A Survey of Sandflies in Fushin Township, Taoyuan County, Taiwan and a PCR Diagnostic Method of Sandfly Infection

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Abstract

Leishmaniasis is caused by the protozoan parasites of *Leishmania* spp., which are transmitted by the bites of female sand flies. In addition, these small flies can also transmit other diseases such as bartonellosis and sandfly fever. A one-time survey was conducted in a house of a 2006 patient and its surroundings at the Fushin Township, Taoyuan County, Taiwan to understand the species of the sand flies and the possible infections of *Leishmania* spp. In a total of 3 teams, each team used two kinds of light traps, castor oil sticky traps, emergence traps, backpack aspirators, and human mouth aspirators. A total of 102 sand flies (62♀ 40♂) were collected and identified as *Sergentomyia iyengari taiwanensis*. CDC light traps (39♀31♂) captured the highest number of sand flies and followed by blacklight traps (16♀5♂), human mouth aspirators (7♀2♂) and caster oil sticky traps (1♂). Forty-seven female sand flies were subject to detect *Leishmania* infection by fluorogenic real-time PCR and all showed negative results. The sensitivity of this test was 3.42 *Leishmania donovani* for one single sand fly.

Keywords: *Leishmania*, sandfly, real-time PCR, Collection methods, Taoyuan County.

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Introduction

The sandflies (class, Insecta; order, Diptera; suborder, Nematocera; family, Psychodidae) are closely related to the mosquitoes (Culicidae) that they belong to the same suborder but different families. These tiny flies are responsible for the transmission of several human diseases, such as Kala-azar (visceral leishmaniasis), cutaneous leishmaniasis, bartonellosis, and sandfly fever. There are about 12 million cases of leishmaniasis worldwide, and the prevalence is increasing at the rate of 1.5 to 2.0 million cases per year (including 1.0 to 1.5 and 0.5 million cases of cutaneous leishmaniasis and Kala-azar, respectively; over 50 thousand of fatal cases). More than 90 percent of cutaneous leishmaniasis cases occurred in Iran, Afghanistan, Syria, Saudi Arabia, Brazil, and Peru [1], and over 90 percent of Kala-azar cases emerged from countries of Bangladesh, Brazil, India, Nepal, Ethiopia, and Sudan [2, 3]. Update, the outbreaks of leishmaniasis are still on going in many regions of Mainland China. In Taiwan, imported cases of Kala-azar were reported between 1942 and 1958. Later, one indigenous case of cutaneous leishmaniasis was reported from Jianshih Township, Hsinchu County in 1968 and 1970, respectively [4]. Recently (in May 2005), another indigenous case of cutaneous leishmaniasis was reported in Fushin Township, Taoyuan County and the causative agent was confirmed to be *Leishmania tropica* Wright [5].

More than 600 sandfly species, which divided into 5 genera, have been described in the world. According to the adult classification made by Chinese researchers, the sandfly species were classified into three genera (*Phlebotomus*, *Sergentomyia*, and *Idiophlebotomus*) in the Old World and two genera in the New World [6]. However, based on the documentation done by Lewis and other

