ANIMAL RESERVOIRS AND POTENTIAL VECTORS OF *LEISHMANIA SIAMENSIS* IN SOUTHERN THAILAND

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Abstract. *Leishmania siamensis* is newly described as the causative pathogen of autochthonous leishmaniasis in Thailand. Potential vectors and animal reservoirs of *L. siamensis* are not thoroughly studied. An environmental survey was carried out in the affected area in two provinces in southern Thailand: Songkhla and Nakhon Si Thammarat. Ninety-nine villagers, 378 sandflies, and potential animal reservoirs were examined. *Leishmania* DNA amplicon was identified in two species of female sandflies, *Sergentomyia (Neophlebotomus)* and *Sergentomyia (Parrotomyia) barraudi*. The DNA amplicon was also identified in black rats (*Rattus rattus*). A phylogenetic tree of confirmed patients, sandflies and black rats fell into a single clade and separate from other *Leishmania* species. This study showed the potential involvement of *R. rattus* and *Sergentomyia* (*Neophlebotomus* and *Parrotomyia*) sandflies in transmission of *L. siamensis.*

Keywords: *Leishmania siamensis*, reservoir, vector, Thailand

INTRODUCTION

The number of reported cases of autochthonous leishmaniasis in Thailand has been rising in recent years. The novel *Leishmania* species, *L. siamensis*, was described as the causative agent in four recent reports (Sukmee *et al*, 2008; Suankratay *et al*, 2010; Bualert *et al*, 2012; Chusri *et al*, 2012). The phylogenetic tree of this new species is closely related to *L. enrietti*, the new world species that distinctively infects guinea pigs and is transmitted by *Lutzomyia* sandflies (Machado *et al*, 1994; Bualert *et al*, 2012). Although *L. siamensis* was previously reported as causing autochthonous cutaneous equine (Müller *et al*, 2009) and bovine (Lobsiger *et al*, 2010) leishmaniasis in central Europe – in addition to reports from Thailand (Sukmee *et al*, 2008; Suankratay *et al*, 2010) – there is only one study identifying *Sergentomyia (Neophlebotomus) gemmea* as the potential vectors of this disease (Kanjanopas *et al*, 2013).

Environmental studies of the vectors of *Leishmania* conducted in central, western, northern, and northeastern regions of Thailand demonstrated distribution of the three most predominant genera of sandfly: *Sergentomyia* (Apiwathnasorn *et al*, 1989), *Phlebotomus* (Polseela *et al*,

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2007), and *Idiophlebotomus* (Polseela *et al*, 2011a, b). A recent cross-sectional survey of sandflies in three affected areas in southern Thailand also demonstrated *S. (Neophlebotomus) gemmea* was the most predominant species in all areas (Sukra *et al*, 2013). According to the reports of autochthonous cutaneous and visceral leishmaniasis in Songkhla and Nakhon Si Thammarat in southern Thailand (Chusri *et al*, 2012), an epidemiological investigation of vectors and animal reservoirs, as well as an active human case finding, were performed in August 2011. The objective of this study was to increase knowledge about the reservoirs and vectors of *L. siamensis* that have emerged in southern Thailand.

MATERIALS AND METHODS

Ethical considerations

The study on animals was carried out according to the protocol approved by the Institution Committee for Experimentation and Care of Research Animals of the Bureau of Epidemiology, Department of Disease Control, the Ministry of Public Health, Thailand, and the study followed the Ethical Principles and Guideline for the Use of Animals (1999) of the National Research Council of Thailand. Animal facilities were supported by the Animal Research Department of The Office of Disease Prevention and Control 12, Songkhla, which was officially established by the Office of the National Committee for Research Animal Development of the National Research Council of Thailand (NRCT). The human and animal protocols for this study were approved by the Research Ethics Committee of Prince of Songkla University (Ref Nº 56-037-14-1-3; 2011 August 1). The villagers, the homeowners, and the owners of animals provided writen informed consent after explanation by the researchers.

