Original article

Survey of sand flies and *Leishmania* infection in Doi Saket District, Chiang Mai Province, Thailand

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Objectives To survey sand flies in areas near the residence of a leishmaniasis patient's home in Doi Saket District, Chiang Mai Province, Thailand and examine the *Leishmania* DNA.

Methods Sand flies were collected using CDC light traps for two consecutive nights between 6 PM and 6 AM from February through September 2016. The study areas included rice fields, banana trees, chicken coops, and stacks of firewood. The *Leishmania* DNA was examined using a PCR method.

Results A total of 863 adult sand flies were collected. Among the 425 females, five species were morphologically identified, i.e., *Phlebotomus stantoni*, *Sergentomyia gemmea*, *S. barraudi*, *S. indica*, and *S. hivernus*. Sergentomyia indica was the predominant species. Most of the flies were collected from chicken coops. The highest density of sand flies captured was in May with an average temperature of about 29 ºC and relative humidity of approximately 61%. No *Leishmania* DNA was detected in any of the sand flies collected in this study.

Conclusions This study provided information on the different distribution patterns of sand fly species in Doi Saket District, Chiang Mai Province, Thailand where the leishmaniasis case was located. No Leishmania DNA was detected in the sand flies collected in this study. Further study is required to better understand the vector status of sand flies in Thailand. **Chiang Mai Medical Journal 2017;56(4):223-30.**

Keywords: Sand fly, Habitat, population density, *Leishmania* DNA, Chiang Mai

Introduction

*Leishmania*sis is a health problem in both tropical and subtropical areas (1). *Leishmania* infections in Thai patients have been reported in six southern, one central, one eastern, and four northern provinces in Thailand. The majority of cases are caused by *Leishmania martiniquensis*; only one case, in the southern province of Trang, is caused by "*Leishmania siamensis*" (2). Recently, Leishmaniasis cases caused by *L. martiniquensis* have been reported in Lamphun and Chiang Mai Provinces in the northern region of Thailand (3,4). In 2015, a man living in Doi Saket District, Chiang Mai Province, was admitted to Maharaj Nakorn Chiang Mai Hospital with symptoms of splenomegaly. After investigation, the patient was diagnosed with Leishmaniasis caused by *L. martiniquensis* (in preparation).

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Received: June 02, 2017, **Accepted:** August 17, 2017.

Phlebotomine sand flies are known to transmit Leishmaniasis in both the old and new worlds. In Thailand, although Leishmaniasis cases are increasing, the disease vector has not been identified and proved. However, Chusri *et al*. (2014) have reported that *Leishmania* DNA amplicon are identified in females of two species of sand flies, *Sergentomyia gemmea* and *Sergentomyia barraudi* (5). Several investigations of sand flies in the central, northern and southern regions of Thailand have been conducted, where four genera, i.e., *Sergentomyia*, *Phlebotomus*, *Chinius*, and *Idio-Phlebotomus*, have been identified and at least 29 sand fly species recorded (6-8). Recently, five species have been reported in Hang Dong District, Chiang Mai Province (9). However, no survey has been conducted of sand flies in Doi Saket District. The objectives of this study were to survey sand flies in Doi Saket District, Chiang Mai Province and to examine sand flies for *Leishmania* DNA in that district.

Methods

The study area and sand fly collection

The area surrounding the Leishmaniasis patient's house in Doi Saket District, Chiang Mai, Thailand (18°49'32N et 99°5'13E) was selected as the study area (Figure 1). Sand flies were collected using five CDC light-traps augmented with dry ice from 6 PM to 6 AM from February through September 2016 for two consecutive nights each month. Temperature and relative humidity were measured using a Thermo-Hygrometer (DeltaTRAK, Pleasanton, CA, USA). Collection of sand flies was carried out within a 200 meter radius of the patient's house. The traps were placed in areas of high humidity, i.e., rice fields, banana trees, chicken coops, and stacks of firewood. Traps were placed at the same locations each month. Sand flies collected were transferred to the laboratory at the Department of Parasitology, Faculty of Medicine, Chiang Mai University, for species identification and examination for *Leishmania* DNA.

