on the sand fly fauna composition of Aichoun.[17]

Regarding the types of biotopes surveyed, *Ph. perniciosus* showed in the biotope which contains an accumulation of large amounts of organic matter and which may provide in the same place with a blood source, resting and breeding sites. In Morocco, several epidemiological and entomological findings, including the high abundance and endophily of this species suggest the capacity of *Ph. perniciosus* to be a vector of *L. infantum*.[6]

Blood meal identification in field-caught sand flies can reflect an association between sand flies and reservoir hosts. The analysis of *Ph. perniciosus* shows: (i) the zoophylic preferences of this sand fly species justified by the not selectivity of an host animal, (ii) that the region continues to be an endemic focus for canine leishmaniasis; cutaneous leishmaniasis due to *L. infantum* occurs sporadically in the north of Morocco as reported by many researches. [6, 18] The dog is the main domestic reservoir of the species.[6]

In this study, the large distribution and the long activity period of *Ph. sergenti* and species of the subgenus *Larroussius* such as *Ph. perniciosus* in Aichoun locality indicate the high potential risk of *L. tropica* and *L. infantum* transmission in this area.[17]

The absence of VL cases does not mean that this locality is VL free, since most *L. infantum* visceral infections remain asymptomatic. Moreover leishmaniasis lesions due to *L. infantum* are morphologically similar to *L. tropica*. Hence, more epidemiological investigations are needed to demonstrate the parasite species circulating in this leishmaniasis endemic area.

These feeding rates should be considered in relation with the constitution of habitats where the engorged species was collected, as most of the fed females were from stable henhouse with the available sources. This vector species is known to be opportunistic feeders rather than exhibiting preferences for any specific animal.[11] our data on both feeding rates and multiple feedings habit of *Ph. perniciosus* confirm its opportunistic behavior. The fed females collected in different stations were found to feed on the respective animals housed in these shelters and when almost animals were present simultaneously. *Ph. perniciosus* feed on all of them, indicating that blood meal is probably associated to its availability rather than to its specific attractiveness. FR values was higher than 1 for different animals. It gave relatively low value only for Sheep (0.27).

Our work confirms the observations on the attraction of *Ph. perniciosus* to large animals in the study performed by G. Bongiorno and al. in Macerata province, central Italy.[11, 14]

In conclusion, we consider that continuous monitoring of sand fly activity as well as the identification of blood meal help improving the understanding the role of domestic animals in transmission of parasite in ZVL and ZCL endemic foci. Only continued surveillance may bring new insights on a global scale, and particularly in respect to this complex biological ecosystem of vector-parasite-host-environment-climate in the regions that have recently become endemic.

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