Study setting

The study was conducted in Na Thawi District (6º 44′ 30″ N, 100º 41′ 30″ E) located in Songkhla Province and Sichon District (8º 56′ 59″ N / 99º 48′ 48″ E) located in Nakhon Si Thammarat Province in southern Thailand. The affected area within a radius of 500 meters from the house of the confirmed cases consisted of 50 houses and 201 residents. Approximately 90% of the area is rubber plantation. The climate is characterized into two seasons: the dry season from March to September and the monsoon season from October to February. The temperature range is 23-38ºC and the average rainfall is 2,093 mm. The relative humidity is approximately 79% (36-92%).

Conduct of study

Active human case surveys were carried out among 99 villagers who live within the affected area by collecting information on present and past histories, and physical examinations. Samples, including 10 ml venous blood, 5 ml saliva specimen, and 5 ml urine, were collected from villagers for laboratory investigation. Blood samples of animals in the affected area–including 28 dogs, 20 cats, 30 black rats (*Rattus rattus*), and 3 Indochinese ground squirrels (*Menetes berdmorei*) were collected, while liver and spleen samples were collected only from black rats and squirrels. There were no domestic animals, such as cows or horses, which are known to be hosts of *Leishmania* species in this area.

The CDC Miniature Light Traps (Model 512) were used to collect sandflies for two consecutive months, August and September 2011. The collection was done indoors and outdoors from 6:00 PM to

6:00 AM. Sandflies were trapped from the villagers' houses, and plantation. Species identification was performed at the Office of Disease Prevention and Control 12. Reservoirs were identified and separated by sex in a field laboratory. Sandflies were stored in 75% ethanol and mounted in Hoyer's medium for species identification. All samples and sandflies were sent to the Department of Parasitology, Chulalongkorn University for Leishmania parasite detection and sandfly species confirmation. Genus and species identification was performed using Lewis's key (Lewis, 1987).

***Leishmania* species identification**

The *Leishmania* species were identified using 18S rRNA gene primer set described by Spanakos *et al* (2008), and by nucleotide sequences of the amplified PCR products of the internal transcribed spacer 1 (ITS1) region of the rRNA gene. PCR amplicons were cloned into TA-cloning vectors pTZ57R/T (InsTAclone™ PCR Cloning Kit; Fermentas, MD) according to the manufacturer's protocol. The recombinant plasmid DNA was extracted using the FastPlasmid™ Mini Kit (Eppendorf, Hamburg, Germaney). Plasmid DNA sequencing was performed using the M13F (-20) primer (5' GTAAAACGACGGCCAGT 3') (1st Base Laboratories, Selangor, Malaysia). Nucleotide sequences were analyzed using BioEdit Sequence Alignment Editor© (ver 7.1.3; Ibis Bioscience, Carlsbad, CA) (Hall, 1999), and the consensus sequences were searched for species identification through the Basic Local Alignment Search Tool (BLAST™) (National Library of Medicine, 2011). A phylogenetic tree was constructed by using a maximum-likelihood phylogenetic tree and Kimura 2-parameters model (K2P) for nucleotide substitution in MEGA 5, evaluated by the bootstrap test (1,000 pseudoreplicates). The best-fitting model of nucleotide substitution was investigated using the MODELTEST function of the MEGA 5 program (Tamura *et al*, 2011).

RESULTS

History reviews and physical examinations of the villagers showed no evidence that could be attributed to leishmaniasis, and PCR assays were negative for *Leishmania* DNA in the blood samples of the subjects. None of animals in the affected area (dogs, cats, black rats, and squirrels) had clinical manifestations compatible with leishmaniasis. The samples (blood, liver and spleen) of two black rats collected from Na Thawi District of Songkhla Province tested positive for *Leishmania* DNA amplicon size 379 bp, which had close similarity to *L. siamensis* (Fig 1).

