รายงานวิจัยฉบับสมบูรณ์

การสำรวจริ้นฝอยทรายพาหะของเชื้อ *Leishmania siamensis* ในประเทศไทย

Survey of sand fly vectors for *Leishmania siamensis* in Thailand

โดย

รองศาสตราจารย์ นายแพทย์ เผด็จ สิริยะเสถียร

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ภาควิชาปรสิตวิทยา
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
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Abstract

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Investigator : Associate Professor Padet Siriyasatien

Department of Parasitology

Faculty of Medicine, Chulalongkorn University

E-mail Address : padet.s@chula.ac.th

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Autochthonous leishmaniasis cases in Thailand are increasing dramatically. The disease was found in both immunocompetent and in immunocompromise hosts especially AIDS patients. The disease is transmitted to human and other vertebrate hosts through the bite of infected female sand fly. In Thailand, data on sand fly vector for leishmaniasis is limited. Objectives of this study are to demonstrate the natural infection of *Leishmania* parasites in sand flies collected in endemic areas, to develop a molecular technique for identification of sand fly species, and to study feeding behavior of sand fly vector. Sand flies were collected from endemic areas of leishmaniasis in southern and northern Thailand. Two sand fly species (*Sergentomyia hivernus* and *Se. khawi*) were described for the first time in the country. *Leishmania* and *Trypanosome* parasites were isolated from sand fly samples from the collection sites. Data obtained from this study could be used for effective control of leishmaniasis and potential emerging diseases such as trypanosomiasis in Thailand.

Keywords : Sand fly, *Leishmania*, *Trypanosome*, Emerging diseases

บทคัดย่อ

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ชื่อโครงการ: การสำรวจริ้นฝอยทรายพาหะของเชื้อ *Leishmania siamensis* ในประเทศไทย

ชื่อนักวิจัย: รองศาตราจารย์ นายแพทย์ เผด็จ สิริยะเสถียร
ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
อีเมล์ padet.s@chula.ac.th

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โรคลิชมาเนียที่เกิดในประเทศไทยมีแนวโน้มมากขึ้นอย่างชัดเจน โดยโรคนี้สามารถพบได้ในผู้ที่มีภาวะภูมิคุ้มกันปกติ และผู้ที่มีภูมิคุ้มกันบกพร่องโดยเฉพาะในผู้ป่วยเอดส์ โรคนี้ติดต่อสู่คนและสัตว์มีกระดูกสันหลังอื่นๆ ผ่านการกัดของริ้นฝอยทรายตัวเมียที่มีเชื้อ ในประเทศไทยข้อมูลเกี่ยวกับริ้นฝอยทรายพาหะนำโรคลิชมาเนียมีอยู่อย่างจำกัด วัตถุประสงค์ของการศึกษานี้จึงต้องการแสดงให้เห็นว่ามีการติดเชื้อลิ-ชมาเนียในธรรมชาติ ในริ้นฝอยทรายที่จับมาจากแหล่งระบาดของโรค พัฒนาการจำแนกชนิดของริ้นฝอยทรายโดยเทคนิคทางอณูวิทยา และศึกษาพฤติกรรมการดูดกินเลือดของริ้นฝอยทราย โดยริ้นฝอยทรายที่ได้จากการศึกษานี้ได้มาจากแหล่งระบาดของโรคในภาคใต้ และภาคเหนือของประเทศ ผู้วิจัยได้บันทึกการค้นพบริ้นฝอยทราย 2 ชนิดคือ *Sergentomyia hivernus* and *Se. khawi* เป็นครั้งแรกในประเทศไทย และสามารถแยกเชื้อปรสิตลิชมาเนีย และทริพพาโนโซม จากริ้นฝอยทรายที่เก็บจากแหล่งระบาดได้ ข้อมูลที่ได้จากการศึกษานี้จะสามรถใช้ในการควบคุมโรคลิชมาเนีย และโรคที่อาจอุบัติใหม่เช่นโรคติดเชื้อทริพพาโนโซมของประเทศไทยต่อไป

คำสำคัญ: ริ้นฝอยทราย ลิชมาเนีย ทริพพาโนโซม โรคอุบัติใหม่

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CONTENTS

LIST OF TABLES

PAGE

LIST OF FIGURES

Introduction to the research problem and its significance

Leishmaniasis is a parasitic disease spread by the bite of infected female sand fly (Lainson and Shaw, 1987). Life cycle of *Leishmania* initiated by the female sand flies feed on an infected vertebrate host blood. Amastigotes of the *Leishmania* parasite transform into promastigotes in the digestive tract of female sand fly and transmitted to a new vertebrate host during next blood feeding (Adler, 1964; Vickerman and Preston, 1976). The diversity of sand fly species can play roles in the maintenance of the transmission cycle. Humans are generally considered as accidental hosts. Reservoir hosts of leishmaniasis are domestic and wild animals such as dog, fox, jackal, rodents, and wolves (Davies et al., 2003). Female sand flies are the most important vector for transmission leishmaniasis worldwide. The clinical syndrome of leishmaniasis is classified into three main forms, which are cutaneous, mucocutaneous, and visceral leishmaniasis (kala azar) (Pearson and De Queiroz Sousa, 1994, Murray et al., 2005). However, the clinical presentation of leishmaniasis depends on the species of *Leishmania* parasites and host's immunity (Roberts, 2006; Berman, 1997). Currently, the disease infects approximately 12 million people and endemic in 88 countries. There are estimated that 1-2 million new cases occurring each year (1.5 million cases of cutaneous leishmaniasis and 500,000 of visceral leishmaniasis) (WHO, 2012). Leishmaniasis is a significant cause of morbidity and mortality in several countries globally (Herwaldt, 1999). The disease is often documented co-infection among HIV, tourists, refugees, military personnel as well as among residents of areas (Pearson and Sousa, 1996; Pavli and Maltezou, 2010). Human infections occur in countries surrounding the Mediterranean Sea, East Africa, the Middle East, South Asia, Europe, and Central and South America (Davies et al., 2003).

Autochthonous leishmaniasis cases in Thailand are dramatically increased in the past few years (Kongkaew et al., 2007; Sukmee et al., 2008; Suankratay et al., 2010; Chusri et al., 2012; Bualert et al., 2012, Phumee et al., 2013). The disease was reported in both immunocompetent and immunocompromise hosts especially in AIDS patients. Approximately 20 cases of autochthonous leishmaniasis have been documented, and most of the cases in Thailand have been reported in southern Thailand (Sukmee et al., 2008; Chusri et al., 2012; Bualert et al., 2012). Sukmee et al. (2008) reported the autochthonous leishmaniasis in Thailand caused by a new species, *Leishmania siamensis*. Patients infected with this *L. siamensis* may present with clinical presentations of leishmaniasis that have been reported

including visceral (Kongkaew et al., 2007; Sukmee et al., 2008; Suankratay et al., 2010), diffuse cutaneous (Bualert et al., 2012; Phumee et al., 2013) and overlapping diffuse cutaneous and visceral forms (Chusri et al., 2012; Phumee et al., 2013). Moreover, *L. siamensis* was also found as a causative agent of cutaneous leishmaniasis in cows and a horse in Germany (Muller et al., 2009), Switzerland (Lobsiger et al., 2010), and the United States (Reuss et al., 2012). All of the report indicated that *L. siamensis* are emerging in many different areas and increasing trend. There is currently no vaccine available for any form of leishmaniasis. Study of sand flies vector is required for alternative control of leishmaniasis. More recently *L. siamensis* infection has been described in Myanmar patients (Phumee et al., 2013).

In Thailand, little is known about survey studies of the distribution of sand fly species and their habitats. There are reported of sand flies in the western, central, northern, and northeastern regions of Thailand which were identified for three genera, such as *Sergentomyia*, *Phlebotomus*, *Idiophlebotomus* and *Chinus* (Apiwathnasorn et al., 1989; Apiwathnasorn et al., 1993). The predominant genus found in all areas of the studies was *Sergentomyia* with a small number of *Phlebotomus argentipes* (1%). In 2009, Sukra and others revealed that genus *Sergentomyia* were mostly identified in three southern provinces (Phangnga, Suratthani, and Nakonsitammarat), which the affected areas of leishmaniasis in Thailand such as *Sergentomyia gemmea* (81.4%), *S. iyengari, S. barraudi, S. indica, S. silvatica*, and *S. perturbans* and another genus, *Phlebotomus (P. argentipes)* (Sukra et al., 2013). Recently, *S. (Neophlebotomus) gemmea* were detected of *L. siamensis* DNA, and then Kanjanopas et al. (2013) revealed that *S. (Neophlebotomus) gemmea* might be a potential vector of *L. siamensis* (Kanjanopas et al., 2013). Therefore, finding naturally infected sand flies is essential for ecology and epidemiology of leishmaniasis. In this study, we investigated for potential vectors of leishmaniasis from the southern and northern regions of the country by culturing and PCR for detection the *L. siamensis* parasites from collected sand flies in autochthonous leishmaniasis reported areas. The benefits of this study are to understand the natural infection rates with *L. siamensis* of sand flies in endemic areas, identify a species by molecular, and feeding behavior of sand flies. Information obtained from the study would be applied to develop the effective control strategies for leishmaniasis in Thailand.

Literature review

Leishmania parasite

Leishmania is parasitic protozoa belonging to the genus *Leishmania*, Family Trypanosomatidae of the Order Kinetoplastida (Figure 1). This protozoan is the parasite responsible for the disease leishmaniasis, which is transmitted mostly by the bite of female sand flies, belonging to 30 sand fly species (WHO, 2007). There are three main types of clinical presentation (Lainson and Shaw, 1987; Maltezou, 2010). Firstly, cutaneous leishmaniasis (CL) show skin ulcers usually form on exposed areas, such as face, arms and legs. CL is endemic in more than 70 countries, with an estimated of 1.5–2 million new cases each year. Afghanistan, Syria, and Brazil are the main foci. CL is caused by *L. tropica, L. aethiopica, L. major,* and *L. infantum* or *L. chagasi* in the Old World and by *L. braziliensis, L. guyanensis, L. panamensis, L. peruviana, L. mexicana, L. amazonensis*, and *L. venezuelensis* in the New World. Secondly, mucocutaneous leishmaniasis (MCL) is endemic in Central and South America, and characterized by destructive metastatic lesions in the mucous membranes of the nose, mouth and throat cavities and surrounding tissues that occur months or years after the onset of the primary cutaneous infection. This disabling form of leishmaniasis can lead to the sufferer being rejected by the community. The last form is visceral leishmaniasis (VL): kala azar, characterized by high fever, substantial weight loss, enlargement of the spleen and liver, and anemia. VL is the most severe form of leishmaniasis. The disease is usually fatal if untreated. VL is endemic in more than 60 countries in tropical and subtropical areas, and in Mediterranean countries; however 90% of the 500,000 new cases that occur every year concern six countries including India, Bangladesh, Nepal, Brazil, Ethiopia, and Sudan. The different pathologies usually correlate with infection by different species (Bañuls et al., 2007).

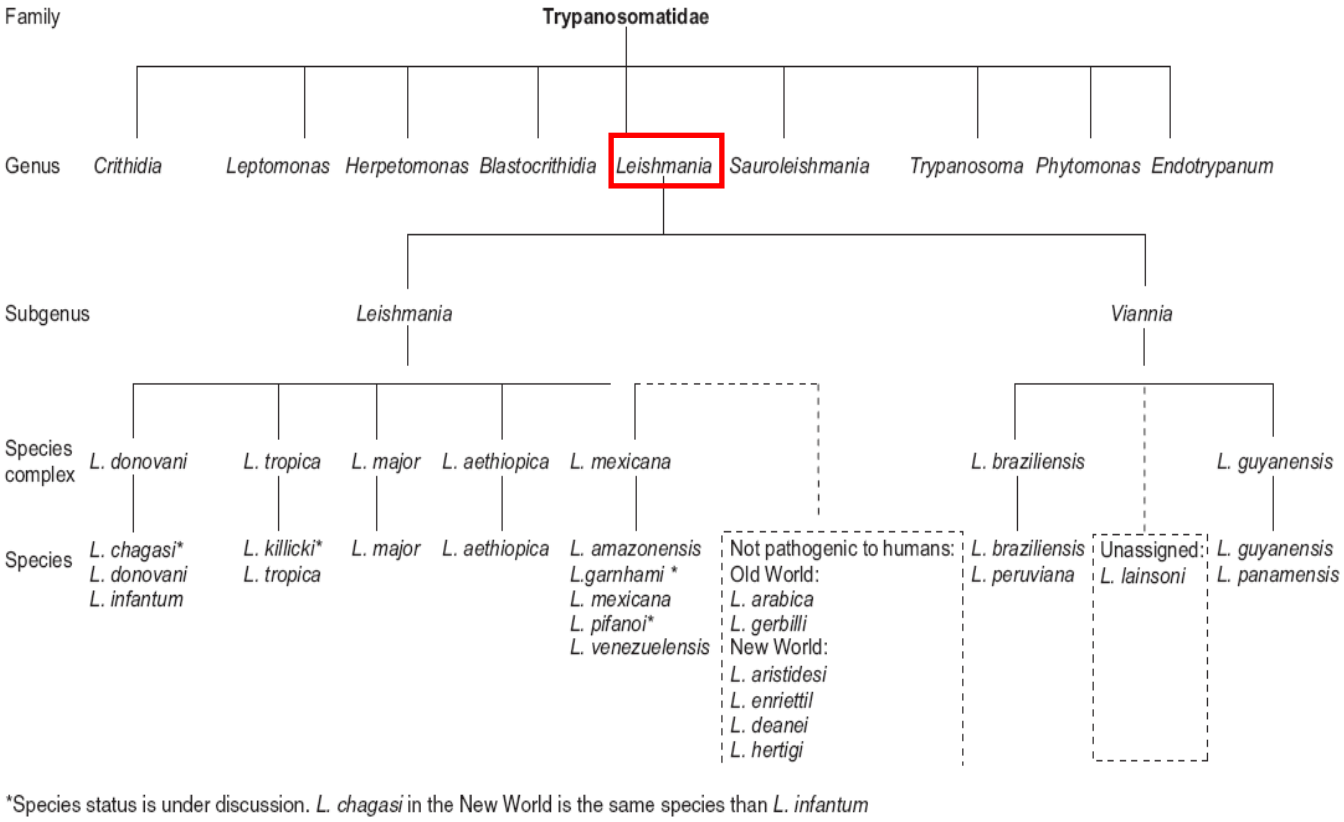

Figure 1 The taxonomy of *Leishmania* (WHO 2012)

Life cycle of *Leishmania* parasites (Figure 2)

Leishmaniasis is a zoonotic disease which includes animal reservoir hosts in its life cycle (Desjeux, 2001). Humans are considered to be an accidental host, although anthroponotic without animal reservoirs is reported in some *Leishmania* species. *Leishmania spp*. have a dimorphic lifecycle consisting of amastigotes with a round or ovoid-shaped and immotile form, live in the cytoplasm of vertebrate macrophage and promastigotes characterized by a spindle-shaped and motile form with an external flagellum, live in female sand fly (Bates, 2007; Kato et al., 2010).

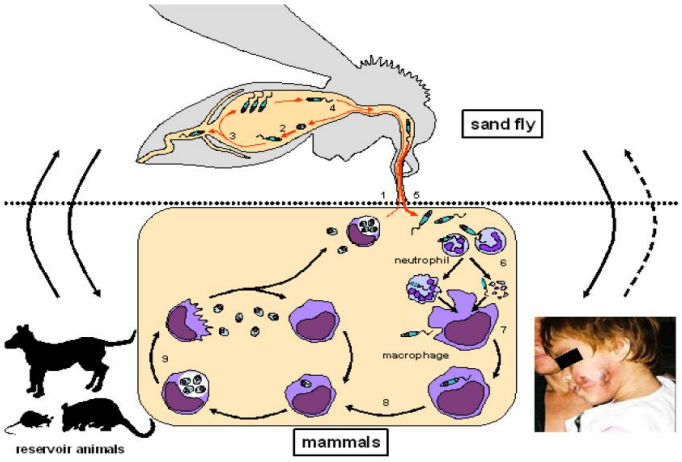

Figure 2. Schematic life cycle of *Leishmania* parasites

Leishmaniasis and HIV/AIDS co-infection

Leishmaniasis and HIV/AIDS co-infection was first reported in 1985, after that in 1994 were found increase infected at the Southern Europe, such as France, Italy, Portugal and Spain. Spain had been reported the most common area of HIV/AIDS co-infection covering approximately 57% of reported cases (Alvar et al., 1997; Desjeux and Alvar, 2003). The study of the spread of leishmaniasis has been reported that co-infected gradually increase worldwide since in 1999 and showed the uncommon symptoms of leishmaniasis (Desjeux, 1998). The co-infection, leishmaniasis accelerates the onset of AIDS by cumulative immunosuppression and stimulation of virus replication (Desjeux, 1998). The report showed this co-infected with more than 35 countries. WHO found that the number of patients who co-infected approximately 39.5 million people, which 1 in 3 of the patients lived in endemic area of leishmaniasis (Alvar et al., 2008). VL (*L. infantum* or *L. donovani*) is a common infection for advanced HIV, which is 77-90% of patients, CD-4 count < 200 cells/mm (Morales et al., 2002) and approximately 63% in the New World (Rabello et al., 2003). For treatment, using same drug which treatment VL, which is not co-infection such as pentavalent antimonials and Liposomal amphotericin B (Lindoso et al., 2012), but found that the treatment is not good and high rates of relapse in VL/HIV co-infected; however, the treatment depends on the species of *Leishmania* and host's immunity.

Autochthonous leishmaniasis in Thailand

In the past, leishmaniasis was found in the Thai people who have a history of returning from endermic areas such as the Middle East. However, recently they are several reports of patients who no history of travelling outside the country were infected with the leishmania parasites. The disease was reported in northern, central and southern provinces of Thailand (Maharom et al., 2008). Interestingly, these patients were from provinces where a potential sand fly vector has never been reported (Apiwathnasorn et al, 1989; Apiwathnasorn et al., 1993). During 1960-1986, 11 cases of sporadic imported VL cases were reported in Thai men who had travelled to endemic areas (Suttinont et al., 1987; Viriyavejakul et al., 1997). From 1996 to 2012 at least 9 cases of autochthonous leishmaniasis in Thailand were reported (Table 1).