Sand fly dissection and identification

The collected sand flies were euthanized by ether and separated by gender and the numbers of males and females were recorded. Abdominal segments VIII-X and the heads of the females were dissected in phosphate-buffered saline (PBS) (10 mM sodium phosphate, 145 mM sodium chloride, pH 7.2) under an Olympus SZ51 stereo microscope (Tokyo, Japan) and kept in 75% ethanol until mounted in Hoyer's medium for species identification. The remaining parts of each sand fly were placed in a 1.5 microcentrifuge tube and kept at -20 °C until used for DNA extraction. Genus and species identification of the sand flies was performed using keys and articles by Theodor (1938) (10), Quate (1962) (11), and Lewis (1978, 1987) (12,13).

Figure 1. Aerial map of the study area in Doi Saket District, Chiang Mai Province, northern Thailand showing the location where sand flies were collected (▼).

Light microscopy

The cibarial teeth and spermathecae were mounted on slides, observed under a light microscope, and photographed using an Olympus microscopy camera using DP2-SAL Firmware Ver.3.3.1.198 (Tokyo, Japan).

DNA extraction and polymerase chain reaction

DNA extraction employed the GeneJET Genomic DNA Purification kit (Thermo Fisher Scientific, MA, USA). In the PCR reaction, a 18S rRNA gene primer set described by Spanakos *et al*. (2008) (14) was used to detect *Leishmania* DNA. Sand fly DNA samples were tested for the presence of *Leishmania* DNA using the primers LEI70R (5'-CGCGGTGCTGGACACA-GGGTA-3') and LEI70L (5'-CGCAACCTCGGTTCG-GTGTG-3'). The PCR reaction was performed in a final volume of 50 µL consisting of 1X PCR buffer, 2.5 mmol/L of MgCl2, 0.2 µmol/L of each dNTP, 0.5 pmoles of each primer, 2 U of Taq DNA polymerase, and 10 µl of DNA template. DNA of *L. martiniquensis* (CM1 strain) (3) was used as a positive control. The PCR was performed with an initial denaturation step at 94 °C for 5 min followed by 40 cycles of 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 1 min then a final elongation step at 72°C for 10 min.

Results

A total of 863 phlebotomine sand flies (425 females and 438 males) were collected. The flies belonged to two genera, *Phlebotomus* and *Sergentomyia*. The female sand flies were identified using morphological characteristics of the cibarial teeth (Figure 2) and spermathecae (Figure 3). Five species were identified from the 425 females, i.e., *P. stantoni* (2.35%), *S. gemmea* (31.06%), *S. barraudi* (16.71%), *S. indica* (45.65%), and *S. hivernus* (4.23%) (Table 1). At the collection site, *S. indica* was found to be the most abundant species, whereas the least common species was *P. stantoni*.

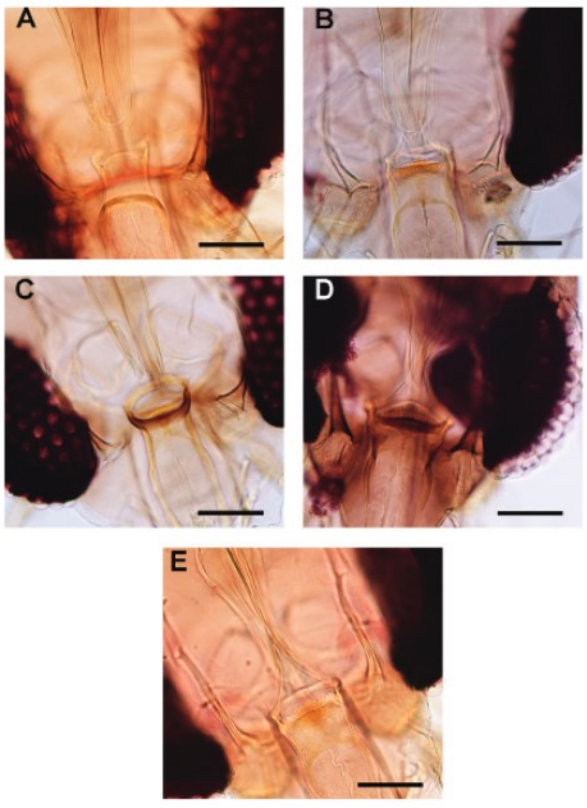

Figure 2. Morphological characteristics of cibarial teeth and pigment patches of female sand flies. (A) *P. stantoni*. (B) *S. gemmea*. (C) *S. barraudi*. (D) *S. indica*. (E) *S. hivernus*. Bar = 50 µm.