Three hundred seventy-eight female and 110 male sandflies were captured. Field-captured sandflies were classified into two species: *S. (Neophlebotomus) gemmea* (465, 95.2%) and *S. (Parrotomyia) barraudi* (23, 4.8%). Sandflies were pooled into nineteen pools (1-7 flies/pool) and were tested for *Leishmania* DNA. Only one pool of female *S. (Neophlebotomus) gemmea* and female *S. (Parrotomyia) barraudi* from Na Thawi District tested positive for *Leishmania* DNA amplicon size 379 bp (Fig 2). The ITS2 sequences of *Leishmania* amplified from sandflies collected from Na Thawi District of Songkhla Province also had close similarity to *L. siamensis* ITS2 sequences obtained from the patient and black rats (Fig 3).

A phylogenetic tree was constructed using the ITS1 region of the rRNA gene sequences of *L. siamensis* from a patient, black rats, and sandflies, and sequences of

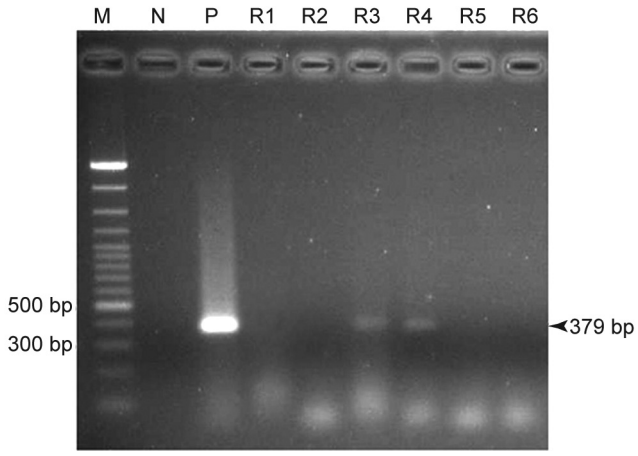

Fig 1–PCR amplification of the ITS1 region of the rRNA gene of *L. siamensis* from the liver and spleen of black rats (lanes R1-R6), Lane M: 100 bp marker; lane N: negative control and lane P: positive control using DNA from cultured *L. siamensis*. Lanes R3, R4: PCR amplification of the ITS1 region of the rRNA gene from 2 black rats.

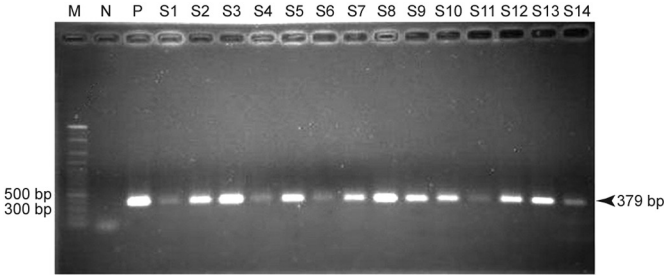

Fig 2–PCR amplification of the ITS1 region of the rRNA gene of *L. siamensis* from pooled female sandflies (lanes S1-S14). Lane M: 100 bp marker; lane N: negative control and lane P: positive control using DNA from cultured *L. siamensis*, Lanes S1-7 : PCR amplification of the ITS1 region of the rRNA gene from female *S. (Neophlebotomus) gemmea* and lanes S 8-14 : PCR amplification of the ITS1 region of the rRNA gene from female *S. (Parrotomyia) barraudi*.

this gene region from other *Leishmania* species from GenBank. The tree shows that *L. siamensis* falls into a single clade, separate from other *Leishmania* species (Fig 4).

DISCUSSION

This study identified black rats (*R. rattus*) as the potential animal reservoir, and *S. (Neophlebotomus) gemmea* and *S. (Parrotomyia) barraudi* as the potential vector for *L. siamensis*.