Table 1 Summary of nine cases of autochthonous leishmaniasis in Thailand

Year/ province/ part of Thailand/ Occupation	**Age (years)/ Sex**	**Underlying disease**	**Form of leishmaniasis/ Clinical features**	**Types of samples**	**Species of *Leishmania*/ Identification methods**	**Reference**
1996/Suratthani/ South/ NA	2/ Female	No	VL/ Fever, hepatosplenomegaly, anemia, thrombocytopenia	Bone marrow aspiration	No species identified	Thisyakorn *et al*., 1999
2005/ Nan/ North/ Construction worker in several provinces	40/ Male	Amphetamine and opium addiction	VL/ Fever, hepatosplenomegaly, pancytopenia, mediastinal mass	Bone marrow	No species identified	Kongkaew *et al*., 2007
2006/ Phangnga/ South/ Worker in rubber plantation	55/ Male	No	VL/ Fever, hepatosplenomegaly, pancytopenia	Bone marrow	*L. siamensis*/ Giemsa's stained BM, PCR of ITS1 region of ssrRNA gene and minicircle kDNA gene followed by DNA sequencing, PCR-RFLP of the ITS1 region with *Hea*III endonuclease and miniexon gene with *Eae*I endonuclease	Sukmee *et al*., 2008

2007/ Bangkok/ Center/ Lumber truck driver	66/ Male	Diabetes, hypertension	VL/ Fever, weight loss, hepatosplenomegaly, pancytopenia	Bone marrow	*L. infantum*/ Giemsa's stained BM, PCR of ITS1 region of the ssrRNA gene and miniexon gene followed by DNA sequencing, PCR-RFLP of the miniexon gene with *Eae*I endonuclease	Maharom *et al*., 2008
2009/ Chantaburi/ East/ Fisherman	37/ Male	AIDS, Chronic HCV infection	VL/ Fever, nephritonephrotic syndrome, hepatosplenomegaly, anemia, thrombocytopenia	Bone marrow	*L. siamensis*/ Giemsa's stained BM, PCR of 18S rRNA gene and ITS1 region of the rRNA gene followed by DNA sequencing	Suanktatay *et al*., 2010
2010/ Trang/ South/ NA	32/ Female	HIV infection	CL, VL/ Subcutaneous nodules, anemia, hepatomegaly	Bone marrow, Blood, Skin biopsy	*L. siamensis*/ Hematoxylin-eosin stained skin biopsy, Culture, PCR of ssrRNA locus and minicircle kDNA followed by DNA sequencing	Bualert *et al.,* 2012

2011/ Songkhla/ South/ Rubber planter	46/ Male	HIV infection, Evans syndrome	VL, CL/ Anemia, thrombocytopenia, splenomegaly, knee ulcer	Bone marrow, Ulcer discharge, Urine, Oral fluid	*L. siamensis*/ Giemsa's stained BM, Culture, PCR of 18S rRNA gene and ITS1 region of the rRNA gene followed by DNA sequencing	Chusri *et al*., 2012
2011/ Trang/ South/ Pet shop owner	30/ Male	HIV infection	CL/ Multiple papules and plaques with ulcers, oozing, developing collarettes of scales, mind hepatosplenomegaly	Bone marrow, Ulcer biopsy, Urine, Oral fluid	*L. siamensis*/ Giemsa's stained BM, PCR of 18S rRNA gene and ITS1 region of the rRNA gene followed by DNA sequencing	Chusri *et al*., 2012
2012/ Lumphun/ North/ farmer	52/ Male	No	progressive anemia, hepatosplenomegaly for a few months	Bone marrow aspiration, Blood, Oral fluid	*L. siamensis*/ Wright's stained BM, PCR of 18S rRNA gene and ITS1 region of the rRNA gene followed by DNA sequencing	Unpublished data

(CL: Cutaneous leishmaniasis, VL: Visceral leishmaniasis, HIV: Human Immunosuppressive Virus, NA: Not available)

Sand flies in Thailand

There are reports of sand flies in the world, which found only 10% of the approximately 600 known species of sand fly are vectors, and only 30 of these are principle vectors for leishmaniasis. Mostly found around human habitations, in dark corners in the crevices of the walls having high humidity and temperature, feces, manure, rodent burrows, leaf litter, caves, and termite mounds. The female sand fly lays the tiny eggs approximately 15 to 80. In adult, the small wings are very hairy and unable to fly (Figure 3).

Figure 3. Structure of sand fly

In Thailand, entomological surveys of sandfly showed the sandfly consist of 24 species in different areas, such as *P. argentipes, P. asperulus, P. barguesae, P. hoepplii, P. major major, P. mascomai, P. philippinensis gouldi, P. stantoni, P. teshi, S. anodontis, S. barraudi, S. bailyi, S. dentata, S. quatei, S. silvatica, S. gemmea, S. hodgsoni hodgsoni, S. iyengari, S. indica, S. mahadevani, S. perturbans, S. punjabensis, Chinius barbazani, and Nemopalpus vietnamensis*; moreover, more female sandflies were collected than males. (Quate, 1962; Causey, 1938; Theodor, 1938; Apiwathnasorn et al, 1989; Apiwathnasorn et al., 1993; Depaquit et al., 2006; Depaquit et al., 2009; Muller et al., 2007; Polseela et al., 2007). In 2012, sukra and others survey of sandflies in the affected areas of *L. siamensis* were conducted in Phang-nga, Suratthani, and Nakonsitammarat province of southern Thailand. The results showed seven species consist of *S. gemmea, S. iyengari, S. barraudi, S. indica, S. silvatica, S. perturbans*, and *Phlebotomus argentipes*. The most predominant species was *S. gemmea* approximately 81.4 % in all the affected areas. Recently, Kanjanopas and others (2013) revealed that four species female sandflies, *S. (Neophlebotomus) gemmea, S. (Neophlebotomus) iyengari, S. (Parrotomyia) barraudi, and P. (Anaphlebotomus) stantoni* from Trang Province, southern Thailand, where *L. siamensis* in an affected area of leishmaniasis; moreover, *L. siamensis* DNA was identified in *S. (Neophlebotomus) gemmea*. This study was a preliminary survey showed the *S. (Neophlebotomus) gemmea* might be a potential vector of *L. siamensis*.

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Objectives

-To collect and identify sandflies from affected areas of leishmaniasis

-To demonstrate alive *L. siamensis* in sand flies from affected areas of leishmaniasis by culturing and determine infection rate in the sand flies by PCR

Methodology

Study Areas

The study areas are selected from previously reported of autochthonous leishmaniasis patients. The areas of our study are Trang and Songkhla (for southern); Lamphun and Chiang Rai (northern).

Sand fly collection

Sand flies were collected around the leishmaniasis patient's home at southern and northern Thailand. The CDC light traps were installed. The traps were hung from tree branches or hooks at a height of 10 m from the trap hood to the ground, and all traps were activated simultaneously from 18:00 to 06:00 for one night. On the next day, insects were collected from the light traps and anesthetized using chloroformsoaked cotton balls. All sand flies were differentiated according to their gender and genus (Phlebotomus and Sergentomyia) based on morphological identification cues, as observed under a stereomicroscope (Olympus, Japan). The head and genitalia of individual sand flies were cut off in a drop of ethanol, cleared, and mounted between slide and cover slip for identification. The body of each specimen was then stored individually in 70% ethanol in a 1.5-ml sterile tube for further PCR amplification (Figure 4). The specimens were identified by observation of the head and genitalia under a BX50 microscope (Olympus, Japan). The identification was performed using the following keys and articles. Measures were obtained using Stream motion software (Olympus, Japan) and a video camera connected to the microscope. Drawings were generated using a camera lucida.

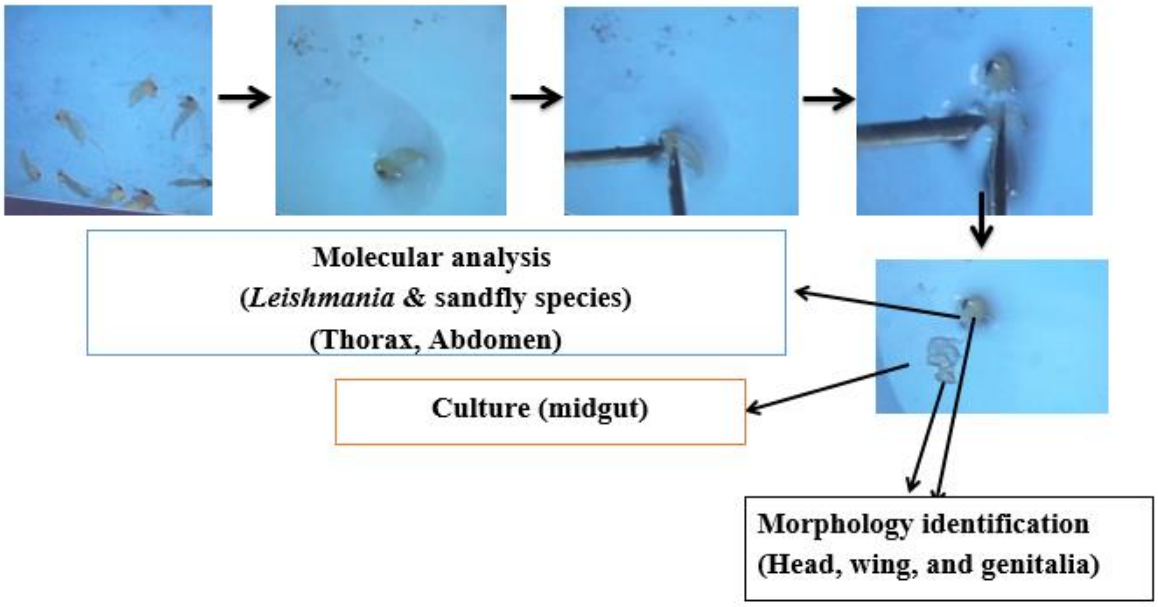

Figure 4 Sand fly sampling for detection *Leishmania*

Culture for *Leishmania* parasites

Schneider's insect medium (Sigma-Aldrich, USA) containing 20% fetal bovine serum and 100 U/100 mg/ml penicillin and streptomycin (Sigma-Aldrich, USA) will be used for culturing *Leishmania* parasites. Female sand fly was grinded in 1XPBS and loaded into 5 ml culture media in a 25 cm^3 flask and maintained at 25±2 ^{0}C. The cultures were inspected for the parasites every 24 hours under an inverted microscope (Olympus, Japan). For continuous maintaining, the cultures passaged every 2-3 days by diluting the original culture with Schneider's media at 1:2 dilutions. Alternatively take 0.5 ml (when enough much cell) of the old culture and inoculate 4.5 ml of fresh medium in a new flask.

DNA extraction

Individual sand fly of each sample was lysed by lysis buffer and placed in liquid nitrogen for 1 minute and then ground with a sterile plastic pestle. Genomic DNA was isolated using DNA extraction kits: Invisorb® Spin Tissue Mini Kit (STRATEC Molecular GmbH, Germany) according to the manufacturer's instructions. The extracted DNA was eluted in 50 µl of elution buffer; the fraction of extracted DNA was spectrophotometrically

quantitated using a Nanodrop 2000c (Thermo-scientific, USA). The extracted DNA samples were kept at -80 ^{0}C for long term storage.

PCR amplification

***Leishmania*, Trypanosome and sand fly DNA detection**

The PCR reaction was set up in a final volume of 25 µl, containing approximately 100 ng of extract DNA. The primers are designed to anneal specific to the ITS1 regions of the rRNA of *Leishmania* parasites which were described by Spanakos *et al*. (2007) (LeF primer: 5' TCC GCC CGA AAG TTC ACC GAT A 3' and LeR primer: 5' CCA AGT CAT CCA TCG CGA CAC G 3'; 379 bp); 379 bp. PCR reactions were performed in a PCR Mastercycler® pro (Eppendorf, Germany). The SSU rDNA gene of trypanosomatids (TRY927F: 5'-GAAACAAGAAACACGGGAG-3' and TRY927R: 5'-CTACTGGGCAGCTTGGA-3') (Noyes et al. 1999). For the amplification of sand fly DNA for species identification, we used primers N1N-PDR: 5′- CAY-ATT-CAA-CCW-GAA-TGA-TA -3′ and C3B-PDR: 5′- GGT-AYW-TTG-CCT-CGA-WTT-CGW-TAT-GA -3′ to amplify CytB gene of sand fly, following a previously published method (Esseghir et al. 1997, Depaquit et al. 2015). PCR were performed in a PCR Mastercycler® Pro (Eppendorf, Germany) with the following conditions: an initial denaturation of 4 minutes at 94°C, followed by 40 cycles of denaturation (94 °C for 1 minute), annealing at 1 minute at 65°C for the ITS1 gene or 51.7 °C for the SSU rDNA gene, extension (72°C for 1 minute) and a final extension at 72°C for 7 minutes. The reaction was electrophoresed on a 2% (w/v) agarose gel, stained with ethidium bromide and visualized with Quantity One Quantification Analysis Software version 4.5.2 (Gel Doc EQ System; Bio-Rad, CA). Double distilled water (ddH2O) was used as a negative control, and DNA from the promastigotes culture of *L. siamensis* was used as the positive control. Aliquots of the PCR amplicons were analyzed on a 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized with Quantity One quantification analysis software version 4.5.2 Gel Doc EQ system (Bio-Rad, USA).

DNA cloning and sequencing

The PCR amplicons were ligated into pGEM-T Easy Vector (Promega, USA). Ligation reactions mixture were composed of 5 µl 2X Rapid ligation buffer, 3 µl of PCR products, and 1 µl pGEM-T Easy Vector. The ligated vectors were transformed into DH5α competent cells and chimeric plasmids were screened by blue-white colony selection system. The suspect positive colonies were cultured and were used for further plasmid DNA extraction by using Invisorb® Spin Plasmid Mini kit (STRATEC Molecular GmbH, Germany) following the manufacturer's instructions. Purified plasmids were sent to sequence by 1st BASE DNA sequencing services (1st base laboratories, Malaysia) using universal forward T7 primer. Nucleotide sequences were analyzed using BioEdit Sequence Alignment Editor Version 7.0.9.0, the consensus sequences were compared with available sequence data in the GenBank by BLAST search (available at http://www.ncbi.nlm.gov/BLAST).

Results and Discussion

Sand fly collection in southern Thailand

In this study, sand flies were collected from the Songkhla province of Southern Thailand in September 2013 using CDC light traps. The traps were installed inside a pile of firewood, in an incinerator, under coconut, bamboo, and banana trees, in termite mounds, and in the space under the home of a patient with leishmaniasis. The traps were left overnight. On the next day, insects were collected from the light traps and anesthetized using chloroform-soaked cotton balls. All sand flies were differentiated according to their gender and genus (*Phlebotomus* and *Sergentomyia*) based on morphological identification cues, as described under a stereomicroscope (Olympus, Japan). The head and genitalia of individual sand flies were cut off in a drop of ethanol, cleared, and mounted between slide and cover slip for identification. The body of each specimen was then stored individually in 70% ethanol in a 1.5 ml sterile tube for further PCR amplification.

The specimens have been identified by observation of the head and genitalia under a BX50 microscope (Olympus, Japan). The identification has been done using the following keys and articles (Raynal 1935, Raynal, 1936, Raynal and Gaschen 1935, Quate 1962, Lewis 1978, Lewis 1987). Measures and have been performed using the Stream motion software (Olympus, Japan) and a video camera connected to the microscope. Drawings have been made using a *camera lucida*.

A total of 45 sand flies, including 21 males and 24 females, were collected (Table 2).

Table 2 Sampling species

Species	Males	Females	Total
Phletotomus stantoni	2	5	7
Sergentomyia “barraudi”	7	7	14
Se. hivernus	3	4	7
Se. indica	4	3	7
Se. khawi	5	5	10
Total	21	24	45

They belonged to the following species: *Phlebotomus stantoni* Newstead, *Sergentomyia barraudi* (Sinton), *Se. khawi* (Raynal), *Se. hibernus* (Raynal & Gaschen) and *Se. indica* (Theodor). Apiwathnasorn et al. (1989) reported that sand flies could be found in different geographical areas of Thailand, such as in caves, termite hills, air-raid shelters, ancient stone sanctuaries, tree hollows, and rock crevices. Some reports have described sand flies inhabiting the Naresuan Cave, Phitsanulok Provinces (northern) (Polseela et al. 2011), caves in the Kanchanaburi Province (western) (Apiwathnasorn et al. 2011), the Tham Phra Phothisat temple, the Saraburi province (central) (Polseela et al. 2011) and the Satun province (Panthawong et al. 2015) of Thailand. Sand flies found in Thailand have been identified to fall within 4 genera: *Sergentomyia*, *Phlebotomus*, *Idiophlebotomus*, and *Chinius*. The most common genera found in the country are the *Sergentomyia* flies, following by the *Phlebotomus* flies; the *Idiophlebotomus* and *Chinius* flies are believed to be restricted largely to caves. In 2009, Sukra et al. reported that *Se. gemmea* (81.4%) were mostly found in the three southern provinces of Thailand (Phangnga, Suratthani, and Nakonsitammarat). Importantly, autochthonous leishmaniasis cases caused by *L. siamensis* and *L. martiniquensis* have also been reported in these areas (Sukra et al. 2009). In this paper, we report two new species for Thailand: *Se. khawi* (Raynal & Gaschen, 1936) previously reported from Cambodia, China and Malaysia (Seccombe et al., 1993) and *Se. hivernus*. The latter species has been described from Vietnam, formerly Indochina, by Raynal and Gaschen (1935) under the name of *Ph. hibernus* then changed in *Ph. hivernus* by Raynal (1935b).