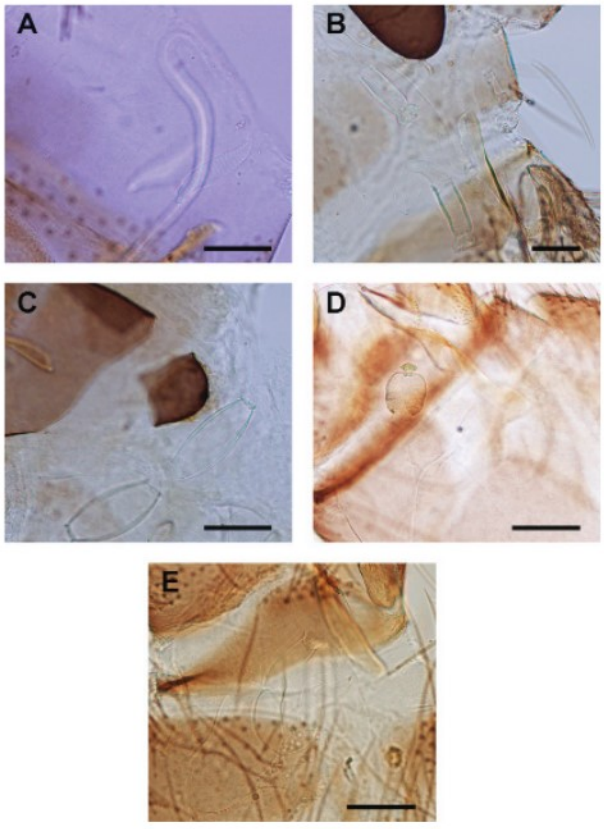

Figure 3. Morphological characteristics of spermatheca of female sand flies. (A) *P. stantoni*. (B) *S. gemmea*. (C) *S. barraudi*. (D) *S. indica*. (E) *S. hivernus*. Bar = 50 µm.

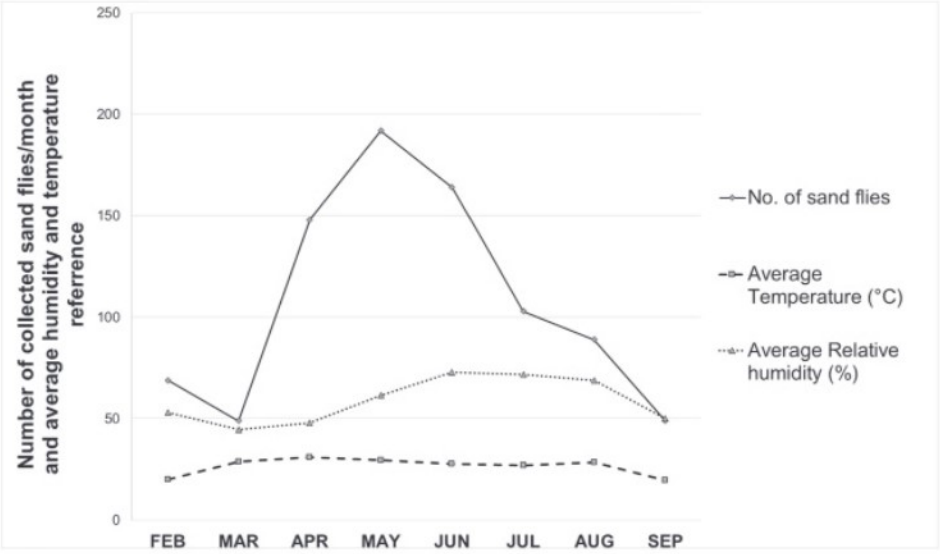

Figure 4. Relationship between sand fly prevalence or density and the climate parameters of relative humidity and temperature in Doi Saket District from February through September 2016

Table 1. Species identification numbers of 425 female sand flies in this study from February through September 2016

Sepsis	Month									Percentage
	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Total	
S. indica	5	8	52	58	49	14	6	1	194	45.65
S. gemmea	18	7	5	17	25	24	18	18	132	31.06
S. barraudi	11	4	7	6	18	9	9	7	71	16.71
S. hivernus	2	0	0	0	9	3	0	4	18	4.23
P. stantoni	0	2	2	1	1	0	2	2	10	2.35
Total	36	21	66	83	102	50	35	32	425	100.00

Habitats of each sand fly species are shown in Table 2. The most common habitat of the sand flies was chicken coops (71.29%), followed by rice fields (12.47%), stacks of firewood (11.30%) and banana trees (4.94%). *S. gemmea*, *S. barraudi*, and *S. indica* were found at all the sand fly trap areas, but most frequently at termite mounds and chicken coops.