For the reservoir of leishmaniasis, animals kept around the house are most important because they tend to live peridomestically and possibly rely on human waste (Abranches *et al*, 1998). Although black rats are not described in the strict definition of the reservoir host (Ashford, 1997), this study showed that they might be one of the foci of infection if potential vectors are present. Similar to the study of *L. tropica* infection in black rats, collected rats in this study did not have any apparent cutaneous lesions (Svobodová *et al*, 2003). *Leishmania* parasites are usually obtainable from blood and visceral organs of asymptomatic rats (Aljeboori and Evans, 1980). This study also supported the potential transmission between vectors and asymptomatic reservoirs, previously reported in *L. chagasi* and *L. infantum* infections (Svobodová *et al*, 2003).

Sandfly species is suspected as the vector of *Leishmania* parasites when the species is predominant and has anthropophilic behavior (Sukra *et al*, 2013). Although the surveys of sandflies in Thailand showed the predominant species was *Phlebotomus* (Apiwathnasorn *et al*, 1993), in this study all the field-captured sandflies

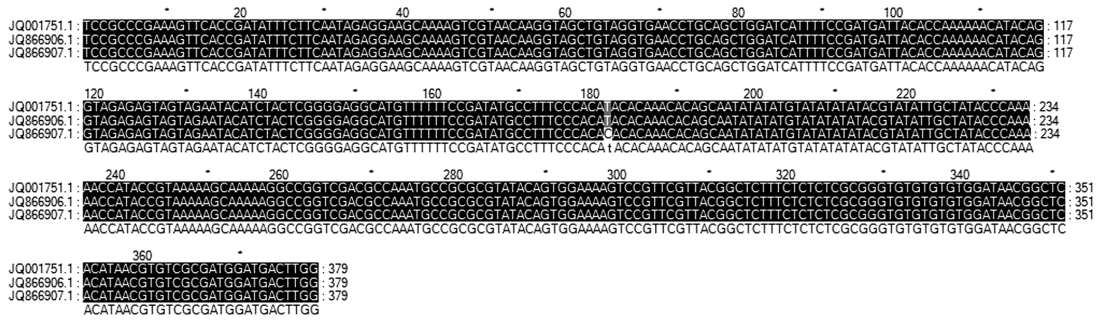

Fig 3–Comparison of ITS1 gene sequences of *L. siamensis* amplified from a patient (JQ001751.1), a black rat (JQ866906.1) and a sandfly (JQ866907.1).

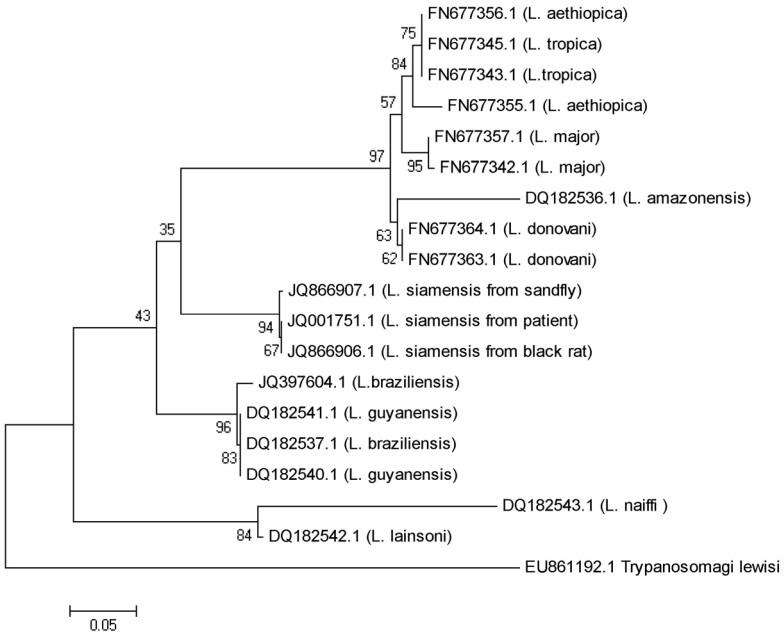

Fig 4–A phylogenetic tree was constructed using a maximum-likelihood phylogenetic tree and Kimura 2-parameters model (K2P) for nucleotide substitution in MEGA 5, evaluated by the bootstrap test (1000 pseudoreplicates).

were *Sergentomyia* (*Neophlebotomus* and *Parrotomyia*) species. Our results are similar to a recent report on the distribution of sandflies in affected areas in southern Thailand (Sukra *et al*, 2013). Previous studies showed anthropophilic behaviors of the *Sergentomyia* species, including reports of human biting, and were naturally infected by human *Leishmania* (Lawyer *et al*, 1990).