It has been surprisingly considered as a junior synonym of *Se. iyengari* by Quate (1962). To our opinion, this synonymy is wrong. The spermathecae of *Se. hivernus* are long, wide, smooth, tubular without limit between body and duct whereas those of *Se. iyengari* are and ii) that *Se. hivernus* exhibits many denticles on the cibarium (about 10 in the original description; 3 to 8 in the present study; mean: 5.4; standard error: 1.95 on one row) whereas *Se. iyengari* has been described without any denticle (Figure 5). The number of teeth of the specimens we observed is ranked from 10 to 16, which is in agreement with the number of 14 recorded in the original description. We noticed the third antennal segment (A III=flagellomere 1) is shorter in our specimens (minimum 218 µm, maximum 239 µm, mean 230 µm, standard error 9.79 µm) than in those described by Raynal (250 to 285 µm long). Consequently, we reinstate *Se. hivernus* we consider as a valid species. By the way, we

consider for *Se. hivernus* a more important range for the number of cibarial teeth (10 to 16) and cibarial denticles (variable 3 to about 10).

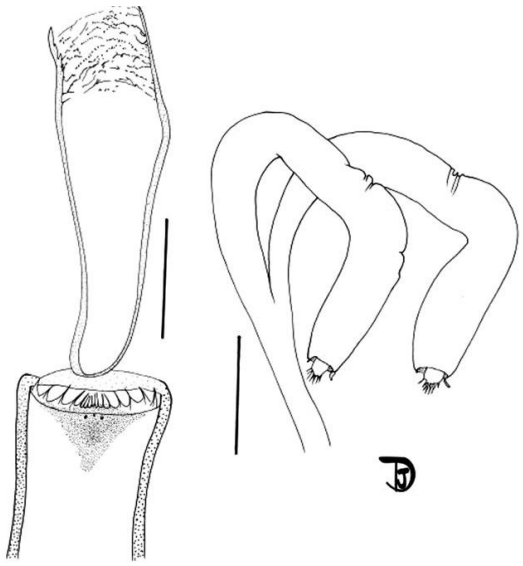

Figure 5 Drawings of *Se. hivernus* from Southern Thailand: pharynx and cibarium (left) and spermathecae (right). Bars = 50 μm

The phylogenetic tree constructed based on the CytB gene showed that there is a strong link between morphological identification and molecular characterization (Figure 6). Our study confirms that the CytB gene sequences are useful for sand fly species identification (Depaquit 2014). The CytB sequences showed that the *Ph. stantoni* sand flies collected from this study were 100%, 99% and 99% identical to the *Ph. stantoni* sand flies collected from Chiang Mai, Thailand (KM409494), Malaysia (KM409495) and Vietnam (KM409497 and KM409498), respectively (Depaquit et al. 2015). Surprisingly, the two populations of *Se. barraudi* could be clearly distinguished based on their CytB sequences without any apparent morphological evidence. Further taxonomic studies need to be carried out to explore this observation. MEGA 6.0 software. The tree shown is based on the Kimura 2-parameter model of nucleotide substitution. Bootstrap values are based on 1,000 replicates. Red triangle indicated sand fly species obtained from this study.

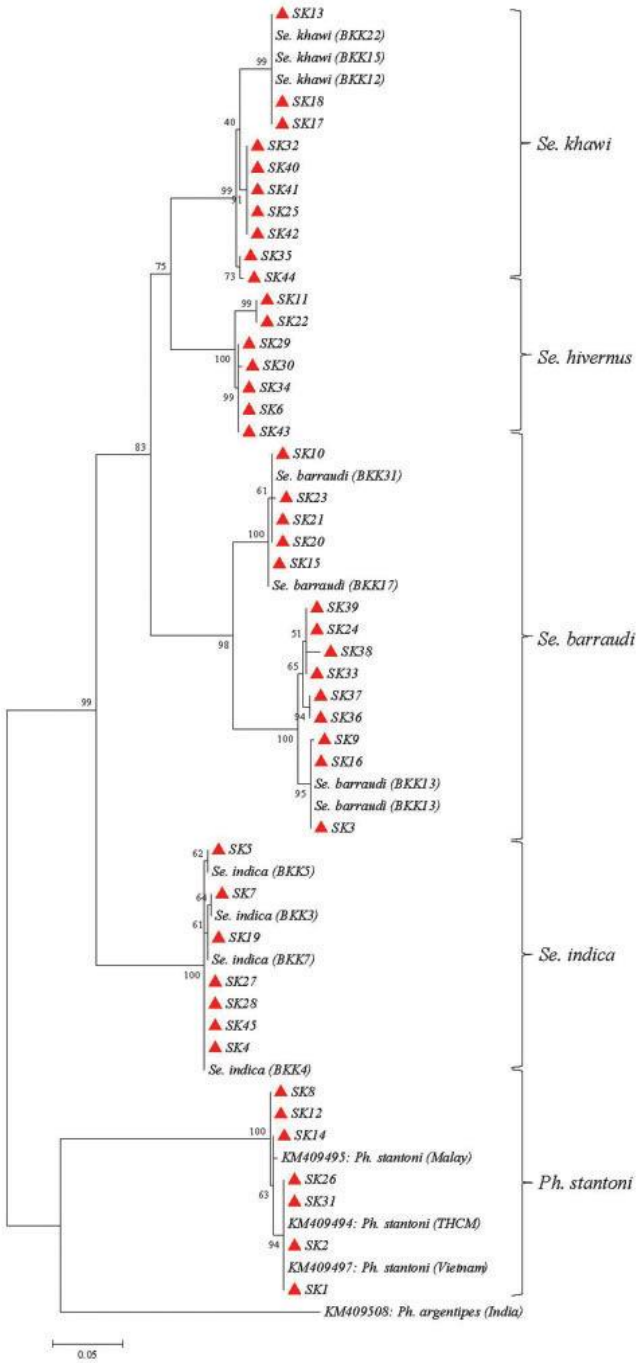

Figure 6 Maximum likelihood tree of the CytB gene sequences of the sand fly using MEGA 6.0 software. The tree shown is based on the Kimura 2-parameter model of nucleotide substitution. Bootstrap values are based on 1,000 replicates. Red triangle indicated the sand fly species that was obtained from this study.

PCR was used to detect Trypanosomatidae parasites in the sand fly samples. One female *Ph. stantoni* sand fly sample was positive for Trypanosoma sp. The PCR amplicons of ITS1 and SSU rDNA amplified from this study were 360 and 939 bp, respectively. The nucleotide sequences of the ITS1 and SSU rDNA gene were submitted to GenBank and assigned the access numbers KJ467211 and KJ467217, respectively. Neither the ITS1 nor SSU rDNA sequences were identical to those of any previously described Trypanosoma sequences available in the database, suggesting that this is instead a novel Trypanosoma species.

A phylogenetic ML tree was constructed using the ITS1 and SSU rDNA regions. The ITS1 gene of the *Trypanosoma sp*. of sand fly from this study showed a unique cluster from other Trypanosoma species obtained in this database (Stevens et al. 1998, Kato et al. 2010, Nzelu et al. 2014), which are closely related to trypanosomes isolated from rodents (Figure 7A). An ML tree constructed from SSU rDNA gene found a close similarity to the ITS1 gene (Figure 7B). The suspected novel Trypanosome species is closed related to *T. microti* and *T. kuseli* (accession numbers AJ009158 and AB175626, respectively), which trypanosomes isolated from rodents. Sequence comparisons between this new species and *T. microti* and *T. kuseli* were 94% identical (Figure 7C).

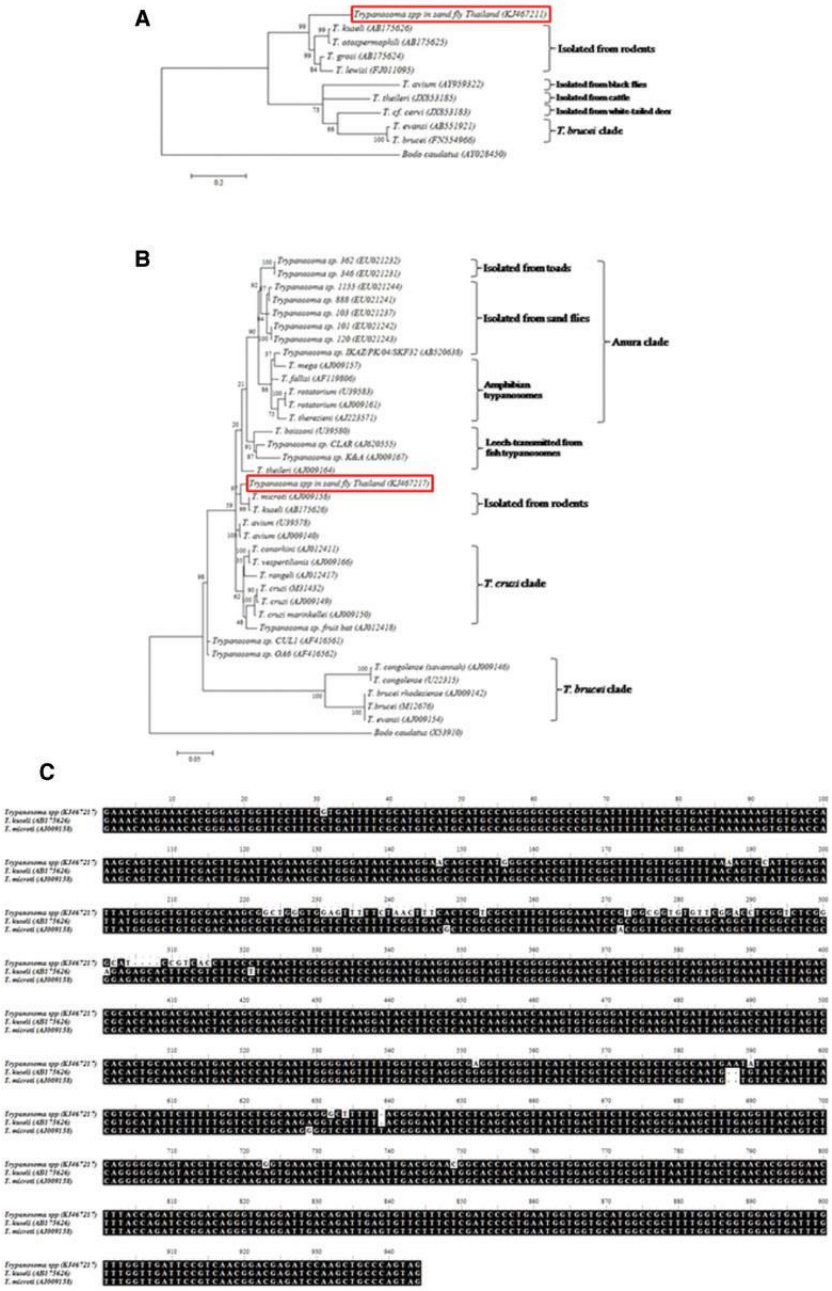

Figure 7 Phylogenetic tree of ITS1 (A) and SSU rDNA (B) gene sequences of Trypanosoma sp. The Kimura-2-parameter model in MEGA6.0 software was used. Bootstrap values based on 1,000 replicates. Their names for this study are genus, species, and accession numbers. ITS1 gene (Bodo caudatus accession no. AY028450 as an outgroup) or SSU rDNA gene (*B. caudatus* accession no. X53910 as an outgroup). Comparison between SSU rDNA sequences obtained from *Trypanosoma sp* from this study, *T. microti* and *T. kuseli* (C).

Previous reports have stated that trypanosome parasites can be found in sand fly vectors. A long time ago, *Se. minuta* (Rondani) was suspected of transmitting Trypanosoma platydactyli Catouillard to the gecko *Tarantola mauretanica* (Adler and Theodor 1935). McConnell and Correa (1964) suggested that most of the trypanosome infections in their study were in *Dampfomyia vespertilionis* (Fairchild & Hertig). Kato et al. (2010) reported a natural infection due to a species of *Trypanosoma* in the sand fly *Ph. kazeruni* in Pakistan. More recently, Nzelu et al. (2014) have found *Trypanosoma* DNA in Se. africana in Ghana. Ferreira et al. (2015) identified *Trypanosoma* spp. in Brazilian *Sciopemyia sordellii*, *Sc. servulolimai*, *Sc. sp.*, and *Evandromyia infraspinosa*.

This is the first report of *Trypanosoma sp*. DNA detected in a *Ph. stantoni* female from Thailand. We suspect that it belongs to a novel species of the genus *Trypanosoma*. Unfortunately, we have not yet isolated, cultivated, or described this putative novel species. Further investigations will be necessary to demonstrate the relationships between trypanosome parasites, sand fly vectors and reservoir hosts

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Sand fly collection in northern Thailand

Results

A total of 824 female sand flies were captured in this survey studies. There are consist of 13 species in 4 genera including *Se. anodontis*, *Sergentomyia* sp., *Se. sylvatica*, *Se. iyengari, Se. indica*, *Se. hivernus, Se. barraudi*, *Ph. barguesae*, *Ph. stantoni*, *Ph. mascomai*, *Ph. kiangsuensis*, *Id. longiforceps*, and *Chinius* sp. (Table 3).

Table 3 Summary of sand flies collected and detection of *Leishmania* parasite by isolation and PCR

Species	Number (%)	Positive for *Leishmania* detection	
		Isolation (n)	PCR (n)
Se. anodontis	275 (33.37)	1	3
Se. sylvatica	77 (9.35)	0	0
Se. barraudi	32 (3.88)	0	0
Se. iyengari	21 (2.55)	0	1
Se. indica	13 (1.58)	0	0
Se. hivernus	2 (0.24)	0	0
Sergentomyia sp.	213 (25.85)	0	1
Ph. barguesae	66 (8.01)	0	0
Ph. stantoni	48 (5.83)	0	0
Ph. mascomai	8 (0.97)	0	0
Ph. kiangsuensis	2 (0.24)	0	0
Id. longiforceps	66 (8.01)	1	9
Chinius sp	1 (0.12)	0	0
Total	**824**	**2**	**14**

Numerous *Leishmania*-like grouped promastigotes were observed in the midgut of one *Id. longiforceps* and one *Se. anodontis* samples (Figure 8A). We were successfully isolated *Leishmania* parasites from these two samples (THCR128 and THCR 115 isolated from *Se. anodontis* and *Id. longiforcep* respectively) (Figure 8B). All samples of female sand flies were screened for *Leishmania* DNA by PCR. Species of *Leishmania* parasites were identified by the ITS1 and HSP70 gene sequences. Sequences of the ITS1 and HSP70 genes were 573 and 1,421 base pairs, respectively. All PCR positive samples including isolates THCR128 and THCR 115 in this study showed 100% sequence identification in both partial ITS and HSP70 genes. Therefore, we used sequences of THCR 115 to represent *Leishmania* parasites found in this study.

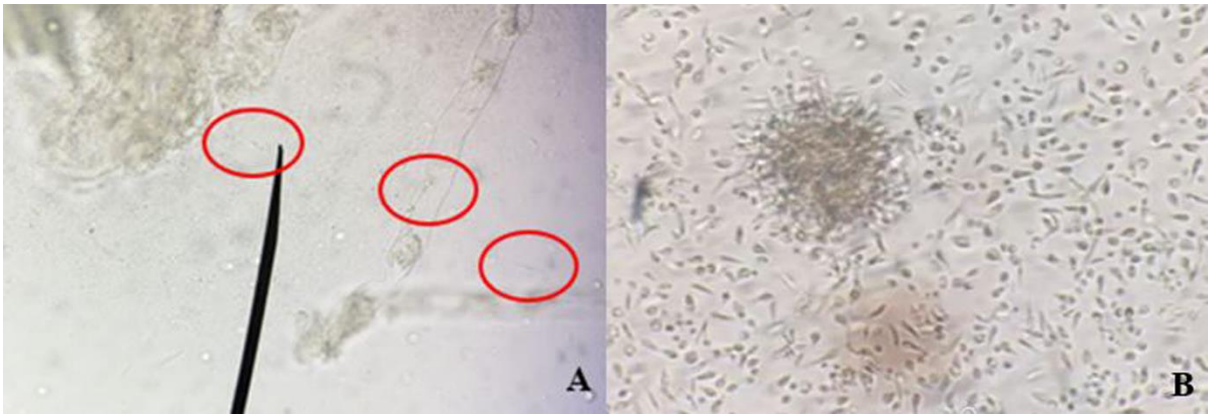

Figure 8 *Leishmania* species isolated from a midgut of *Id. longiforceps* (A). Rosettes of parasites with varying in morphology were cultured in Schneider's insect medium (B).

The partial sequence of the ITS1 using the BLAST program showed that the sequences was similar to the *Trypanosoma* sp. accession number KJ467211 (99% identity and only 17% of query cover) and all *Leishmania* sp. sequence deposited in GenBank (100% identity and only 17% of query cover) (Figure 9). The partial sequence of the HSP70 using the BLAST program showed that the sequences was similar to the *Trypanosoma lewisi*, accession number KP208748 (90% identity and 95% of query cover), *L. braziliensis*, accession number XM001566275 (89% identity and 100% of query cover), and *L. panamensis* accession number XM010702330 (89% identity and 100% of query cover)

(Figure 10). Phylogenetic analysis using ITS1 and HSP70 sequences by MEGA 7, a neighbor-joining algorithm, and Kimura 2-parameter correction confirmed that our sample (THCR115) was closely related to *Leishmania* sp. more than *Trypanosoma* sp. in both genes when comparison with reference DNA sequences (Figure 11).