Western researchers, they recognize two genera (*Phlebotomus*, and *Sergentomyia*) in the Old World and three genera (*Lutzomyia*, *Brumptomyia*, and *Warileya*) in the New World [7-9]. Among them, *Phlebotomu*s and *Lutzomyia* Sandflies feed on warm-blooded animals (such as humans and mammals) and, therefore, serve as potential vectors of human diseases. They are about 30 vector species worldwide. *Sergentomyia* and *Idiophlebotomus* sandflies feed on cold-blooded animals and bats, respectively. In Mainland China, more than 40 species of sandflies have been identified and the major vectors of leishmaniasis are *Phlebotomus chinensis* Newstead, *P. wui* Yang and Xiong, *P. alexandri* Sinton, and *P. longiductus* Nitzulescu [6]. In Taiwan, the first report of sandflies was the description of a male sandfly, which was collected in Datung Township, Yilan County in 1940. Later in 1966, another report described the sandfly collection methods and the results in a survey of 19 Townships in 6 Counties in Taiwan. The collection methods included light traps, human mouth aspirators on resting sites, monkey-baited traps, Magoon traps, and Malaise traps. A total of 1,558 sandflies belonging to 8 species were collected in this study. The 8 species were *P. kiangsuensis* Yao and Wu, *Sergentomyia iyengari taiwanensis* Cates and Lien, *S. barraudi* Sinton, *S. souamipleuris* Newstead, [10] and other 4 unidentified species [4]. The latest survey was conducted between July 1995 and June 1996 in 16 Townships of 9 Counties in Taiwan. The methods used to capture sandflies in this survey were 3 types of light traps, Malaise traps, and human collection. A total of 979 sandflies representing 6 species were collected. The 6 species were *S. iyengari taiwanensis* Cates and Lien, *S. souamipleuris* Newstead, *S. barraudi* Sinton, and 3 unrecorded species [11].

Because of the wider breeding distribution and large species diversity of sandflies, it is important to combine multiple collection methods in a survey. The

commonly used methods include castor oil sticky traps, light traps, emergence traps, Shannon traps, human bait landing collection, human mouth aspirators on resting sites, household insecticide knock down collection, and Malaise traps. Of these methods, light traps were ranked the most efficient [12]. In Taiwan, studies suggested that light traps attracted more sandflies, while Malaise Trap captured more species [11]. Conventional microscopes were commonly used to identify the sandfly species and to detect sandfly infection. In recent years, molecular techniques have been used to differentiate the sibling species of sandflies that are similar in morphology [13]. Furthermore, this technique was also used to detect sandfly infection of parasites, such as kDNA polymerase chain reaction assay and fluorescent quantitative polymerase chain reaction [14-16]. Although three indigenous cases of leishmaniasis were found in Taiwan, no vector species of sandflies have been collected. Therefore, we conducted this survey by using multiple collection methods to understand the sandfly species and their abundance around the house of the case in Taoyuan. In addition, a real time PCR was used to detect the sandfly infection of *Leishmania*.

Materials and Methods

1. Description of the survey site

The survey site (24° 44’ N and 121° 20’ E) was located in a mountain area in Fusing Township, Taoyung County and included the house of the leishmaniasis case in 2005 (Figure 1A). No one was residing in the house at the time of survey. Near the house is a small grocery store that usually opens until 9 p.m. The opposite side of the house, across the mountain trail passing right by the house's front door, is a mountain terrain. In the terrain, there is a house with dogs and chickens around.

(1) (2)

Figure 1. (1) The empty house of the leishmaniasis patient (a) and its surrounding areas in Fushin Township, Taoyuan County. (2) A sandfly of *Sergentomyia iyengari taiwanensis*, resting on the foot of the wall marked in (b) on the left picture.

2. Sandfly collection and species identification

The survey was carried out in the most abundance month (July) of sandflies. All investigators were divided into three groups with six to seven persons for each group. In order to collect more species, six collection methods were included. They are 1) castor oil sticky paper traps (20 x 21 cm). The paper was fixed using long bamboo stick at 20 cm above the ground outside overnight to trap the sandflies emerging from rodent burrows. Five traps were used for each group with a total of fifteen traps. 2) CDC miniature light traps baited with carbon dioxide (Model 512, John W. Hock, Gainesville, FL). The traps were hung 50-75 cm above the floor outside overnight. One light trap was used for each group with a total of three traps. 3) CDC updraft blacklight traps baited with carbon dioxide (Model 1312, John W. Hock, Gainesville, FL). These traps were hung downward 50-75 cm above the floor outside overnight. One light trap was used for each group with a total of three traps. 4) Emergence traps. A big funnel (20 cm