In this study, the sequencing of nucleotide sequences of the 18S rRNA gene and the ITS1 region of the rRNA gene extracted from sandflies were identical to those from the reported patients. Similar to the recent study in Trang Province, one of the affected areas in southern Thailand, which also identified *S.* (*Neophlebotomus*) *gemmea* as a potential vector of leishmaniasis with this identical gene (Kanjanopas *et al*, 2013). Taken together, the data suggests that *S.* (*Neophlebotomus*) *gemmea* and *S.* (*Parrotomyia*) *barraudi* might be potential vectors for *L. siamensis*.

The following findings in this study supported *L. siamensis* infection as a potential zoonotic disease. First, the phylogenetic analysis of *L. siamensis* is closely related to *L. enrietti*, the zoonotic leish-

maniasis infection in guinea pigs (Tamura *et al*, 2011). Second, *S*. (*Neophlebotomus*) *gemmea* and *S.* (*Parrotomyia*) *barraudi* that were identified as the potential vectors are recognized as a human and animal biting sandflies (Apiwathnasorn *et al*, 1993). Third, *L. siamensis* ITS2 sequences from infected patients, potential animal reservoirs, and sandfly vectors had very close similarity.

This study had several limitations. First, there was no previously demonstrated reservoir animals including horses and cattle in the study site. Second, the study was conducted during a short period, there was a possibility of different species distribution in this area. Third, we were not able to provide data if sandflies were blood fed or not. Last, this study was unable to demonstrate live *L. siamensis* in reservoirs and vectors because organism isolation by conventional culture was negative.

With the increasing number of patients with autochthonous leishmaniasis, involving *L. siamensis* in addition to the presence of naturally infected animal reservoirs and sandfly vectors and the potential to be a zoonotic disease, leishmaniasis has the potential to increase in Thailand. Further study of specific vector and animal reservoir control is needed for appropriate management.

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REFERENCES

Abranches P, Campino L, Santos-Gomes GM. Canine leishmaniasis. New concepts of epidemiology and immunopathology: their impact in the control of human visceral leishmaniasis. *Acta Med Port* 1998; 11: 871-5.

Aljeboori TI, Evans DA. *Leishmania* spp in Iraq. Electrophoretic isoenzyme patterns. I. Visceral leishmaniasis. *Trans R Soc Trop Med Hyg* 1980; 74: 169-77.

Apiwathnasorn C, Sucharit S, Rongsriyam Y, *et al*. A brief survey of phlebotomine sandflies in Thailand. *Southeast Asian J Trop Med Public Health* 1989; 20: 429-32.

Apiwathnasorn C, Sucharit S, Surathin K, Deesin T. Anthropophilic and zoophilic phlebotomine sand flies (*Diptera: Psychodidae*) from Thailand. *J Am Mosq Control Assoc* 1993; 9: 135-7.

Ashford RW. What it takes to be a reservoir host. *Belg J Zool* 1997; 127: 85-90.

Bualert L, Charungkiattikul W, Thongsuksai P, *et al*. Autochthonous disseminated dermal and visceral leishmaniasis in an AIDS patient, southern Thailand, caused by *Leishmania siamensis* [case report]. *Am J Trop Med Hyg* 2012; 86: 821-4.