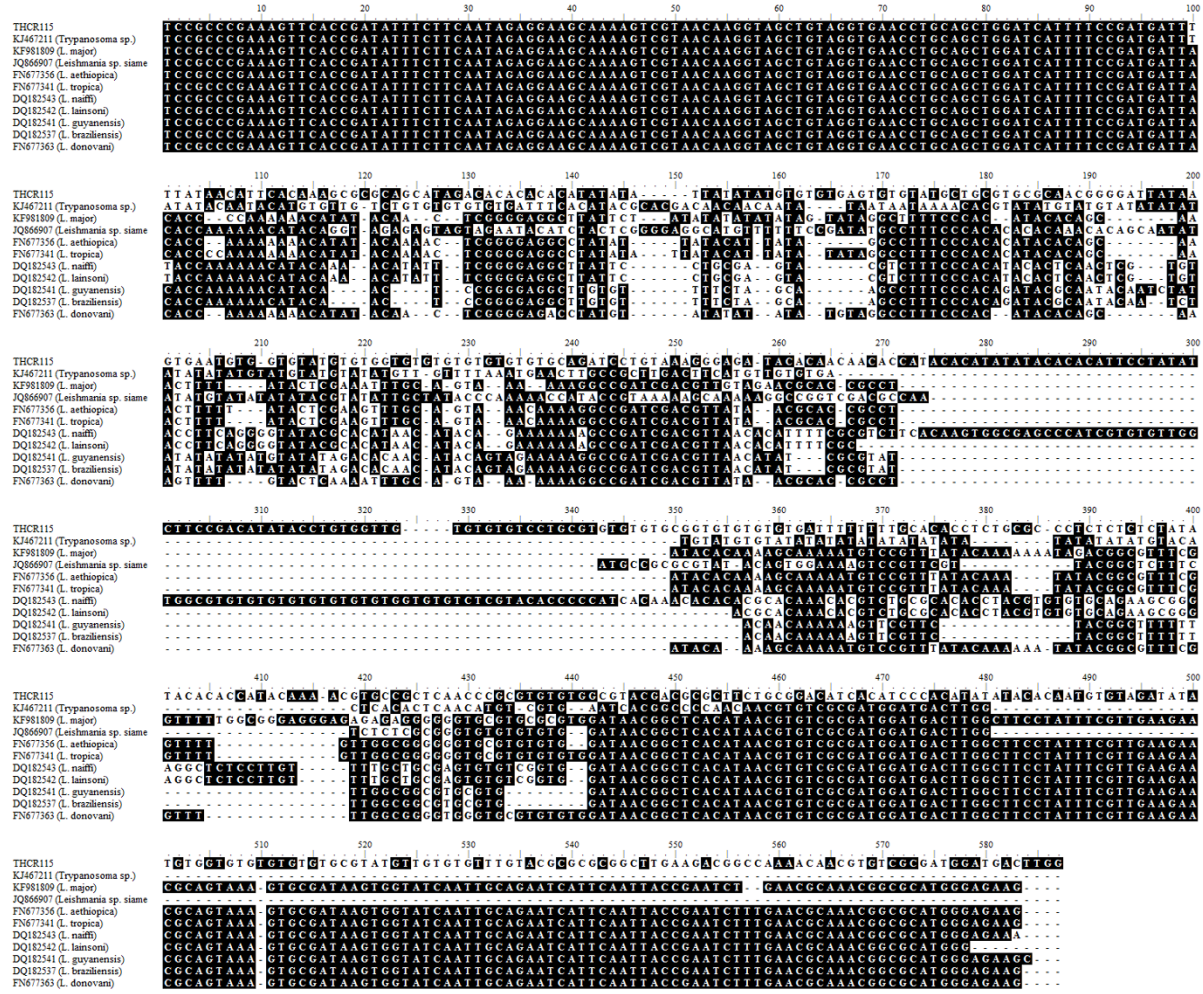

Figure 9 Comparison between ITS1 sequences obtained from this study (THCR115) and references sequences form GenBank

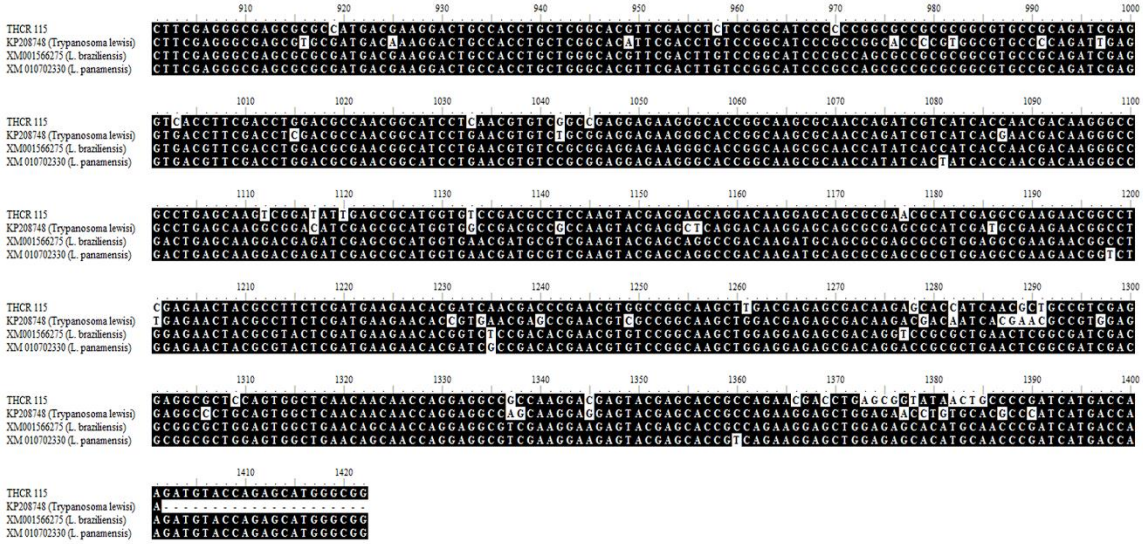

Figure 10 Comparison between HSP70 sequences obtained from this study and *Trypanosoma lewisi* (KP208748), *L. braziliensis* (XM001566275), and *L. panamensis* (XM010702330) form GenBank

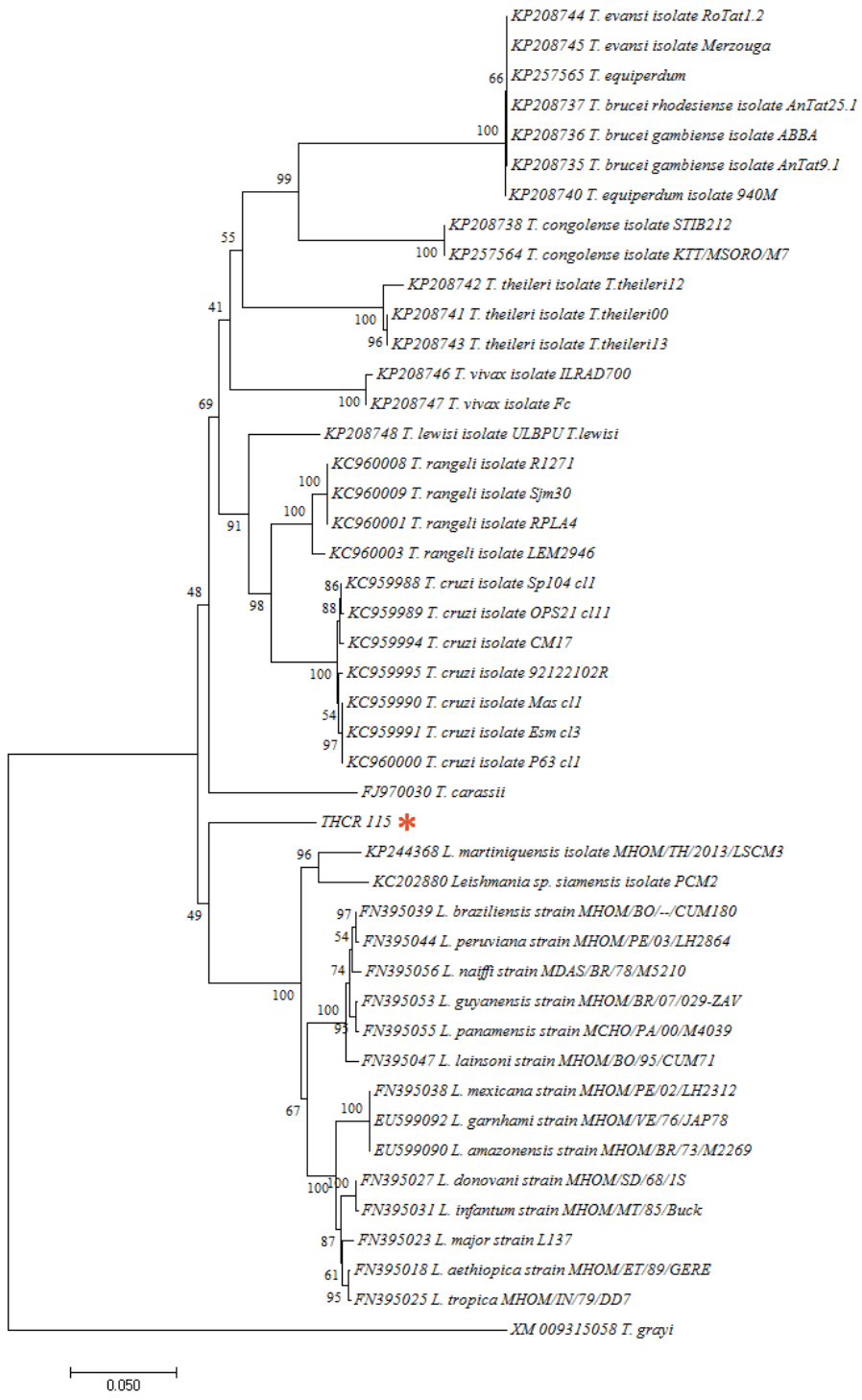

Figure 11 Phylogeny of parasite based on HSP70 gene sequences. Bootstrap confidence values (1000 replicates). Red asterisk indicated the positive sample (THCR115) that was obtained from this study.

Discussion

Autochthonous leishmaniasis in Thailand is caused by two major *Leishmania* species; *L. siamensis* and *L. martiniquensis* [3-14]. For the north of Thailand, there are five leishmaniasis cases (2 case from Lamphun, 1 case from Chiang Rai, 1 case from Chiang Mai, and 1 case from Nan) caused by *L. martiniquensis* were reported [4, 9, 11, 24, 25]. This study investigated sand fly vectors of leishmaniasis in Chiang Rai, northern, Thailand. Among the 824 samples examined *Sergentomyia* (76.82%) is the predominate species followed by *Phlebotomus* (15.05%), *Idiophlebotomus* (8.01%), and *Chinius* (0.12%). Polseela et al (2011) revealed that 13 sand fly species collected from Naresuan Cave, Phitsanulok Province, northern Thailand, including *Nemopalpus vietnamensis* (49.15%), *Ph. argentipes* (20.15%), *C. barbazani* (15.79%), *Ph. teshi* (9.53%), and *Se. anodontis* (3.21%), *Se. barraudi* (0.63%), *Ph. stantoni* (0.57%), *Se. dentata* (0.49%), *Se. quatei* (0.17%), *Ph. philippinensis gouldi* (0.12%), *Se. sylvatica* (0.10%), *Se. gemmea* (0.05%), and *Se. iyengari* (0.04%) [26]. Previously reports, *Ph. argentipes, Se. barraudi, Se. indica,* and *Se. iyengari* can found at Chiang Rai Province, moreover *Ph. argentipes, Ph. stantoni, Se. indica, Se. anodontis, Se. iyengari, Se. silvatica, Se. barraudi,* and *Se. bailyi* have been reported in Chiang Mai Province [27]. Recently, Polseela et al. (2016) described the *Se. phadangensis* n. sp, a new species of sand fly in Thailand by studying morphology identification and molecular analysis of cytochrome b rDNA [28]. *Id. longiforceps* flies were reported in Khao Tham Khun Chorn cave in Ratchaburi province, central of Thailand [29, 30]. More recently, Phumee et al. (2017) described the first detection of *Se. hivernus* in southern Thailand [31], which this species also found in this study.

As described previously, the DNA of *Leishmania* parasites has been detected in sand flies collected from endemic areas, southern Thailand [21]. However, alive *Leishmania* parasites in sand flies have never been demonstrated. This report is the first demonstration of natural *Leishmania* parasites infection in *Id. longiforceps and Se. anodontis.* Reports as *Sergentomyia* species for the potential role as a vector of leishmaniasis have been described [32]. For example, *L. donovani* DNA has been detected in *Se. babu* in India [33], *L. major* DNA has been detected in *Se. sintoni* in Iran [34], in *Se. minuta* form Portugal [35], and *Se. clydei* [36] and *Se. minuta* [37] from Tunisia. In addition, *L. infantum* DNA has been detected in *Se. dubia*, *Se. magna* and *Se. schwetzi* in Senegal [38], whereas "*L. siamensis*" DNA has been

found in *Se. gemmea* and *Se. barraudi* from Thailand [39]. Futhermore, promastigotes of *Leishmania* have been observed in *Se. antennata*, *Se. clydei*, *Se. bedfordi*, *Se. africana*, *Se. garnhami*, *Se. graingeri*, *Se. ingrami*, *Se. kirki*, and *S. schwetzi* in Kenya [40] and in Ethiopia [41]; however, they were unable to identify the *Leishmania* species. From our knowledge, detection of *Leishmania* parasites in *Idiophlebotomus* sand flies has never been reported. Data from this survey shows that *Leishmania sp*. infection rate in the genera *Idiophlebotomus* (1.1 %) which is higher than the genera *Sergentomyia* (0.5 %). Although, we were able to demonstrate alive *Leishmania* parasites in both female *Id. longiforceps* and *Se. anodontis*, but the *Leishmania sp*. in this study has never been report as a causative agent of leishmaniasis in Thailand. Sequence analysis of ITS1 and HSP70 of this *Leishmania sp*. showed that it is not related to the causative agents of autochthonous leishmaniasis in Thailand (*L.siamensis* and *L. maetiniquensis*). ITS1 is usually used for evolution and identification of *Leishmania* because high sequence variations. Therefore, ITS1 sequences of many *Leishmania* species were available from GenBank [22], such as *L. infantum*, *L. donovani*, *L. major*, *L. siamensis*, *L. martiniquensis*, *L. tropica* and other leishmanial parasites [42-45]. The HSP70 gene sequences are commonly also used for *Leishmania* species identification. This gene region can be used for discrimination between *L. lainsoni*, *L. amazonensis*, *L. infantum*, the *L. braziliensis/L. peruviana* complex, and the *L. guyanensis/L. panamensis* complex [23]. Furthermore, HSP70 usually used for study of *Trypanosome* evolution including *T. cruzi*, *T. lewisi, T. carassi*, *T. congolense*, *T. grayi*, *T. rangeli*, *T. theileri*, *T. vivax*, and *Trypanozoon*, which could be clearly distinguished [46]. Therefore, further studies of potential of this parasites as a caused for leishmaniasis in vertebrate hosts is required.

Apart from *Leishmania*, several reports also described the *Trypanosome* DNA in sand flies [31, 36, 47, 48]. Nzelu et al. (2014) revealed that *L. tropica* and *L. major* DNA and *Trypanosoma* DNA can detected in *Sergentomyia* sand flies in Ghana [47]. However, Kato et al. (2010) report of live naturally *Trypanosoma* species infected from phlebotomine sandflies in Pakistan [48]. More recently, Barratt et al. (2017) isolated novel Trypanosomatid, *Zelonia australiensis* sp. nov. (Kinetoplastida: Trypanosomatidae) in black fly, *Simulium* (*Morops*) *dycei* Colbo, 1976 [49]. Phumee et al. (2017) also reported that *Trypanosoma* sp. DNA was detected in a *Ph. stantoni* collected from southern Thailand [31].

The origin, evolution, and distribution of *Leishmania* and sand flies in the Old and New World are ambiguous. Recently, Akhoundi et al. (2016) described information in relation to both the *Leishmania* species they transmit and the animal reservoirs of the parasites [50]. The detection of parasite closely related to *Leishmania* sp. in *Id. longiforceps* and *Se. anodontis* is novel knowledge of Thailand. Sand flies are distributed throughout the country, extensive survey of sand flies in Thailand and pathogens they transmit are required to monitor the possible outbreak of emerging diseases caused by novel pathogens transmitted by sand flies.

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Development of loop-mediated isothermal amplification (LAMP) for simple detection of *Leishmania* infection

Chaichontat Sriworarat[1], Atchara Phumee[2], Mathirut Mungthin[3], Saovanee Leelayoova[3] and Padet Siriyasatien[2,4*]

Abstract

Background: Leishmaniasis is a neglected tropical disease that is caused by an obligate intracellular protozoan of the genus *Leishmania*. Recently, an increasing number of autochthonous leishmaniasis cases caused by *L. martiniquensis* and the novel species *L. siamensis* have been described in Thailand, rendering an accurate diagnosis of this disease critical. However, only a few laboratories are capable of diagnosing leishmaniasis in Thailand. To expand leishmaniasis diagnostic capabilities, we developed a simple colorimetric loop-mediated isothermal amplification (LAMP) technique for the direct detection of *Leishmania* DNA.

Methods: LAMP was performed for 75 min using four primers targeting the conserved region of the18S ribosomal RNA gene, and the DNA indicator used was malachite green (MG). To simulate crude samples, cultured promastigotes of *L. siamensis* were mixed with blood or saliva. Also, clinical samples (blood, saliva, and tissue biopsies) were obtained from patients with cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL). All samples were boiled for 10 min and introduced directly into the LAMP reaction mixture without DNA purification.

Results: The use of MG resulted in an unambiguous differentiation of positive and negative controls. For *L. siamensis*, the detection limit was 10^3 parasites/mL or 2.5 parasites/tube. Saliva, tissue biopsies, and whole blood were indicative of active *Leishmania* infection, and their direct usages did not adversely affect the detection limit. In addition, this LAMP assay could detect DNA from multiple *Leishmania* species other than *L. siamensis* and *L. martiniquensis*, including *L. aethiopica*, *L. braziliensis*, *L. donovani* and *L. tropica*.

Conclusions: The simplicity and sensitivity of LAMP in detecting active *Leishmania* infection could enable the rapid diagnosis of leishmaniasis, thereby facilitating the survey and control of leishmaniasis in Thailand. However, our limited number of samples warranted a further validation with a larger cohort of patients before this assay could be deployed.

Keywords: *Leishmania martiniquensis*, *L. siamensis*, LAMP, Malachite green, Diagnosis

Background

Causing more than 50,000 deaths annually [1], leishmaniasis is one of the most debilitating poverty-related diseases and presents a severe threat to socioeconomic development. This disease is caused by more than 20 species of the obligate intracellular protozoa *Leishmania* [2]. These are transmitted to humans through the bites of female sand flies [1]. Upon infection, three main clinical forms can be recognized: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL). CL, the most common form, is characterized by the presence of various ulcerative lesions, which lead to disfiguring and/or disabling scars [3, 4]. Consequently, patients with CL often live in obscurity [5], thereby preventing expeditious treatment and increasing the probability of transmission. MCL is described by a severe destruction of mucosal regions (nose, mouth, and throat) [4]. VL is an infection of the internal organs that is characterized by prolonged fever, anemia, hepatosplenomegaly, and weight loss. VL is fatal if left untreated [6].