Figure 4 shows variation of the sand fly density by month in the study area. The density of captured female sand flies was high in warm and humid periods between April and August. The highest density was noted in May when an average temperature was 29°C and the relative humidity was approximately 61%; the lowest density occurred in March. However, *S. indica*, *S. gemmea*, and *S. barraudi* were found throughout the study period.

No *Leishmania* DNA was detected in any of the sand flies collected in this study (data not shown).

Discussion

Results of this study provide information on the different distribution patterns of sand fly species in Doi Saket District, Chiang Mai, Thailand where the Leishmaniasis case was found. The female:male ratio (approximately 1:1) of the sand flies collected in this study was consistent with previous reports on southern Thailand (16,17). *S. indica* was the most abundant

Table 2. Species and habitats of trapped female sand flies in the study area of Doi Saket, Chiang Mai Province, Thailand

Sand fly Species	Areas of sand fly collection				Total
	Rice fields	Banana trees	Chicken stall	Stack of woods	
S. indica	15	5	158	16	194
S. gemmea	16	12	87	17	132
S. barraudi	16	4	41	10	71
S. hivernus	6	0	8	4	18
P. stantoni	0	0	9	1	10
Total	53	21	303	48	425
Percentage	12.47	4.94	71.29	11.30	100

species (46%), followed by *S. gemmea* and *S. barraudi*. The study area was mostly surrounded by rice fields. This study differs from a previous report on Hang Dong District, Chiang Mai Province, where *S. gemmea* was the predominant species (35.36%) in an area surrounded by dense bamboo trees (9). Previous surveys of the areas near *Leishmania*sis patients' homes located in a palm tree plantation, a rubber tree plantation, and a bamboo tree plantation reported that *S. gemmea* was the most abundant species in Phang-Nga, Suratthani, Nakonsritammarat, Trang, Songkhla, and Lumphun Provinces. Moreover, *S. indica*, *S. gemmea*, and *S. barraudi* were reported to be abundant in chicken coops (15-17,5,8). In other studies, *S. gemmea*, *S. iyengari*, and *S. barraudi* have been found to be abundant at cattle corrals and chicken coops (16,17). A study to identify blood meals in sand flies using cyt-b DNA reported that DNA of various mammals, including humans, cattle, Indian gerbils, and rats, was detected in *S. clydei*, *S. dubia*, and *S. ghesquierei* sand flies, indicating that these species feed primarily on mammals (18). In addition, Maia *et al*. (2015) analyzed cyt-b DNA amplified from the blood-meals detected in engorged female sand fly specimens and reported that *P. perniciosus* fed on a wide range of domestic animals including horses, rabbits, cattle, chickens, pigs, and sheep, while human and lizard DNA have been detected in engorged *S. minuta* (19). During this study, no blood fed sand flies were found in any of the collection areas. An explanation may be that the sand flies might have been attracted to the traps before feeding on blood. Perhaps dry ice made the traps more attractive than their usual source of a blood meal. The results of the current study suggest that chickens may be the major blood source for *S. indica*, *S. gemmea*, and *S. barraudi*.

In this study, calculation of the population density of sand flies was conducted between February and September 2016. The density of sand flies was high in April to June, with the highest density in May at an average temperature of about 29 ºC and a relative humidity of approximately 61%. Results of this study are similar to previous studies conducted in three provinces in southern Thailand, Phang-Nga, Suratthani, and Nakonsritammarat (16), and in Hang Dong District, Chiang Mai Province (9). Variations in species abundance in different areas might be associated with differing environmental conditions, e.g., some species of plants may be more or less suitable for sugar feeding, resting, and as breeding sites for each of the different sand fly species. Since *Sergentomyia* sand flies are zoophilic (18), they might have a role in the transmission of zoonotic Leishmaniasis.

Although no *Leishmania* DNA was detected in the sand flies collected in this study, further investigation of the vector status of *S. gemmea* and *S. barraudi* is needed in all transmission areas in Thailand. In that regard, Seblova *et al*, (2015) have demonstrated that the Culicoides sonorensis midge can transmit *Leishmania enriettii* (20). Furthermore, Dougall *et al*. (2011) have detected Australian *Leishmania* DNA in the biting midge, *Forcipomyia* (*Lasio-*

helea) sp.1, and possibly *Forcipomyia* (*Lasiohelea*) *peregrinator* (21). As *L. martiniquensis* and "L. *siamensis*" have been described as members of the *L. enriettii* complex (22-24), extensive surveys of midges and investigation of their vector status should be conducted as well. Regarding potential vectors of Leishmaniasis, black rats (*Rattus rattus*) have been identified as a potential animal reservoir (5). No investigation of animal reservoirs was performed in this study; identification of animal reservoirs is also needed.