diameter) that was attached by a plastic bag was placed right above the entrance of the rodent burrows overnight. Two traps were used for each group with a total of six traps. Unfortunately, two traps in one group were either missing or damaged because the traps were exposed at the roadside. 5) Backpack aspirator (John W. Hock, Gainesville, FL). Before sunset, one aspirator was used to sample sandflies resting on walls of the houses, plants, and stonewall for 30 minutes per group. 6) Human mouth aspirators. After dark, investigators used flashlights to look for sandflies on the wall inside and outside of the houses and stonewall for one hour per group. Sandflies were collected by human mouth aspirators and were placed into paper cups. Later, 10% sugar solution on a cotton ball was provided. Those sandflies collected by other methods were stored in a dry ice box, and carried back to laboratory for further identification.

In laboratory, live sandflies were frozen to death. Later, all sandflies were stained with 20% carbal fuchsin solution and, then, identified for species by microscopes based on some typical characteristics (such as hairs on abdominal tergites, buccal capsule and pigment patch, pharyngeal basket, female spermatheca, and male ectgenitalia) [6, 10, 17]. After species identification, all specimens were stored at - 20°C for subsequent *Leishmania* detection. In addition, sandfly data were pooled over groups because of the small samples in each group. A χ^2-test with an assumption of equal effectiveness of each method was used to detect the capture difference of the collection methods.

3. *Leishmania* detection

The protocols of Wortmann et al. [18] and Gomez-saladin et al. [14] to extract DNA and fluorogenic real-time PCR assay were modified to detect *Leishmania* protozoa on field-caught sandflies. Briefly, individual female sandfly was put into a 1.5 ml centrifuge tube, and, then, a glass bead and PBS buffer

solution were added. Sandfly specimen was homogenous by a tissue lyser (Retsch, Haan, Germany). DNA from *Leishmania* was extracted using DNeasy® blood and tissue DNA extraction kit (Cat no. 69506, Qiagen, Austin, Texas) and the protocol described by the manufacturer was followed. All DNA extracts were stored at 2-8°C for subsequent fluorogenic real-time PCR assay.

The PCR amplifications were conducted in a solution containing 2μl DNA extracts, Taqman® universal PCR master mix, genus primers of *Leishmania* (0.5μM LEISU1 5'-AAGTGCTTTCCCATCGCAACT-3' and 0.5μM LEISL1 5'-GACGCACTAAACCCCTCCAA-3') (Mission Biotech, Taipei, Taiwan), and fluorescent probe (0.1μM LEISP1 5'-CGGTTCGGTGTGTGGCGCC-3') (PE Applied Biosystems, Foster City, CA). Reaction using a sequence detector (ABI PRISM 7000, PE Applied Biosystems, Foster City, CA) began with incubation at 50°C for 2 minutes, then elevated to 95°C for 10 minutes for polymerase activation, and followed by 45 PCR cycles (95°C for 15 seconds and 60°C for 1 minute). When the target segments (16s rRNA gene of *Leishmania* protozoa) exist, the probe will attached to this segment and release fluorescence. The detector receives the fluorescence signal and the intensity of signal increases with the number of PCR cycles. Because male sandflies do not bite, they will not be infected with *Leishmania*. Therefore, a single male sandfly was chosen as a negative control and the mixture of a single male sandfly and 180μl *Leishmania* (1.89x10^{3} *L. donovani* per ml, which is provided by Dr. Lee's Laboratory of Academia Sinica) as a positive control in the entire process. In addition, we performed a serial 5-fold dilution of *Leishmania* genomic DNA and used the diluents to demonstrate the sensitivity of fluorogenic real-time PCR. Based on this result, the concentration of positive controls was set as 38μg/μl or 190μg/μl. Reaction mixtures without DNA template were used as negative controls in each

PCR.