* Correspondence: padet.s@chula.ac.th
[2]Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand
[4]Excellence Center for Emerging Infectious Diseases, King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Bangkok 10330, Thailand
Full list of author information is available at the end of the article

Since the first case report in 1996 [7], indigenous leishmaniasis has been increasingly prevalent in Thailand, especially in HIV patients. Case reports include both VL [7–12] and CL [10, 11, 13–15] and are concentrated in the northern and southern part of Thailand. In contrast to other regions in Asia, most cases in Thailand are caused by either *L. martiniquensis* or *L. siamensis* [12, 16]. As early detection is one of the most important aspects of disease containment, the need for a robust and rapid diagnostic method has never been higher. To summarize, currently available methods can be divided into three groups: parasitological methods, serological methods, and molecular methods, each of which presents various advantages and disadvantages.

Parasitological methods, which include microscopy and parasite culturing, have been considered the gold standard in diagnosing leishmaniasis. In Thailand, however, only a handful of laboratories could culture parasites. Also, serological diagnostic methods with comparable sensitivity to parasitological methods have been developed; however, most of them (enzyme-linked immunosorbent assay (ELISA) [17, 18], immunofluorescence antibody test (IFA) [19], and western blotting [20]) require sophisticated instruments, limiting their usages in healthcare environments. Moreover, these serological techniques have never been evaluated for the diagnosis of leishmaniasis in Thailand.

Molecular methods with great sensitivities and specificities have also been developed to diagnose leishmaniasis. One of the most classical techniques, polymerase chain reaction (PCR), is widely used [21–24]. However, the requirements for expensive equipment, DNA purification, and gel visualization have forestalled its utilization in field settings.

In 2000, Notomi et al. [25] developed the loop-mediated isothermal amplification (LAMP) method. In short, this method uses several complex primers and a strand-displacement polymerase to achieve amplification. Though conceptually challenging, LAMP has several advantages over PCR from a field diagnostics point of view. 1) The reaction proceeds isothermally, thereby obviating the need for expensive thermal cyclers [25]. 2) Crude DNA extracts can be used directly without purification [25, 26]. 3) The products can be detected visually using multiple parameters, including turbidity, fluorescence, and color.

Nevertheless, turbidity is challenging to discern and is unstable over time. Fluorescence measurement requires costly dyes (SYBR Green I [27], calcein [28], propidium iodide [29]) and is technically inconvenient due to its requirement for UV illumination. Due to their inhibitory effects, these dyes must be introduced post-reaction, increasing contamination risks. Colorimetric measurements are among the most straightforward of all of the detection methods. Several dyes have been reported to be useful, such as hydroxynaphthol blue (HNB) [30] and malachite green (MG) [31]. HNB requires the operator to distinguish between blue and violet, which can be ambiguous. In contrast, MG only requires distinction between blue and transparency.

LAMP has also been applied for the detection of *L. infantum*, both in dogs and humans [32, 33]. Pan-leishmania LAMP is also reported by Karani et al., Mikita et al. and Nzelu et al. [26, 31, 34]. However, *Leishmania*–specific colorimetric LAMP from clinical samples has never been described.Therefore, in this study, we developed a LAMP method using MG to detect *Leishmania* DNA from crude clinical samples. These data can be useful for the deployment of LAMP in field settings and can further enable detailed surveying of *L. siamensis* and *L. martiniquensis* in Thailand.

Methods

Primer design

To develop a pan-leishmania assay, we chose the highly conserved 18S ribosomal RNA gene, as in previous pan-leishmania assays [26, 31, 34]. A consensus sequence was made from nine different *Leishmania* species, including *L. tropica* [GenBank: KF041809.1], *L. martiniquensis* [KJ467218.1], *L. mexicana* [KF041806.1], *L. hertigi* [KF041804.1], *L. donovani* [KF041801.1], *L. chagasi* (syn. *L. infantum*) [KF041797.1], *L. infantum* [KF302752.1], *L. amazonensis* [KF302746.1], and *L. enriettii* [KF041798.1]. However, *L. siamensis* was not included in this process due to the absence of its sequences on GenBank. The consensus sequence was imported into the PrimerExplorer version 4 software (http://primerexplorer.jp/elamp4.0.0/index.html), and primers were designed to avoid any mutations that were presented. To ensure optimality, primers with the lowest change in Gibb's free energy (ΔG) for dimer formation and the highest change in hybridization ΔG were chosen. The primers' thermodynamic properties were further validated using the OligoCalc software (http://www.basic.northwestern.edu/biotools/oligocalc.html). A final verification of specificity was performed using Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis against human DNA and any other organisms included in the differential diagnosis of leishmaniasis. The resulting primers are shown in Table 1 and Fig. 1, and were synthesized by BioDesign Co., Ltd, Pathumthani, Thailand.

Quantitative polymerase chain reaction

The 25 μl reaction mixture contained 12.5 μl of 2× reaction mix from the SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit (Life Technologies, USA), 0.08 μM of F3 primer, 0.08 μM of B3 primers, and 1 U of BIOTAQ™ DNA Polymerase (Bioline, Germany). The reaction was performed using the CFX96™ real-time PCR system (Bio-Rad

Table 1 Primer sequences used in the study

Primer	Sequence (5'→ 3')
FIP (F1c-F2)	GTCAAATTAAACCGCACGCTCCACGGGGGAGTACGTTCGCAA
BIP (B1c-B2)	TCAACACGGGGAACTTTACCAGATCACCACCATTCAGGGAATCGA
F3	CGAAAGCTTTGAGGTTACAGTCT
B3	CAAACAAATCACTCCACCGAC

Laboratories, USA) and was programmed as followed: 3 min of initial denaturation at 95 °C, 40 cycles of denaturation at 95 °C for 20 sec, annealing at 50 °C for 30 sec, extension at 72 °C for 40 sec, and fluorescence data acquisition at 77 °C. Upon completion, a final extension at 72 °C for 10 min ensued. The C_q (quantification cycle) was defined to be 10 times the standard deviation of the baseline. A standard curve was generated using DNA dilutions in triplicates, and the efficiency was determined to be 98.44 % with a linear range spanning 6 orders of magnitudes. Specificities of all samples were verified using melting curve analyses, which demonstrated a single peak at 83.5 °C.

Loop-mediated isothermal amplification

The LAMP reaction mixtures (25 μl) were based on that described by Tomita et al. [28], which contained 1× Isothermal Amplification Buffer (New England Biolabs, USA), 8 mM $MgSO_4$, 0.8 M Betaine (Sigma-Aldrich, USA), 1.4 mM each of dATP, dCTP, dGTP, and dTTP (SibEnzyme, Russia), 40 pmol of FIP primer, 40 pmol of BIP primer, 10 pmol of F3 primer, 10 pmol of B3 primer, and 8 U of *Bst* 2.0 WarmStart® DNA Polymerase (New England Biolabs, USA). In addition, the colorimetric indicator, 0.008 % MG (Sigma-Aldrich, USA) was added. The reaction was performed at 65 °C for 75 min using the Veriti® 96-well Thermal Cycler (Life Technologies, USA). Visualization of the LAMP products was performed using 2.5 % agarose gel electrophoresis at 10 V/cm in 1× TAE buffer.

Promastigote culture

L. siamensis isolate PCM2 and *L. martiniquensis* isolate CU1 were used in this study and were derived from bone marrow aspirates of infected patients. The culture media was Schneider's insect medium (Sigma-Aldrich, USA) supplemented with 10 % fetal bovine serum, 100 U of penicillin (Sigma-Aldrich, USA), and 100 μg/ml streptomycin (Sigma-Aldrich, USA). The cultures were incubated at 25 ± 2 °C, and subculturing was done every 2–3 days at a ratio of 1:2.

Reference DNA

All *Leishmania* DNA samples used in this study were extracted using the Invisorb® Spin Tissue Mini Kit (Stratec Biomedical AG, Germany). While *L. siamensis* and *L. martiniquensis* DNA were derived from cell culturing, *L. aethiopica*, *L. braziliensis*, *L. donovani*, and *L. tropica* DNA were derived from tissue biopsies of patients with imported leishmaniasis. *Trypanosoma brucei* DNA was extracted from a permanent slide sample, while *Trichomonas vaginalis* and *Giardia lamblia* DNA were isolated from infected patient samples at King Chulalongkorn Memorial Hospital. All DNA samples were of sufficient quality, as indicated by their optimal 260/280 and 260/230 ratios.

Detection limit

To generate standard parasite concentrations and assess LAMP's tolerance to inhibitors that can be presented in the saliva, 10-fold dilutions of *L. siamensis* from 10^7–10^0 parasites/ml were made using either 1× phosphate-buffered saline (PBS) or human saliva. Each dilution was divided into two portions. One portion was extracted for

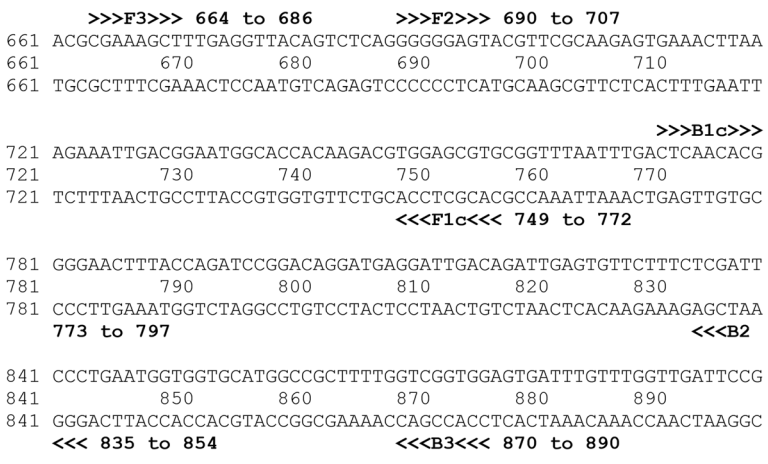

Fig. 1 Targeted Amplification Region on the 18S Ribosomal RNA Gene of *L. martiniquensis* (KJ467218.1)

DNA using the Invisorb® Spin Tissue Mini Kit (Stratec Biomedical AG, Germany), and the other was boiled at 100 °C for 10 min, as described previously [26].

To simulate infected blood, 100 μl dilutions of *L. siamensis* in human blood were made as described above and divided into two portions. The first portion was added with Triton X-100 to a final concentration of 1 %, and boiled at 100 °C for 10 min, which resulted in a coagulum. Next, 50 μl of ddH_2O was added, and the coagulum was broken up by vigorous agitation with a pipette tip. The second portion was subjected to DNA extraction using the Invisorb® Spin Blood DNA Mini Kit (Stratec Biomedical AG, Germany); 2.5 μl of each resultant was directly introduced to both qPCR and LAMP.

Clinical samples

Peripheral blood and saliva were obtained from two patients.

Patient 1 was initially reported by Phumee et al. [35]. In short, the patient was a 49-year-old man who was HIV positive and presented with multiple nodules on his body. Microscopy and cell culturing revealed the presence of *Leishmania* parasites in the nodules, and molecular analysis confirmed *L. martiniquensis* infection [16]. He was successfully treated with amphotericin B and itraconazole. Blood, saliva, and tissue biopsy were obtained. The blood was treated in the same manner as described above, while the saliva and tissue biopsy (drenched in 1× PBS) were boiled at 100 °C for 10 min. 2.5 μl of the supernatant were used as the template.

Patient 2 was described by Chusri et al. [10]. He was a 30-year-old man who was HIV positive. Similar to Patient 1, he had multiple papules on his skin, but this patient also had internal organ involvements. *Leishmania* parasites were microscopically confirmed to be infiltrating the bone marrow and ulcers. Further molecular data confirmed this *Leishmania* species to be *L. martiniquensis* [16]. He was also successfully treated with amphotericin B and itraconazole. Blood, saliva, and bone marrow biopsy were obtained. The saliva and blood was treated as described earlier, and the bone marrow biopsy was treated in the same manner as the blood.

Ethical statement

This study was approved by the Institutional Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University (COA No. 725/2013).

Results

Both qPCR and LAMP successfully amplified regions that were specified by the newly designed primers. For LAMP, successful amplification was associated with MG's characteristic light blue color, whereas failed amplification was associated with transparency (Fig. 2). Gel electrophoresis of the LAMP products also exhibited their characteristic "mixture of stem–loop DNAs with various stem lengths, and cauliflower-like structures with multiple loops" [25], which further confirmed their successful amplifications.

The detection limits (log_{10} parasites/ml) of *L. siamensis* for LAMP, as defined by the appearance of its light blue color, and qPCR, as defined by fluorescence above the C_t, are shown in Table 2.

For qPCR, our results indicated that the direct use of the samples was possible but was associated with increases in the detection limits. However, whole blood could not be used, due to the total inhibition of *Taq* polymerase.

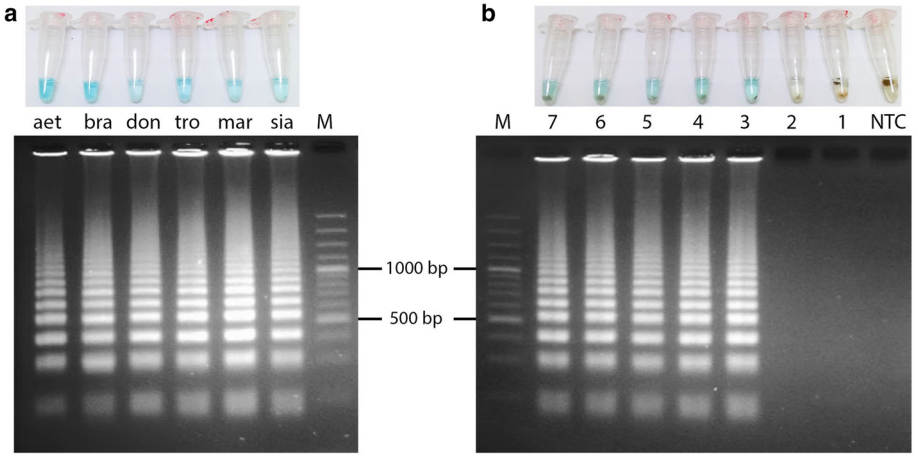

Fig. 2 Detection of LAMP Products by MG-based Colorimetric Changes and Gel Electrophoresis. **a** LAMP was able to detect multiple species of *Leishmania*. (aet = *L. aethiopica*; bra = *L. braziliensis*; don = *L. donovani*; tro = *L. tropica*; mar = *L. martiniquensis*; sia = *L.siamensis*) **b** LAMP could detect *L. siamensis* in the presence of whole blood. Ten-fold dilutions of *L. siamensis* in whole blood (log parasites/ml) were lysed, boiled, and subjected to LAMP. The black precipitates are coagulated blood

Table 2 Detection limits of LAMP and qPCR under various conditions (log parasites/ml) (F* = Fail to amplify)

Diluent	Method	LAMP	qPCR
1X PBS	Boiled	3	5
	Extracted	3	4
Saliva	Boiled	3	4
	Extracted	4	4
Whole blood	Boiled	3	F*
	Extracted	4	5

In the case of LAMP, which is more tolerant of PCR inhibitors, removal of the extraction process allowed a lower detection limit of 10^3 parasites/ml across all samples. Crude samples did not affect the properties of MG as all of the positive samples displayed MG's characteristic color. The use of whole blood did shift the color toward a greenish tone, whereas the negative samples were yellow in color (Fig. 2). However, an excessive amount of whole blood or the presence of uncoagulated blood could prevent the discrimination between positive and negative results, as hemoglobin absorption spectrum overlapped that of MG.

Clinical samples also yielded useful information in microscopically diagnosed patients. Patient 1, who had CL, had detectable *Leishmania* DNA in his saliva and tissue biopsy. Patient 2, in contrast, who had CL and VL, had the DNA presented in his bone marrow, blood, and saliva (Fig. 3).

Discussion

With an increasing number of cases, leishmaniasis is now an emerging infectious disease in Thailand. However, current diagnostic methods require experienced personnel, advanced facilities, and a large amount of time. Thus, simplification is now critical to bring diagnostics to point-of-care settings. Therefore, we developed the LAMP method to complement leishmaniasis diagnostic process and to facilitate epidemiological studies of leishmaniasis in Thailand.

Molecular techniques have exploited multiple genes to detect *Leishmania*, most of which are high-copy-number genes, including cysteine protease B [36], *gp63* [37], internal transcribed spacer 1 (ITS1) [38], 18S ribosomal RNA [26, 31], and minicircle kinetoplast DNA [24]. As expected, comparative studies have shown that the minicircles, which have the highest copy number, yield the highest sensitivity [39, 40]. However, minicircles are highly variable and due to LAMP's requirement of six conserved regions, it is not feasible to design pan-leishmania LAMP primers that target minicircles. In our approach, we chose the 18S ribosomal RNA gene, which represents a balanced trade-off between copy number and variability. As Karani et al., Mikita et al. and Nzelu et al. recently reported, LAMP has been shown to detect most species of *Leishmania* [26, 31, 34]. Here, we also experimentally confirmed the detection of *L. martiniquensis* and *L. siamensis*. Our detection limit was comparable to that of Mikita et al. (10^3 parasites/ml), but was 10-fold higher than that of Nzelu et al. (10^2 parasites/ml) [26, 31].

We detected *Leishmania* DNA in the blood and bone marrow, and in the tissue and saliva of the VL and CL patient, respectively. Our findings agreed with others that *Leishmania* DNA can be found in multiple noninvasive sources, including saliva (CL, VL) [10, 12, 41], skin swabs (CL) [26] and peripheral blood (VL) [42, 43]. We recommend the use of multiple DNA sources to reduce the probability of false negatives.