Chusri S, Hortiwakul T, Silpapojakul K, Siriyasatien P. Consecutive cutaneous and visceral leishmaniasis manifestations involving a novel leishmania species in two HIV patients in Thailand [case report]. *Am J Trop Med Hyg* 2012; 87: 76-80.

Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser* 1999; 41: 95-8.

Kanjanopas K, Siripattanapipong S, Ninsaeng U, *et al*. *Sergentomyia (Neophlebotomus) gemmea*, a potential vector of *Leishmania siamensis* in southern Thailand. *BMC Infect Dis* 2013; 13: 333.

Lawyer PG, Ngumbi PM, Anjili CO, *et al*. Development of *Leishmania major* in *Phlebotomus duboscqi* and *Sergentomyia schwetzi* (*Diptera: Psychodidae*). *Am J Trop Med Hyg* 1990; 1: 31-43.

Lewis DJ. Phlebotomine sandflies (*Diptera: Psychodidae*) from the oriental region. *Syst Entomol* 1987; 12: 163-80.

Lobsiger L, Muller N, Schweizer T, *et al*. An autochthonous case of cutaneous bovine leishmaniasis in Switzerland. *Vet Parasitol* 2010; 169: 408-14.

Machado MI, Milder RV, Pacheco RS, Silva M, Braga RR, Lainson R. Naturally acquired infections with *Leishmania enrietti* Muniz and Medina 1948 in guinea-pigs from São Paulo, Brazil. *Parasitology* 1994; 109:135-8.

Müller N, Welle M, Lobsiger L, *et al*. Occurrence of *Leishmania* sp. in cutaneous lesions of horses in Central Europe. *Vet Parasitol* 2009; 166: 346-51.

National Library of Medicine. BLAST [Internet database]. Basic Local Alignment Search Tool, 2011. Available from: URL: http://blast.ncbi.nlm.nih.gov/Blast.cgi

Polseela R, Apiwathnasorn C, Samung Y. Seasonal variation of cave-dwelling Phlebotomine sandflies (*Diptera:Psychodidae*) in Phra Phothisat Cave, Saraburi Province, Thailand. *Southeast Asian J Trop Med Public Health* 2007; 38: 1011-5.

Polseela R, Apiwathnasorn C, Samung Y. Seasonal distribution of phlebotomine sand flies (*Diptera: Psychodidae*) in Tham Phra Phothisat temple, Saraburi Province, Thailand. *Trop Biomed* 2011a; 28: 366-75.

Polseela R, Vitta A, Nateeworanart S, Apiwathnasorn C. Distribution of cave-dwelling phlebotomine sand flies and their nocturnal and diurnal activity in Phitsanulok Province, Thailand. *Southeast Asian J Trop Med Public Health* 2011b; 42: 1395-404.

Spanakos G, Piperaki ET, Menounos PG, *et al*. Detection and species identification of Old World *Leishmania* in clinical samples using a PCR-based method. *Trans R Soc Trop Med Hyg* 2008; 102: 46-53.

Suankratay C, Suwanpimolkul G, Wilde H, Siriyasatien P. Autochthonous visceral leishmaniasis in a human immunodeficiency virus (HIV)-infected patient: the first in Thailand and review of the literature. *Am J Trop Med Hyg* 2010; 82: 4-8.

Sukmee T, Siripattanapipong S, Mungthin M, *et al*. A suspected new species of *Leishmania*, the causative agent of visceral leishmaniasis in a Thai patient. *Int J Parasitol* 2008; 38: 617-22.

Sukra K, Kanjanopas K, Amsakul S, Rittaton V, Mungthin M, Leelayoova S. A survey of sandflies in the affected areas of leishmaniasis, southern Thailand. *Parasitol Res* 2013; 112: 297-302.

Svobodová M, Votypka J, Nicolas L, Volf P. *Leishmania tropica* in the black rat (*Rattus rattus*): persistence and transmission from asymptomatic host to sand fly vector *Phlebotomus sergenti*. *Microbes Infect* 2003; 5: 361-4.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011; 28: 2731-39.