Results and Discussion

A total of 102 sandflies (62 females and 40 males) were captured in this study and all of them belong to one single species, *Sergentomyia iyengari taiwanensis* Cates (Figure 1B and Table 1), the same species collected in the previous surveys at the same township in 1966 and 1995. Furthermore, these surveys also collected the other species, *S. barraudi* Sinton, and the 1985 survey did not perform species identification [4, 10, 11]. Although the indigenous cases of cutaneous leishmaniasis were found in Jianshih Township, Hsinchu County and Fushin Township, Taoyuan County and a three-year surveillance on human and environments (including specimen from dogs, cats, rats, birds, reptiles, amphibians, and sandflies) were conducted, all results were negative [4]. Species identification of sandflies usually depends on their morphology under microscopes, which is a labor-intensive procedure and needs references. Therefore, the future research should focus on using molecular techniques to identify species and blood-meal sources, and to conduct laboratory diagnosis of patients in a long-term study. In addition, the real time PCR can also be used in detecting sandfly infection.

Table 1. Number of sandflies and mosquitoes collected by different collection methods in Fushin Township, Taoyuan County in July 2007

Collection methods	Group no.	Trap /aspirator no.	Sampling period	*Sergentomyia iyengari taiwanensis*		*Aedes albopictus*		*Uranotaenia nivipleura*		*Culex quinquefasciatus*		Others *	
				F	M	F	M	F	M	F	M	F	M
CDC light traps (CO_2)	3	1	1 night	39	31	10	4	14	0	2	0	7	0
Blacklight traps (CO_2)	3	1	1 night	16	5	10	1	1	0	8	0	9	1
Human mouth aspirator	3	6	60 min	7	3	0	0	0	0	0	0	0	0
Castor oil paper traps	3	5	1 night	0	1	0	0	0	0	0	0	1	0
Backpack aspirator	3	1	30 min	0	0	12	10	0	0	0	0	1	0
Emergence traps	2	2	1 night	0	0	0	0	0	0	0	0	0	0
Total				62	40	32	15	15	0	10	0	18	1

* other mosquito species include *Armigeres subalbatus* Coquillett, *Armigeres omissus* Edwards, *Ochlerotatus albolateralis* Theobald, *Aedes malikul*i Huang, *Culex tritaeniorhync*hus Giles, *Culex pallidothorax* Theobald, and *Tripteroides bambusa* Yamada.

In this survey, the capture effectiveness of six collection methods was significantly different ($\chi^2 = 218$,df = 5, $P < 0.001$). Light traps baited with carbon dioxide collected highest number of sandflies. CDC light traps and blacklight traps hang downward captured 70 sandflies (39 females and 31 males) and 21 sandflies (16 females and 5 males), respectively. Human mouth aspirators (7 females and 2 males) came in second and followed by castor oil sticky paper traps (1 male). Surprisingly, backpack aspirator sampling between 16:00 and 17:00 p.m., did not collect any sandflies. This suggested that the sampling sites (including rock crevices along the roadside, wild bushes, and house walls) are not resting sites of adult sandflies. However, backpack aspirators will be an effective method if it is applied in the peak of sandfly activity (after dark, i.e. 19:00 p.m.) to collect sandflies at foots of the walls inside and outside building, along the

riverbanks, rocks, and the wild bushes. In addition, this study also found that the pores in the mesh net must be fine enough to prevent the escape of this tiny sandflies (1.5-5.0 mm) for data accuracy.