Our results also suggested that LAMP could be used to detect *Leishmania* DNA from crude clinical samples without compromising the detection limit. Furthermore, the use of crude samples even lowered the detection limit by 10-fold (10^3 parasites/ml), but the same could not be said with qPCR, which increased this limit by 10-fold. We suspected that in the case of LAMP, the removal of inhibitors did not compensate for the loss of

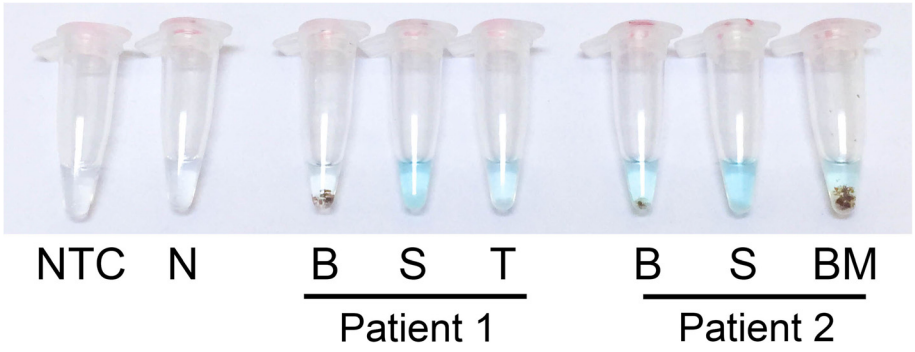

Fig. 3 LAMP Could Be Used Directly with Clinical Samples. Crude clinical samples were directly introduced into the LAMP reaction after being subjected to heating. (NTC = no template control; N = healthy patient control; B = blood; S = saliva; T = tissue biopsy; BM = bone marrow)

DNA from the extraction process, as in the case of qPCR. These results are in concordance with other studies that used boiled samples in LAMP [26, 44, 45]. In addition, of all of the DNA preparation methods compared, Sun et al. [46] reported that simple boiling results in the highest sensitivity.

Furthermore, the use of MG greatly facilitates the interpretation of results as it is highly discernable and consistent [31]. Because MG is inexpensive and can be stored at room temperature, this dye could tremendously increase the applicability of LAMP in the field.

Earlier attempts to couple direct blood samples with a colorimetric detection method were unsuccessful, due to the intense color of hemoglobin. To solve this problem, we induced the precipitation of the blood using its own coagulation system. We initially lysed the blood and its accompanying parasites with Triton X-100 and then promoted coagulation by boiling the sample. After boiling the sample, the coagulum was pulverized using a pipette tip. From this approach, we could clearly distinguish the results using MG, even with 2.5 μl of whole blood.

Because LAMP can be performed isothermally using a simple heat block, stable electricity is not required. Recently, using phase-change material, LaBarre et al. [47] developed a stable heat block that did not require electricity. Microfluidics lab-on-a-chip for LAMP has also been developed and has enabled highly multiplexed reactions and further simplification [48]. Moreover, Tanner et al. [49] reported that the LAMP reaction mixture can be left at 37 °C for two hours without compromising the detection limit. LAMP mixtures have also been lyophilized with a reported storage time at room temperature of at least seven months [50].

Nevertheless, because of the low prevalence of leishmaniasis in Thailand, we could only obtain a small number of microscopically diagnosed patients. Future work in a statistically significant group of patients is required to warrant the performance of this method.

Also, LAMP is particularly prone to contamination due to the large amount of DNA that it can generate, and its capability to amplify minute amounts of DNA. During this study, we nevertheless encountered multiple contamination issues. Therefore, we recommend that post-amplification reaction mixtures should not be opened due to aerosolization risks, and that all proper precautions be taken [51]. To prevent false positive results, no template control and healthy patient control should also be used. We also tested our assay specificity with *T. vaginalis* and *G. lamblia* DNA, with satisfying results. However, our assay did cross-react with DNA from *Trypanosoma* sp., a closely related protozoon (data not shown). Nevertheless, as each protozoon has a distinct set of clinical presentations, this cross-reaction would not impose a significant risk of misdiagnosis.

Conclusion

Sensitive molecular techniques to diagnose leishmaniasis have been introduced; nevertheless, due to their complexities, they have not yet been in widespread use. However, as our results demonstrated, LAMP could be used with unpurified clinical samples without compromising sensitivity and specificity, and its results could be unambiguously interpreted using MG.

With the advent of LAMP, molecular techniques can now be seamlessly integrated with field diagnostics. We anticipate that this combination will be crucial for surveying and controlling leishmaniasis in Thailand. However, further evaluation with a large cohort of patients will be required before the assay can be confidently deployed.

Abbreviations

CL: Cutaneous leishmaniasis; DAT: Direct agglutination test; ELISA: Enzyme-linked immunosorbent assay; ICT: Immunochromatographic test; IFA: Indirect fluorescence antibody; LAMP: Loop-mediated isothermal amplification; MCL: Mucocutaneous leishmaniasis; MG: Malachite green; qPCR: Quantitative polymerase chain reaction; VL: Visceral leishmaniasis.

Competing interests

We declare that we have no competing interests.

Authors' contributions

Designed the experiments, conducted molecular laboratory work, and wrote the manuscript: CS, AP, and PS. Sample Collection: CS, AP, MM, SL, and PS. All authors read and approved the final manuscript.

Authors' information

Mr. Chaichontat Sriworarat is a student at Bangkok Christian College.

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Author details

[1]Bangkok Christian College, Bangkok 10500, Thailand. [2]Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand. [3]Department of Parasitology, Phramongkutklao College of Medicine, Bangkok 10400, Thailand. [4]Excellence Center for Emerging Infectious Diseases, King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Bangkok 10330, Thailand.

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Early detection of novel *Leishmania* species DNA in the saliva of two HIV-infected patients

Padet Siriyasatien[1,2], Sarunyou Chusri[3], Kanyarat Kraivichian[1], Narissara Jariyapan[4], Thanaporn Hortiwakul[3], Khachornsakdi Silpapojakul[3], Adam M. Pym[5] and Atchara Phumee[1*]

Abstract

Background: Leishmaniasis caused by two new species of *Leishmania*; *L. siamensis* and *L. martiniquensis* have been recently described in Thailand. The disease has mainly been documented in AIDS patients from southern Thailand. In this study, polymerase chain reaction (PCR) was used to determine HIV-*Leishmania* co-infection in southern Thailand.

Methods: One ml of saliva and 3 ml of EDTA blood were collected from HIV-infected patients for PCR detection of *Leishmania* DNA, cloning and sequencing. The positive PCR samples were then cultured on Schneider's insect medium.

Results: Three out of 316 saliva samples collected from HIV-infected patients were found to be positive for *Leishmania* DNA (0.95 %). Among the positive samples, one patient was observed with disseminated cutaneous lesions and also tested positive via saliva, whole blood and buffy coat in PCR. The second case presenting with nodular lesions also gave a positive saliva test via PCR two months prior to buffy coat. This diagnosis was confirmed by microscopic examination and a culture of biopsy samples from a nodule. The last case was an asymptomatic *Leishmania* infection which tested PCR positive only in saliva with a consecutive sample collection conducted for three months.

Conclusions: The prevalence of *Leishmania* infection in HIV infected patients within this study is 0.95 %. *Leishmania* DNA was detected in saliva by PCR prior to blood and buffy coat of two HIV infected patients. Early detection of *Leishmania* DNA in saliva would be beneficial for the follow up of asymptomatic *Leishmania* infected patients, the early treatment of leishmaniasis and for surveillance survey purpose. However, full evaluation of sensitivity and specificity of this technique with a large cohort of patients is required before deployment.

Keywords: *Leishmania*, Saliva, HIV-infection, PCR

Background

Autochthonous leishmaniasis cases in Thailand have been increasingly diagnosed in recent years. The disease was described in both immunocompetent and immunocompromised patients, such as those with AIDS [1] and in systemic steroid therapy [2]. Approximately 20 cases of autochthonous leishmaniasis have now been documented, with most found in the south of Thailand [1–9]. Sukmee and others first reported a suspected new *Leishmania* species from Thailand (2008) [3] which was named *L. siamensis* [7]. A report by Leelayoova et al. (2013) [10] demonstrated that *L. siamensis* in Thailand has two lineages: the PG lineage or PCM1 isolate (Accession no JX195640) [3] and TR lineage or PCM2 isolate (Accession no EF200012) [7]. However, more recently Pothirat et al. (2014) [11] identified a PCM1 and a new isolate from northern Thailand LSCM1 (Accession no JX898938) which are *L. martiniquensis* as described by Desbois et al. (2014) [12], and only the PCM2 isolate was identified as *L. siamensis*. They also reiterated that most cases of leishmaniasis are caused by *L. martiniquensis* in Thailand. More recently, Chiewchanvit et al. (2015) also

* Correspondence: amphumee@gmail.com
[1]Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand
Full list of author information is available at the end of the article

described a case of HIV and *L. martiniquensis* co-infection in northern Thailand who presented with chronic generalized fibrotic skin lesions [13].

In other parts of the world such as isolates from Myanmar patients (Accession no KF211417) [2], cows in Switzerland (Accession no GQ281282), a horse in Germany (Accession no GQ281278) and a horse in the USA (Accession no JQ617283) [14–16] may be *L. martiniquensis*. Liautaud et al. (2015) reported the first case of visceral leishmaniasis caused by *L. martiniquensis* from the Caribbean [17]. This indicates that *L. martiniquensis* has a worldwide distribution while *L. siamensis* is limited in its geographic distribution.

Three clinical forms of these novel *Leishmania* species have been described: visceral, disseminated cutaneous, and combined disseminated cutaneous with visceral [1–9, 11, 13]. The disease has been described mostly in immunocompromised patients, especially those with AIDS. Apart from *L. martiniquensis* and *L. siamensis*, an autochthonous leishmaniasis case caused by *L. infantum* was also reported from Thailand [18].

The prevalence of leishmaniasis in Thailand has never been fully studied. Screening tests for leishmaniasis, such as Enzyme-linked immunosorbent assay (ELISA), Direct antiglobulin test (DAT) and rK39 dipsticks, are not generally available. Microscopic examination and culture are time-consuming and require expertise to be reliable. Microscopy, culture and PCR are generally the methods of choice used for diagnosis [1].

PCR has been developed to detect *Leishmania* DNA, and *Leishmania* species were identified by a sequence analysis [19–21]. PCR has high sensitivity and specificity for detecting *Leishmania* DNA [22, 23] and has been used for detection from various clinical samples including blood, bone marrow, tissue, saliva, and urine [1–6]. Saliva has been shown to be a good source for the detection of the new *Leishmania* species DNA [1–6].

Several previous studies demonstrated that *Leishmania* DNA and antibodies were present in oral secretions and saliva, such as *L. braziliensis* DNA from Brazil [24], *L. donovani* from China [25] and *L. infantum* from Tunisia [26]. In Thailand, Phumee et al. (2013) demonstrated that saliva is a good source for PCR detection of novel *Leishmania* species DNA in Thailand [1, 2, 4–6, 9]. They also showed that the *Leishmania* DNA levels in saliva decreased after treatment [1]. Saliva could be used as a biomarker to detect the new *Leishmania* species infection. Furthermore, the collection of saliva is non-invasive, requires no special equipment, and is suitable for children and elders [27, 28].

The prevalence of the disease in Thailand has never been fully investigated. This study's objectives are to determine the prevalence of *Leishmania* infection in HIV-infected Thai patients from southern Thailand through PCR analysis of saliva and blood samples.

Methods

Study design

The study was conducted in southern Thailand from June to September 2013. A total of 316 HIV-infected patients who came for HIV treatment were enrolled in the study at the Division of Infectious Diseases of Faculty of Medicine, Prince of Songkla University. One ml of saliva and 3 ml of EDTA blood were collected for PCR detection of *Leishmania* DNA.

Ethics approval

Informed consent was obtained from all subjects according to protocols approved by the Institutional Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University (COA No. 768/2012).

Study population

Blood and saliva samples were collected from HIV-infected patients who resided in southern Thailand. A total of 316 HIV-infected patients involved in treatment at the Division of Infectious Diseases were enrolled in the study.

DNA extraction

One ml of whole saliva was used to extract DNA from the tissue using the Invisorb® Spin Tissue Mini Kit (STRATEC

Table 1 Clinical presentations, CD4+ T cell levels, PCR, and Culture/Tissue biopsy for *Leishmania* parasite[a]

Patient	Clinical Presentation	CD4+ T cell count (cells/mm^3)	Results of PCR for *Leishmania*									Culture/Tissue biopsy for *Leishmania*
			First sample collection			Second sample collection			Third sample collection			
			S	B	BF	S	B	BF	S	B	BF	
32 year old Male	Relapse disseminated CL 2 years after treatment	110	+	+	+	Not collected			Not collected			+/+
48 year old Male	Nodular CL, relapse 2 years after treatment for disseminated CL	207	+	-	-	+	-	-	+	-	+	+/+
28 year old Female	Asymptomatic	617	+	-	-	+	-	-	+	-	-	-/N/A

[a]*S* Saliva; *B* Blood; *BF* Buffy coat; +: positive; –: Negative; N/A: not available; *CL*: Cutaneous leishmaniasis

Molecular GmbH, Germany) according to the manufacturer's instructions. To extract the DNA, 200 μl of EDTA blood and 50 μl of buffy coat were used with the extraction kit, Invisorb® Spin blood Mini Kit (STRATEC Molecular GmbH, Germany). Extracted DNA was eluted in 50 μl of elution buffer. The quantity and quality of the extracted DNA were determined using a Nanodrop 2000c (Thermo Scientific, Singapore). Extracted DNA samples were kept at −80 °C for long-term storage.

PCR amplification

Amplification was performed in a PCR Mastercycler® pro (Eppendorf, Germany) with conditions as follows; denaturation at 94 °C for 4 min, followed by 40 cycles of 94 °C for 1 min'; 65 °C for 1 min; and 72 °C for 1 min, with the final extension at 72 °C for 7 min. The forward and reverse ITS1 regions of the rRNA of *Leishmania* parasite primers were LeF: 5′ TCC GCC CGA AAG TTC ACC GAT A 3' and LeR: 5′ CCA AGT CAT CCA TCG CGA CAC G 3', respectively [29]. In order to maintain that the template DNA had been extracted properly, primers that anneal to human DNA (UNFOR403: 5'-TGA GGA CAA ATA TCA TTC TGA GG-3' and UNREV1025: 5'-GGT TGT CCT CCA ATT CAT GTT A-3') were used [30]. Therefore, clinical samples which contain human DNA should show the PCR products of 628 bps. The products were analyzed on 1.5 % agarose gel electrophoresis, stained with 0.5 μg/ml ethidium bromide and visualized with Quantity One quantification analysis software, version 4.5.2 Gel Doc EQ system (Bio-Rad, USA). DNA from cultured *Leishmania* promastigotes isolated from a patient [5] was used as the positive control. DNA from saliva and EDTA blood from a healthy individual who had never traveled into endemic areas were used as negative controls.

Cloning, sequencing and nucleotide analysis

The study was designed to use cloning for sequencing rather than direct sequencing because the ITS1 primers used in this study can amplify closely *L. martiniquensis* and *L. siamensis* at 379 and 371 bps, respectively. Moreover, PCR products obtained from some reactions contained small amount of DNA, while direct sequencing requires at least 30–50 ng/μl of DNA. Amplified PCR products were ligated into pGEM-T Easy Vector (Promega, USA). The ligated vectors were transformed into DH5α competent cells and screened through the blue-white colony selection system. The suspected positive colonies were cultured for further plasmid DNA extraction using the Invisorb® Spin Plasmid Mini kit (STRATEC Molecular GmbH, Germany), following the manufacturer's instructions. Purification was performed according to the 1st BASE DNA sequencing system (1st base laboratories, Malaysia) using universal forward T7 primer. Nucleotide sequences were analyzed using the BioEdit Sequence Alignment Editor Version 7.0.9.0. The consensus sequences were compared with available sequence data in GenBank using BLAST search (available at http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences obtained from this study were submitted to GenBank to be assigned accession numbers.

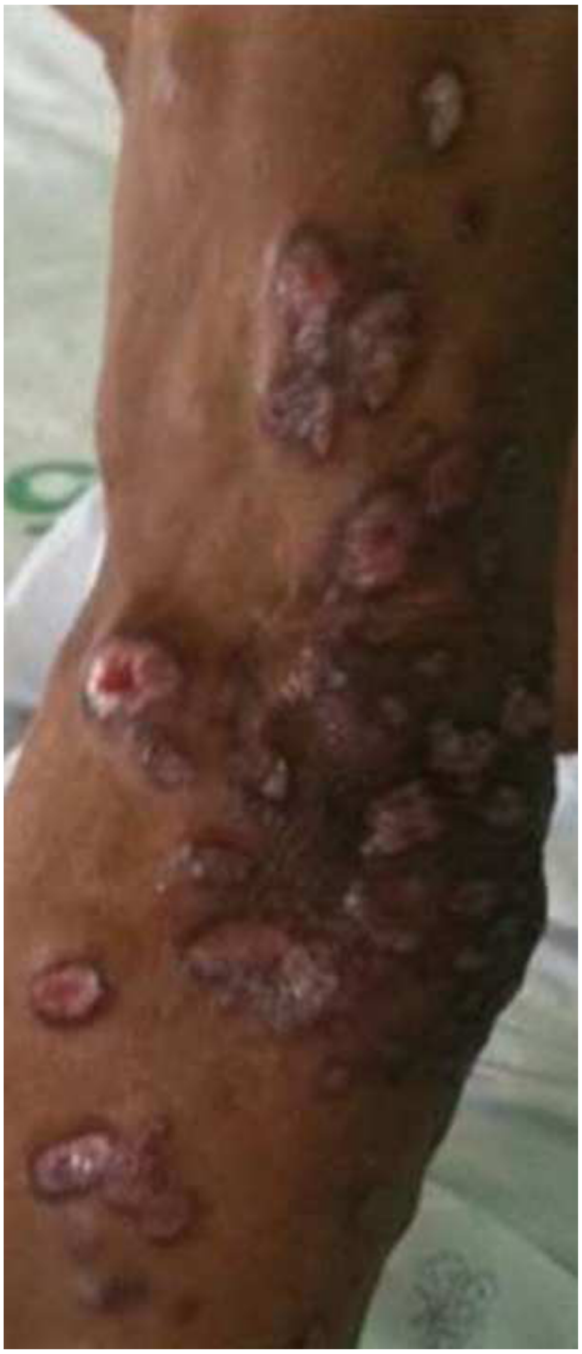

Fig. 1 Cutaneous leishmaniasis lesions of the patient 1

Phylogenetic tree construction

A phylogenetic tree was constructed by Maximum-likelihood method using the Kimura's 2-parameter model implemented in MEGA6 version 6.06 and the tree was tested using 1000 bootstrap replicates. ITS1 sequences of confirmed *L. martiniquensis* (KM677931) [10] and *L. siamensis* (JX195640) [9] were used to compared with ITS1 sequences of our study. *Bodo caudatus* accession no. AY028450 was used as an outgroup.