Conclusions

Distribution patterns of sand fly species in Doi Saket District, Chiang Mai Province, Thailand were determined in this study. Five sand fly species were identified, including *P. stantoni*, *S. gemmea*, *S. barraudi*, *S. indica*, and *S. hivernus*. *S. indica* was the predominant species and a chicken coops were the most common habitat in the study area. Although no *Leishmania* DNA was detected, further investigation of the vector status of sand flies is needed in all infected areas in Thailand.

Acknowledgements

We would like to thank Chiang Mai University for the award of a Post-Doctoral Fellowship 2016 to Pradya Somboon and Sriwatrapron Sor-suwan and a Newton Fund-TRF Travel Grant for PhD Supervisor Year 2014/15 to Narissara Jariyapan. We would also like to thank the Korean International Cooperation for Infectious Diseases (KOICID) for their support of this project.

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การสำรวจริ้นฝอยทราย และการตรวจหาลิชมาเนีย ในอำเภอดอยสะเก็ด เชียงใหม่ ประเทศไทย

ศรีวตาภรณ์ ส.สุวรรณ์, นริศรา จริยะพันธุ์, ชลลดา มะโน และ ปรัชญา สมบรูณ์
ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

วัตถุประสงค์ เพื่อสำรวจริ้นฝอยทรายในพื้นที่บริเวณใกล้กับบ้านผู้ป่วยโรคลิชมาเนีย ในอำเภอดอยสะเก็ด จังหวัดเชียงใหม่ และตรวจหา DNA ของเชื้อ *Leishmania*

วิธีการ ทำการสำรวจริ้นฝอยทราย โดยทำการดักริ้นฝอยทราย ด้วยการใช้อุปกรณ์ดักแมลง CDC light traps ติดตั้งสองคืนติดต่อกัน ระหว่างเวลาหกโมงเย็นจนถึงหกโมงเช้า ตั้งแต่เดือนกุมภาพันธ์ถึงเดือนกันยายน พ.ศ. 2559 พื้นที่บริเวณที่ทำการศึกษาประกอบด้วย ทุ่งนา ต้นกล้วย เล้าไก่ และกองไม้ ทำการตรวจหา DNA ของเชื้อ *Leishmania* ด้วยวิธี PCR

ผลการศึกษา จากการสำรวจพบว่ามีริ้นฝอยทรายทั้งหมด 863 ตัว เป็นเพศเมียจำนวน 425 ตัว ซึ่งจำแนกชนิดได้เป็น 5 ชนิด ได้แก่ *Sergentomyia gemmea S. barraudi S. indica S. hivernus* และ *Phlebotomus stantoni* โดยชนิดที่พบเป็นจำนวนมากที่สุดคือ *S. indica* บริเวณที่พบจำนวนแมลงมากที่สุดคือ เล้าไก่ ความหนาแน่นของริ้นฝอยทรายที่จับในบริเวณนี้สูงสุดในเดือนพฤษภาคม ซึ่งมีอุณหภูมิเฉลี่ยประมาณ 29 °ซ และความชื้นสัมพัทธ์ร้อยละ 61 ในการศึกษานี้ไม่พบ *Leishmania* DNA ในริ้นฝอยทรายที่จับได้ทั้งหมด

สรุปผลการศึกษา ผลการศึกษานี้ได้ให้ข้อมูลของรูปแบบการกระจายตัวที่แตกต่างกันของริ้นฝอยทรายในอำเภอดอยสะเก็ด จังหวัดเชียงใหม่ ที่ซึ่งพบผู้ป่วยโรคลิชมาเนีย แม้ว่าไม่พบ DNA ของเชื้อ *Leishmania* ในริ้นฝอยทรายที่ดักจับได้ในการศึกษานี้ การศึกษาเพิ่มเติมถึงสถานภาพการเป็นพาหะนำโรคของริ้นฝอยทรายยังจำเป็นต้องทำต่อไปในประเทศไทย **เชียงใหม่เวชสาร 2560;56(4):223-30.**

คำสำคัญ: ริ้นฝอยทราย แหล่งอาศัย ความหนาแน่น, DNA ของเชื้อ *Leishmania* จังหวัดเชียงใหม่