A total of 47 female sandflies were analyzed for *leishmania* infection by the fluorogenic real-time PCR and all were negative (Figure 2). The fluorescence intensity of positive control (Genomic DNA) started to increase at cycle 23-24 and cycle 26-27 and the parallel cutoff cycle threshold (Ct) values were 23.85 and 26.66, respectively. The fluorescence intensity of positive control (single male sandfly and *Leishmania* protozoa) in all reactions started to increase at cycle 34-35 and Ct value was 34.57. The reactions for others were lower than the background value. In addition, the fluorogenic real-time PCR amplification for the serial 5-fold dilutions of *Leishmania* genomic DNA suggest that 5-fold diluents (equal to 4.75 DNA μg/ml) can be detected at cycle 18-19 and the minimum detectable concentration was the 5^9 fold diluents (equal to 1.22×10^{-2} DNA μg/μl) which can be detected at cycle 37-38. This concentration is equivalent to 3.42 protozoas per sandfly (Figure 3). In this study, Ct values were linearly decreased by DNA concentration (Figure 4) and the estimated function was Y= 29.7796 – 3.4112 X (R^2= 0.9981). Although fluorogenic real-time PCR method is very sensitive, the infection rate of sandflies, even in epidemic areas, was very low (0.18-7.7%) [15, 19]. The strategy of mass screening on sandflies to detect infection of *Leishmania* is recommended [16], especially on the areas where human and animal cases occur. If PCR is positive, comparing the sequence of the PCR product will identify the *Leishmania* species.

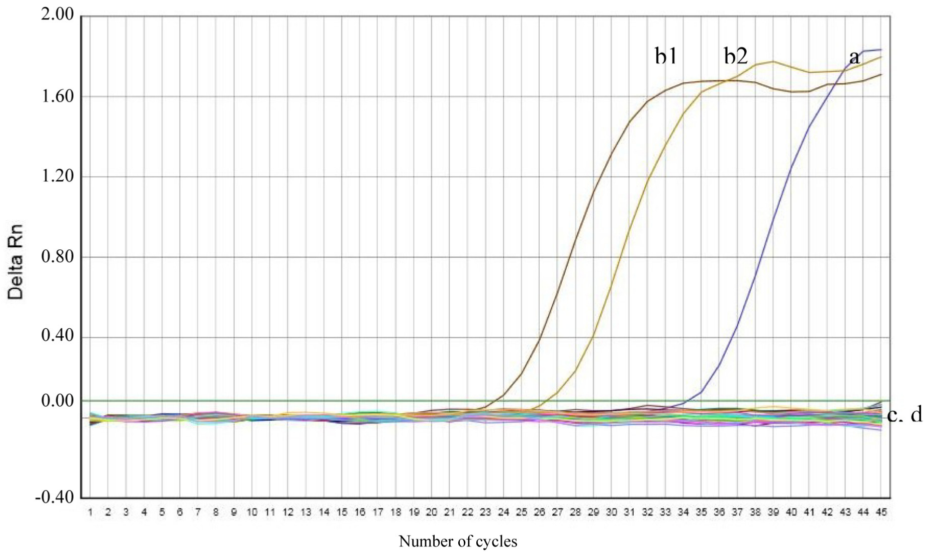

Figure 2. Results from fluorogenic real-time PCR amplifications of 47 sandfly specimens collected in Fushin Township, Taoyuan County in July 2007. a: positive control in entire process (mixture of male sandfly and *Leishmania* protozoa), b: positive control in fluorogenic real-time PCR assays (genomic DNA concentration b1: 0.19μg/ml, b2: 38μg/μl), c: negative control in entire process (single male sandfly), d: no-template negative control in PCR assays, other background curves: negative results of 47 sandfly specimens.