Culture of *Leishmania* parasite

Positive PCR samples were cultured on Schneider's insect medium (Sigma-Aldrich, USA), which contained 10 % fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Sigma-Aldrich, USA). The samples were then incubated at 25 ± 2 °C. The promastigotes were observed daily under an inverted microscopy (Olympus, Japan).

Tissue biopsy and staining

A tissue biopsy was performed on an ulcer or nodule from the PCR-positive study patients. Tissue sections were stained with Hematoxylin and Eosin (H&E) and examined under a light microscope (Olympus, Japan) at 100X magnification.

Results

Saliva and blood samples were tested with *Leishmania*-specific primers, ITS1 gene by PCR. Three of the 316 saliva samples were positive for *Leishmania* species DNA (0.95 %). Among these three positive cases, two had been diagnosed as leishmaniasis two years previously (Table 1). The first of these cases, involving a 32-year-old male, was diagnosed for disseminated CL 2 years previously. He was treated with amphotericin B deoxycholate and itraconazole, following which his lesions regressed and all samples tested by PCR were negative for *Leishmania* [1–6]. However, in June 2013, he developed multiple papules and ulcers (Fig. 1). A CD4+ T-cell count revealed 110 cells/mm^3 and he was started on tenofovir, lamivudine and nevirapine for treatment of HIV. Saliva, whole blood, buffy coats, and tissue biopsy were also positive for *Leishmania* DNA. Both culture and H&E stains confirmed the recurrent diagnosis by showing *Leishmania* in a skin biopsy (Table 1).

Two other cases were positive only in saliva from the first PCR sample collected (Table 1). One of these cases was a 48-year-old male who was diagnosed two years previously with disseminated leishmaniasis [5] and was treated with liposomal amphotericin B, followed by itraconazole. His clinical status improved and blood and saliva samples were negative after treatment. He received boosted lopinavir and lamivudine for HIV. Blood and saliva samples were collected for a *Leishmania* PCR in July 2013 with only saliva testing positive (Table 1 and Fig. 2a). He developed nodules on his brow, left second toe, left ring finger, and left elbow. His lesions were described by Phumee et al. (2014) [9]. Blood and saliva samples were then collected for two consecutive months (August and September 2013). PCR was positive in buffy coat and saliva samples two months after the first collection (September 2013), (Table 1 and Fig. 2a). A tissue biopsy was performed at a nodule from his brow in September 2013. A PCR of the biopsy sample was positive for the novel *Leishmania* species. (Table 1 and Fig. 2a). The first and second cases of leishmaniasis relapsed approximately 2 years after the treatment [5].

The last case was a 28-year-old female who was asymptomatic but whose PCR was positive in saliva (July 2013) and had a CD4+ T-cell count of 617 cells/mm^3.

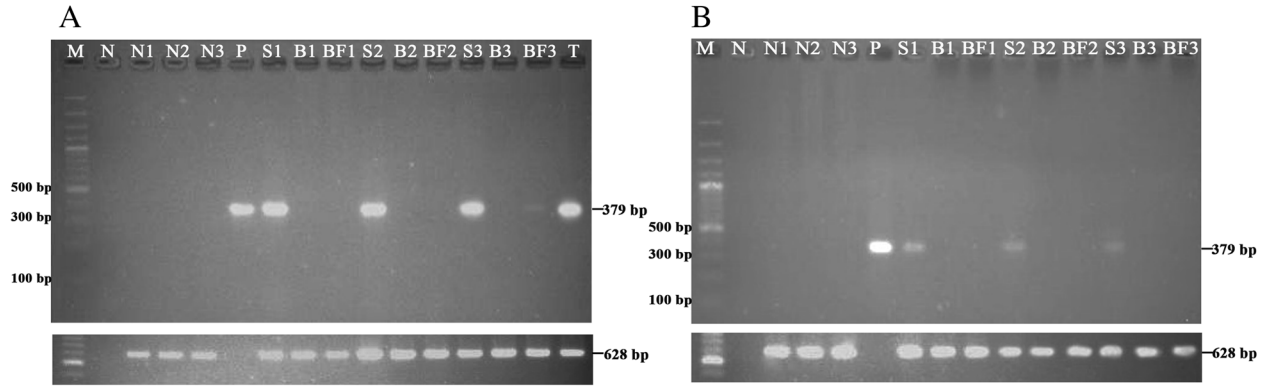

Fig. 2 PCR amplification of the ITS1 gene against saliva, buffy coat, blood, and tissue samples of nodular leishmaniasis case (**a**) and asymptomatic case (**b**). PCR amplicons were analyzed by electrophoresis on a 1.5 % agarose gel and stained with ethidium bromide. Lane S1, B1 and BF1: first saliva, blood and buffy coat collection, respectively; lane S2, B2 and BF2:second saliva, blood and buffy coat collection, respectively; and lane S3,B3 and BF3: third saliva, blood and buffy coat collection, respectively; T: tissue, lane M: molecular mass marker (100 basepairs [bp]); lane P: positive control containing extracted DNA from cultured *L. martiniquensis*-produced fragments of 379 bp, lane N: negative control (no DNA template: double-distilled water); lanes N1–N3: negative control (DNA template from non-infected saliva, blood, and buffy coat, respectively); and a PCR for template DNA control shown below (628 bp)

She did not receive any treatment for HIV. Blood and saliva collected for two consecutive months (August and September 2013) were negative, but PCR remained positive (Table 1 and Fig. 2b).

Amplified sequences obtained from saliva, blood, buffy coat, and tissue of the patient 1 were assigned for accession numbers KU050856-KU050859 respectively. Amplified sequences obtained from saliva, buffy coat, and tissue of patient 2 were assigned for accession numbers KU050860-KU050862, while the amplified sequence from saliva of patient 3 was assigned accession number of KU050863.

The nucleotide sequencing of all PCR-positive samples were 100 % identical to *L. martiniquensis* (Fig. 3a and b). The UNFOR403 and UNREV1025 primers which were annealed specifically to human DNA gave positive results for all clinical samples (Fig. 2a and b). This showed that all extracted DNA from clinical samples were extracted properly.

Discussion

This study identified *Leishmania* co-infections in HIV patients using saliva and blood samples for PCR within

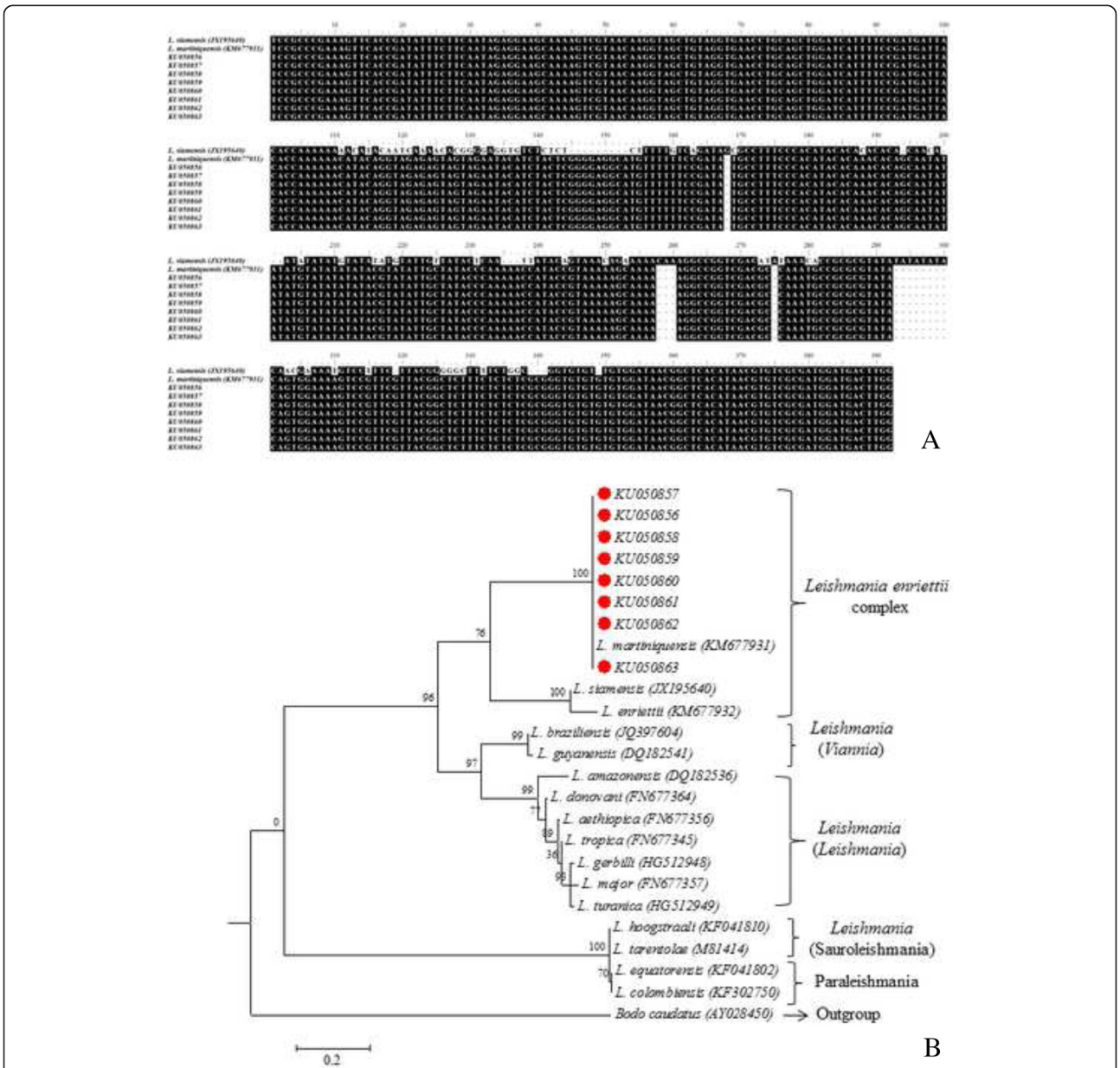

Fig. 3 Sequence comparison between *L. martiniquensis* and *L. siamensis*, the different in size and sequences were observed. Red circle indicated *L. martiniquensis* obtained from this study (**a**). A phylogenetic tree showed that both *L. martiniquensis* and *L. siamensis* were classified into *L. enriettii* complex and were discriminate to other *Leishmania* species (**b**)

an endemic area of Thailand. Three out of 316 saliva samples were positive for PCR Prevalence of *Leishmania* infection in HIV infected patients of this study was 0.95 %. In 2012, Orsini and others investigated prevalence of *Leishmania* infection among 381 HIV-infected patients who living in endemic areas of Brazil. The results showed positive for *Leishmania* in blood samples by using PCR targeted to kDNA region, ELISA, Indirect fluorescent antibody test (IFAT), and rK39 at 6.3 %, 10.8 %, 3.9 %, 0.8 % [30] respectively. Comparing the PCR results, the prevalence of *Leishmania* infection in HIV patients of our study is lower than the result reported by Orsini et al. (2012) [31].

Interestingly in this study, *Leishmania* DNA was detected in saliva prior to appearing in buffy coat in patient two and was also detected only in saliva for patient three. A definite diagnosis was confirmed using microscopy and a culture of tissue biopsy from a nodular lesion. Sequence analysis of amplified PCR products were 100 % identical to *L. martiniquensis* (Accession no KM677931).

Sequence analysis demonstrated that the amplified ITS1 gene region in this study was able to discriminate between *L. martiniquensis* and *L. siamensis* (Fig. 3a). Phylogenetic tree construction showed that both *L. martiniquensis* and *L. siamensis* were classified into the *L. enriettii* complex (Fig. 3b), a result that is similar to that previously reported by Pothirat et al. (2014) [11]. Again, similar to the result of Pothirat et al. (2014) [11] which mentioned that most cases of leishmaniasis in Thailand are caused by *L. martiniquensis*, all three cases of this study were also infected by *L. martiniquensis.*

Patients infected with leishmaniasis in Thailand often have diffuse cutaneous [7, 9], visceral leishmaniasis [1, 3–8] or overlapping diffuse cutaneous and visceral forms [1, 5–9]. Two leishmaniasis cases of this study were presented with cutaneous lesion, one case had diffuse cutaneous lesion (Fig. 1) while another presented with multiple nodular lesions [9].

This study also demonstrated the first asymptomatic *L. martiniquensis* infection in Thailand. Clinical samples were consecutively collected for two months. *Leishmania* DNA was still detected only in the saliva of the asymptomatic case. A study by Phumee et al. (2013) previously demonstrated that *Leishmania* DNA was detected in saliva and buffy coat in all of their cases [1]. However, in this study we found that it was detected only in saliva two months prior to buffy coat, in a patient presenting with nodular leishmaniasis. More recently, Sriworarat et al. (2015) also demonstrated that *L. martiniquensis* DNA was present in saliva prior to blood sample via the use of loop mediated isothermal amplification (LAMP) technique [32]. In this study, *Leishmania* DNA was also detected only in the saliva of an asymptomatic patient. HIV and leishmaniasis co-infection has been previously reported from Thailand [1, 3–9]. Most of these cases were diagnosed from bone marrow or tissue biopsies, and some cases died soon after without therapy [1, 7].

Conclusions

Our findings showed that early detection of *Leishmania* DNA was found when conducting a PCR from the saliva of two HIV infected patients. This could result in the closer follow up of asymptomatic infected patients and lead to earlier treatment of symptomatic leishmaniasis which could decrease morbidity and mortality rates. This could aid the development of disease surveillance tools, especially in asymptomatic cases therefore improving the design of control strategies. However, before the technique can be deployed, sensitivity and specificity of the test should be evaluated with the larger number of patients.

Competing interests

The authors have declared that no competing interests exist.

Authors' contributions

Sample collected data for the experiment: SC, TH, KS. Designed the experiments and wrote the paper: AP, PS, AMP. Involved in the discussions and data analysis: AP, PS, KK, NJ. All authors read and approved the final manuscript.

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Author details

[1]Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand. [2]Excellence Center for Emerging Infectious Disease, King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Bangkok 10330, Thailand. [3]Division of Infectious Diseases, Department of Internal Medicine, Faculty of Medicine, Prince of Songkla University, Songkhla 90110, Thailand. [4]Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand. [5]School of Life Sciences, University of Liverpool, Liverpool, UK.

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Research article

Vector/Pathogen/Host Interaction, Transmission

OXFORD

Detection of an Unknown *Trypanosoma* DNA in a *Phlebotomus stantoni* (Diptera: Psychodidae) Collected From Southern Thailand and Records of New Sand Flies With Reinstatement of *Sergentomyia hivernus* Raynal & Gaschen, 1935 (Diptera: Psychodidae)

Atchara Phumee,[1] Apiwat Tawatsin,[2] Usavadee Thavara,[2] Theerakamol Pengsakul,[3] Suwich Thammapalo,[4] Jérôme Depaquit,[5] Frédérick Gay,[6] and Padet Siriyasatien[1,7,8]

[1]Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (amphumee@gmail.com; padet.s@chula.ac.th), [2]National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand (atawatsin@gmail.com; usavadee99@gmail.com), [3]Faculty of Medical Technology, Prince of Songkla University, Songkhla, Thailand (theerakamol.p@psu.ac.th), [4]The Office of Disease Prevention and Control 12, Songkhla, Thailand (sthammapalo@yahoo.com), [5]Université de Reims Champagne Ardenne, ANSES, SFR Cap santé, EA 4688 – USC "transmission vectorielle et épidemiosurveillance de maladies parasitaires (VECPAR)", Reims, France (jerome.depaquit@univ-reims.fr), [6]Université Pierre et Marie Curie-Paris 6, CHU Pitié-Salpêtrière, AP-HP, Groupe Hospitalier Pitié-Salpêtrière, Service Parasitologie-Mycologie, Paris, France (fredogay@yahoo.fr), [7]Excellence Center for Emerging Infectious Diseases, King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Bangkok, Thailand, and [8]Corresponding author, e-mail: padet.s@chula.ac.th

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Abstract

Although female sand flies are best known as the vectors of *Leishmania* parasites and viruses, several previous reports have demonstrated that these insects can also act as vectors for the trypanosomes of bats, lizards, and snakes. In this report, we created an inventory of Phlebotomine sand flies from southern Thailand. A novel trypanosome was found in a specimen of *Phlebotomus stantoni*, and two sand fly species newly recorded in the country, *Sergentomyia khawi* and *Sergentomyia hivernus*, were described. PCR primer pairs specific for the internal transcribed spacer 1 (*ITS1*) and the small subunit ribosomal DNA (*SSU rDNA*) gene of trypanosomatids were used to demonstrate the presence of the parasite in the sand fly. In addition, the Cytochrome b (*CytB*) gene was used to identify the sand fly species. Among the 45 samples of the sand fly that were collected, seven samples were *Ph. stantoni* sand flies and a single sample was positive for *Trypanosoma* sp. through PCR analysis. This study represents the first detection of *Trypanosoma* sp. in a sand fly from Thailand. The *ITS1* and *SSU rDNA* sequences indicated that this specimen is suspected to be a novel *Trypanosoma* species. Further studies of this suspected new *Trypanosoma* species, including its vertebrate hosts and pathogenic potential, are therefore necessary.

Key words: sand fly, *Trypanosoma* sp., PCR, ITS1, SSU rDNA

Trypanosoma is a flagellate protozoa belonging to the order Kinetoplastida and family Trypanosomatidae. These parasites cause trypanosomiases in humans and animals. The parasites are widely distributed in Asia, Africa, and South America (Luckins 1988). In Asia, trypanosomes can infect cattle, rats, deer, and humans (Shrivastava and Shrivastava 1974, Sarataphan et al. 2007, Hatama et al. 2007, Jittapalapong et al. 2008, Lee et al. 2010, Tang et al. 2012). *Trypanosoma evansi* and *Trypanosoma lewisi* are the most common species found in Asia (Luckins 1988, Jittapalapong et al. 2008). *Trypanosoma* spp. are transmitted by a range of hematophagous arthropods, such as tsetse flies, kissing bugs, mosquitoes, and blood-sucking leeches (Desser 2001, Hamilton et al. 2007). Sand flies are believed to be the vector for trypanosomes that affect bats (Zeledon and Rosabal 1969), lizards (Ayala and McKay 1971, Gramiccia et al. 1989), and snakes (Viola et al. 2008).