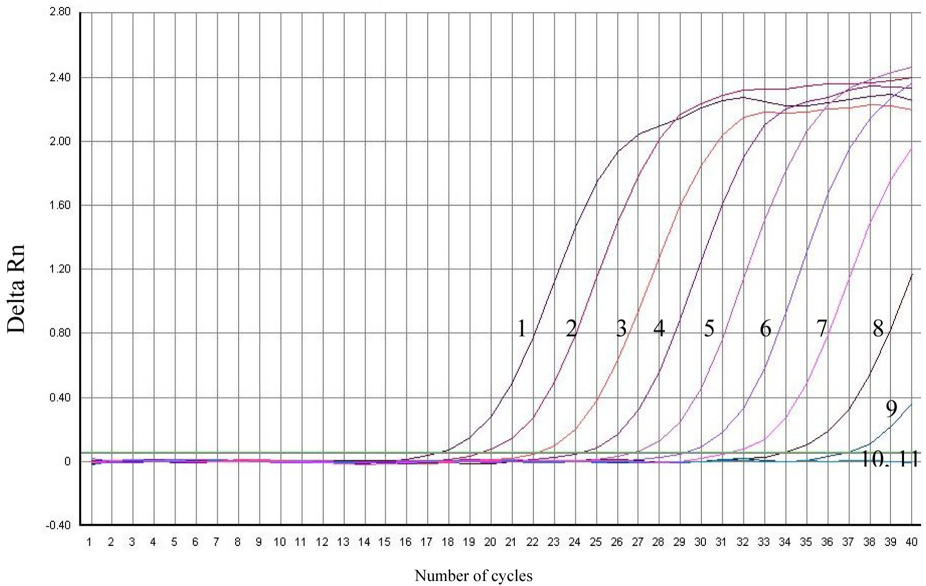

Figure 3. Sensitivity test of fluorogenic real-time PCR assays with different concentrations (serial 5-fold dilution) of Genomic DNA of *Leishmania* protozoa. DNA concentrations were : (1) 4.75 μg/ml (2) 0.95 μg/ml (3) 0.19 μg/ml (4) 38 μg/ μl (5) 7.6 μg/ μl (6) 1.52 μg/ μl (7) 0.304 μg/ μl (8) $6.08x10^{-2}$ μg/ μl (9) $1.216x10^{-2}$ μg/ μl (10) $2.432x10^{-3}$ μg/ μl (11) 4.864 $x10^{-4}$ μg/ μl.

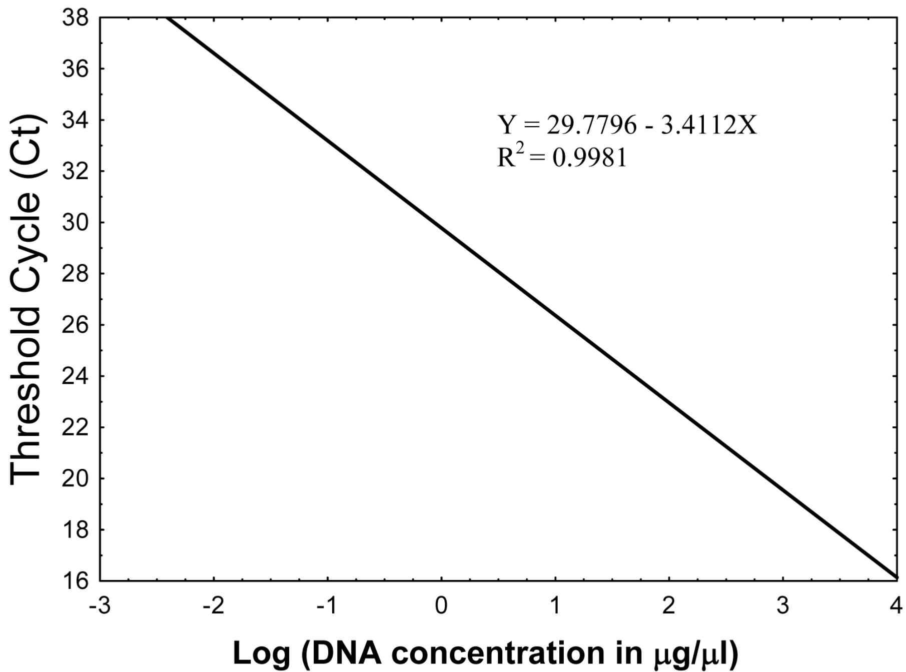

Figure 4. The relationship of Ct values against genomic DNA concentration of Leishmania protozoa in a fluorogenic real-time PCR assay.

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Cited References

1. Volf P, Ozbel Y, Akkafa F, et al. Sand flies (Diptera: Phlebotominae) in Sanliurfa, Turkey: Relationship of *Phlebotomus sergenti* with the epidemic of anthroponotic cutaneous leishmaniasis. J Med Entomol 2002; 39: 12-5.
2. World Health Organization. Urbanization: an increasing risk factor for leishmaniasis. Wkly Epidemiol Rec 2002; 44: 365-72.
3. Chappuis F, Sundar S, Hailu A, et al. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? Nature reviews/Microbiology 2007; 5: S7-16.
4. Cross JH, Gunning JJ, Drutz DJ, et al. 1985. Autochthonous cutaneous-subcutaneous leishmaniasis on Taiwan. Am J Trop Med Hyg 1985; 34: 254-6.
5. Wang JR, Lee ST, Juan WH, et al. Indigenous leishmaniasis in Taiwan: report of a case. International J Dermatology 2008; 47: 40-3.
6. Lu PL, Wu HY. Classification and identification of important medical insects of China. Henan Science and Technology Publishing House, Henan, China. 2003; 229-57.
7. Lewis DJ, Young DG, Fairchild GB, et al. Proposals for a stable classification of the phlebotominae sand flies (Diptera: Psychodidae). Syst Entomol 1977; 2: 319-32.
8. Rispail P, Leger N. Numerical taxonomy of old world Phlebotominae (Diptera: Psychodidae). 2. restatement of classification upon subgeneric morphological characters. Mem Inst Oswaldo Cruz, Rio de Janeiro 1998; 93: 787-93.
9. Tesh RB, Guzman H. Sand flies and the agents they transmit. In: Beaty B, Marquardt WC, Eds. The biology of disease vectors. University Press of Colorado, Niwot, Colorado. 1996; 117-27.
10. Cates MD, Lien JC. The Phebotomus of Taiwan. J Med Entomol 1970; 7:

529-43.

11, Lin TH, Chung CL, Lu LC, et al. The distribution of sandfly on Taiwan. Annual reports of the National Institute of Preventive Medicine, Department of Health, 1996. 1997; 210-8.

12. Toprak S, Ozer N. Distribution of sand fly (Diptera: Psychodidae) species and efficiency of capturing methods in Sanhurfa Province, Turkey. J Med Entomol 2007; 44: 23-8.

13. Mukhopadhyay J, Ghosh K, Braig HR. Identification of cutaneous Leishmaniasis vectors, *Phlebotomus papatasi* and *P. duboscqi* using random amplified polymorphic DNA. ActaTropica 2000; 76: 277-83.

14. Gomez-saladin E, Doud CW, Maroli M. Short report: surveillance of *Leishmania* sp. among sand flies in sicily (Italy) using a fluorogenic real-time polymerase chain reaction. Am J Trop Med Hyg 2005; 72: 138-41.

15. Guerbouj S, Chemkhi J, Kaabi B, et al. Natural infection of *Phlebotomus* (Larroussius) *langeroni* (Diptera: Psychodidae) with *Leishmania infantum* in Tunisia. Trans Royal Trop Med Hyg 2007; 101: 372-7.

16. Kato H, Uezato H, Gomez EA, et al. Establishment of a mass screening method of sand fly vectors for *Leishmeania* infection by molecular biological methods. Am J Trop Med Hyg 2007; 77: 324-9.

17. Sawalha SS, Shtayeh MS, Khanfar HM, et al. Phlebotomine sand flies (Diptera: Psychodidae) of the Palestinian west bank: potential vectors of leishmaniasis. J Med Entomol 2003; 40: 321-8.

18. Wortmann G, Sweeney C, Houng HS, et al. Rapid diagnosis of Leishmaniasis by fluorogenic polymerase chain reaction. Am J Trop Med Hyg 2001; 65: 583-7.

19. Hashiguchi Y, Gomez EAL, de Coronel VV, et al. Natural infections with promastigotes in man-biting species of sand flies in leishmaniasis-endemic areas of Ecuador. Am J Trop Med Hyg 1985; 34: 440-6.