In Thailand, *T. evansi* and *T. lewisi* have been reported in a wide range of domestic animals, including rodents, cattle, buffalos, and pigs (Milocco et al. 2013). However, no reports of trypanosomes in the sand fly species have been published in Thailand, where only a few studies have been performed on Phlebotomine sandflies.

Table 1. Sampling species

Species	Males	Females	Total
Phlebotomus stantoni	2	5	7
Sergentomyia barraudi s. l.	7	7	14
Se. hivernus	3	4	7
Se. indica	4	3	7
Se. khawi	5	5	10
Total	21	24	45

Materials and Methods

In this study, sand flies were collected around the leishmaniasis patient's home at Songkhla province in southern Thailand (Chusri et al. 2012), in September 2013. A total of 17 CDC light traps were installed (two traps inside a pile of firewood, two traps around an incinerator, two traps under coconut trees, three traps under bamboo trees, two traps near banana trees, two traps around termite mounds, and four traps in the space under the house). The traps were hung from tree branches or hooks at a height of 10 m from the trap hood to the ground, and all traps were activated simultaneously from 18:00 to 06:00 for one night. On the next day, insects were collected from the light traps and anesthetized using chloroform-soaked cotton balls. All sand flies were differentiated according to their gender and genus (*Phlebotomus* and *Sergentomyia*) based on morphological identification cues, as observed under a stereomicroscope (Olympus, Japan). The head and genitalia of individual sand flies were cut off in a drop of ethanol, cleared, and mounted between slide and cover slip for identification. The body of each specimen was then stored individually in 70% ethanol in a 1.5-ml sterile tube for further PCR amplification.

The specimens were identified by observation of the head and genitalia under a BX50 microscope (Olympus, Japan). The identification was performed using the following keys and articles (Raynal 1935, Raynal 1936, Raynal and Gaschen 1935, Quate 1962, Lewis 1978, and Lewis 1987). Measures were obtained using Stream motion software (Olympus, Japan) and a video camera connected to the microscope. Drawings were generated using a *camera lucida*.

DNA was extracted from individual sand flies. The flies were left to dry at room temperature and were lysed in 200 µl lysis buffer containing 20 µl of proteinase K. The samples were placed in liquid nitrogen for 1 min and ground with a sterile plastic pestle. DNA extraction was performed using an Invisorb Spin Tissue Mini Kit (STRATEC Molecular, Germany) following the manufacturer's instructions. The DNA was eluted in 50 µl of elution buffer and quantified using a Nanodrop 2000c (Thermo-scientific, USA). The extracted DNA samples were kept at –80 °C for long-term storage.

PCR was performed in a total volume of 25 µl supplemented with ~50 ng of extracted sand fly DNA. The reaction contained 10 mM of each primer, 25 mM of $MgCl_2$, 2 mM of dNTPs, and 1 unit of *Taq* DNA polymerase (Fermentas, PA). PCR was performed using primers designed to amplify the *ITS1* region of the rRNA gene (LeR: 5′- CCA-AGT-CAT-CCA-TCG-CGA-CAC-G- 3′ and LeF: 5′- TCC-GCC-CGA-AAG-TTC-ACC-GAT-A- 3′) of *Leishmania* parasites. However, this primer set also recognizes other Trypanosomatidae sequences (Spanakos et al. 2008). When the sample was suspected to be a *Trypanosome* parasite, the primers that

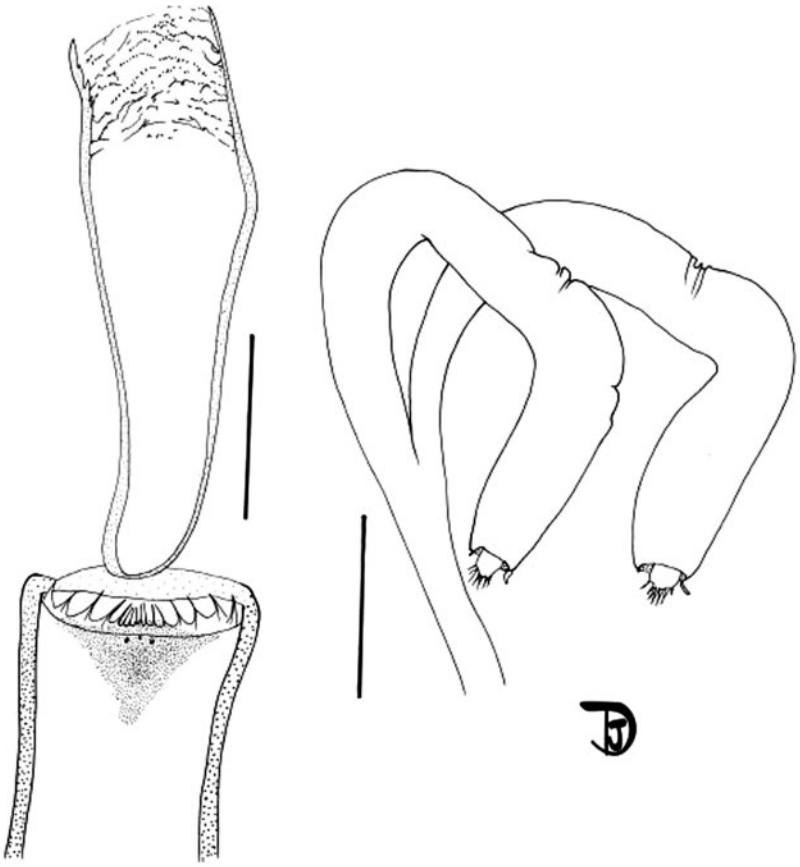

Fig. 1. Drawings of *Se. hivernus* from Southern Thailand: pharynx and cibarium (left) and spermathecae (right). Bars = 50 µm.

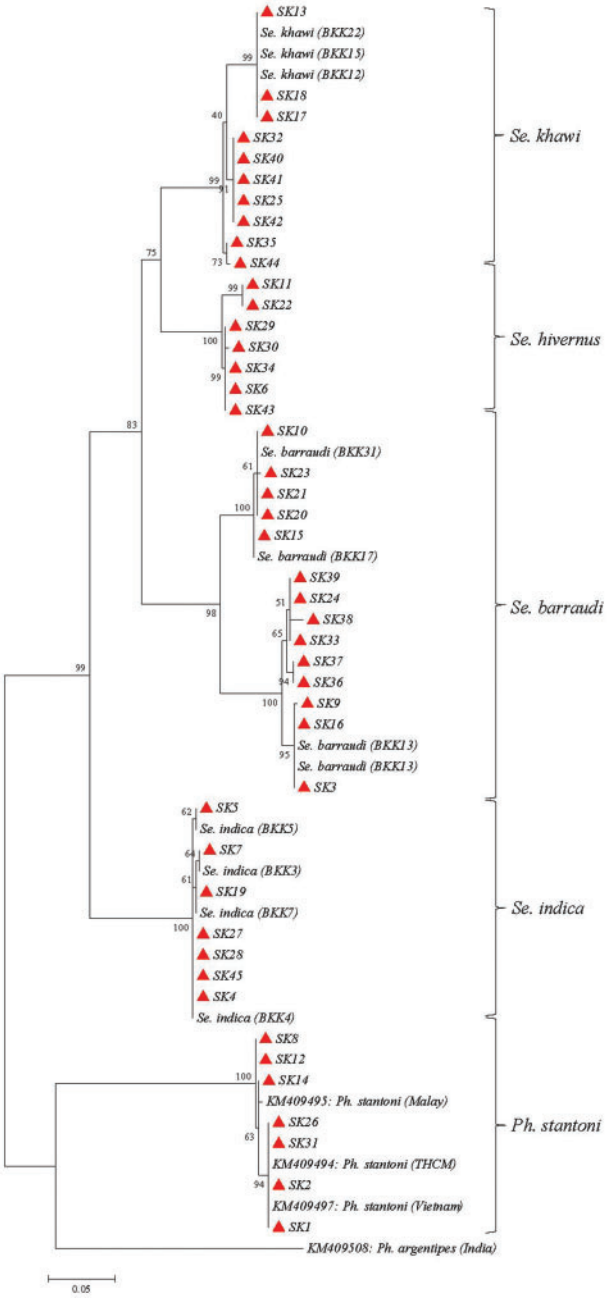

Fig. 2. Maximum likelihood tree of the *CytB* gene sequences of the sand fly using MEGA 6.0 software. The tree shown is based on the Kimura 2-parameter model of nucleotide substitution. Bootstrap values are based on 1,000 replicates. Red triangle indicated the sand fly species that was obtained from this study.

anneal specifically to the *SSU rDNA* gene of trypanosomatids (TRY927F: 5′- GAA-ACA-AGA-AAC-ACG-GGA-G- 3′ and TRY927R: 5′- CTA-CTG-GGC-AGC-TTG-GA- 3′) (Noyes et al. 1999) were used. For the amplification of sand fly DNA for species identification, we used primers N1N-PDR: 5′- CAY-ATT-CAA-CCW-GAA-TGA-TA -3′ and C3B-PDR: 5′- GGT-AYW-TTG-CCT-CGA-WTT-CGW-TAT-GA -3′ to amplify the *CytB* gene of the sand fly following a previously published method (Esseghir et al. 1997, Depaquit et al. 2015). PCR were performed in a PCR Mastercycler Pro (Eppendorf, Germany) with the following conditions: an initial denaturation of 4 min at 94 °C; followed by 40 cycles of denaturation (94 °C for 1 min), annealing at 1 min at 65 °C for the *ITS1* gene or 51.7 °C for the *SSU rDNA* gene, and extension (72 °C for 1 min); and a final extension at 72 °C for 7 min. The reaction was electrophoresed on a 2% (w/v) agarose gel, stained with ethidium bromide and visualized with Quantity One Quantification Analysis Software version 4.5.2 (Gel Doc EQ System; Bio-Rad, CA). Double distilled water (ddH_2O) was used as a negative control, and DNA from the promastigotes culture of *L. siamensis* (MHOM/TH/2010/PCM2; Trang) was used as the positive. Because the aim of this study was to identify potential vectors for leishmaniasis, the positive control of *Trypanosoma* sp. was not used for PCR reactions.

The PCR products of *ITS1*, *SSU rDNA*, and *CytB* were cloned into the pGEM-T Easy Vector (Promega, Madison, WI). The ligated vectors were transformed into *Escherichia coli* DH5α competent cells, and the recombinant plasmids were screened using the blue-white colony selection system. Suspected positive colonies were cultured and subjected to plasmid DNA extraction with the Invisorb Spin Plasmid Mini Kit (STRATEC Molecular, Germany) following the manufacturer's instructions. Purified plasmids were sequenced by 1st Base Laboratories, Malaysia.

Nucleotide sequences were analyzed by comparison with the GenBank database using a BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The *ITS1* and *SSU rDNA* sequences of *Bodo caudatus* Duj. (family: Bodonidae) (with accession no. AY028450 and X53910, respectively) were used as outgroups. The sequences were aligned using BioEdit Sequence Alignment Editor Version 7.1.9. A phylogenetic tree was constructed using the maximum likelihood method with Kimura's 2-parameter and bootstrap analysis with 1,000 replications in MEGA version 6.0.

Results and Discussion

A total of 45 sand flies (21 males and 24 females) were collected from traps (Table 1), and 20 flies were collected from a pile of firewood that was ~100 m away from the patient's home. Ten, eight, four, and three sand flies were collected from an incinerator, around banana trees, around termite mounds and under the patient home, respectively. No sand flies were found in the traps installed under coconut trees and around bamboo trees. The samples belonged to the following species: *Phlebotomus stantoni* Newstead, *Sergentomyia barraudi* (Sinton), *Se. khawi* (Raynal), *Se. hibernus* (Raynal & Gaschen), and *Se. indica* (Theodor). Apiwathnasorn et al. (1989) reported that sand flies could be found in different geographical areas of Thailand, such as caves, termite hills, air-raid shelters, ancient stone sanctuaries, tree hollows, and rock crevices. Some reports have described sand flies inhabiting the Naresuan Cave, Phitsanulok Provinces (northern) (Polseela et al. 2011a), the caves in the Kanchanaburi Province (western) (Apiwathnasorn et al. 2011), the Tham Phra Phothisat temple, the Saraburi province (central) (Polseela et al. 2011b), and the Satun province (Panthawong et al. 2015) of Thailand.

Sand flies found in Thailand are categorized within four genera, *Sergentomyia*, *Phlebotomus*, *Idiophlebotomus*, and *Chinius*, according to Artemiev (1991), Marcondes (2007), or Galati (2013). The most common genera found in the country are *Sergentomyia* flies followed by *Phlebotomus* flies. *Idiophlebotomus* and *Chinius* flies are believed to be largely restricted to caves. In 2013, Sukra et al. reported that *Se. gemmea* (81.4%) were mostly found in the three southern provinces of Thailand (Phangnga, Suratthani, and Nakonsitammarat). Importantly, autochthonous leishmaniasis cases

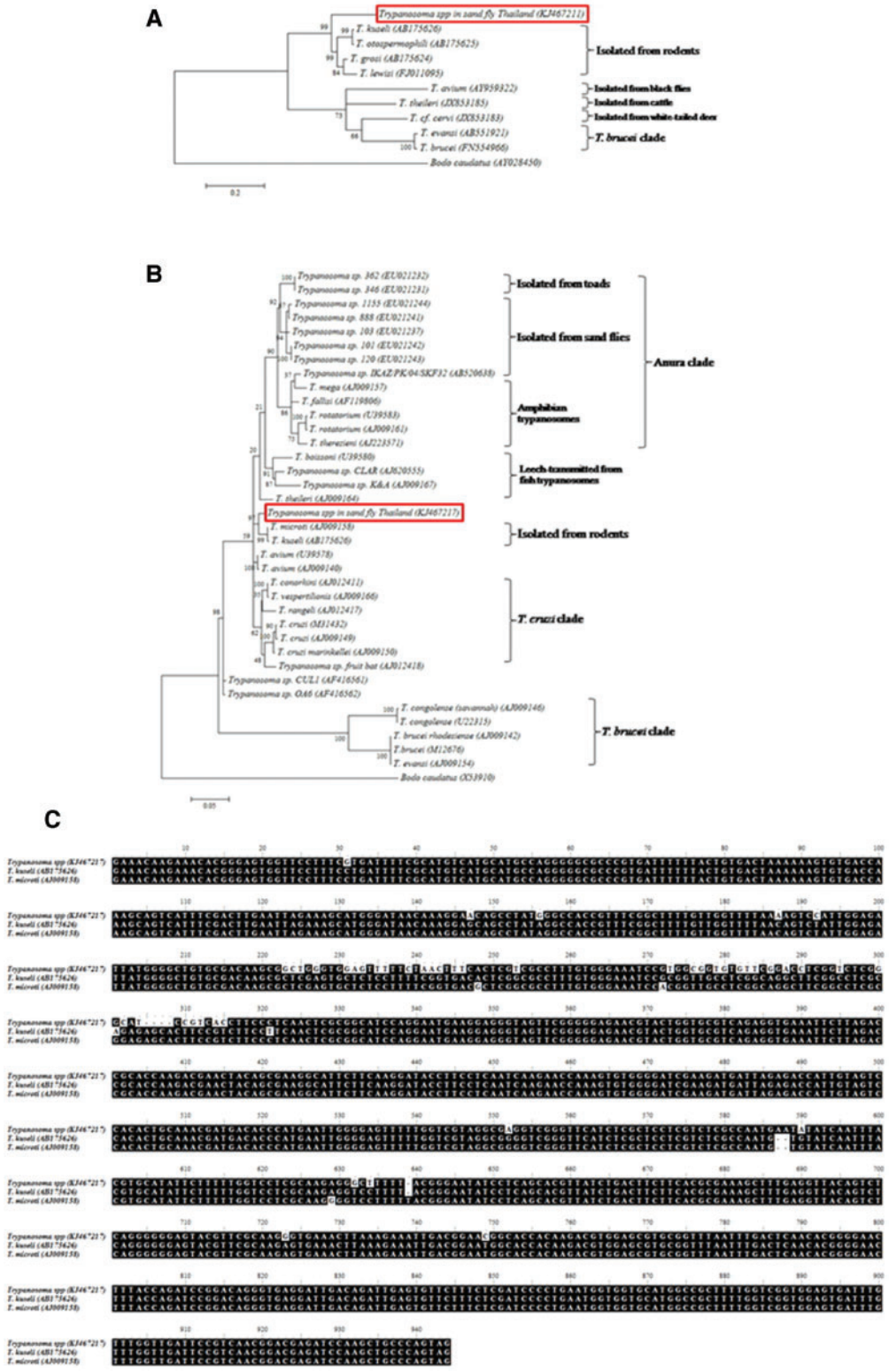

Fig. 3. Phylogenetic tree of *ITS1* (**A**) and *SSU rDNA* (**B**) gene sequences of *Trypanosoma* sp. The Kimura-2-parameter model in MEGA6.0 software was used. Bootstrap values based on 1,000 replicates. The names for this study include genus, species, and accession numbers. *ITS1* gene (*Bodo caudatus* accession no. AY028450 as an outgroup) or *SSU rDNA* gene (*B. caudatus* accession no. X53910 as an outgroup). Comparison between *SSU rDNA* sequences obtained from *Trypanosoma* sp. from this study, *T. microti* and *T. kuseli* (**C**).

caused by *L. siamensis* and *L. martiniquensis* have also been reported in these areas (Sukra et al. 2013). In this paper, we report two new country records: *Se. khawi* (Raynal & Gaschen, 1936) previously reported from Cambodia, China and Malaysia (Seccombe et al. 1993) and *Se. hivernus*. The latter species was described from Vietnam, formerly Indochina, by Raynal and Gaschen (1935) under the name of *Ph. hibernus*, which was then changed in *Ph. hivernus* by Raynal (1935). This species was, surprisingly, considered as a junior synonym

of *Se. iyengari* by Quate (1962). In our opinion, this synonymy is wrong because the spermathecae of *Se. hivernus* are long, wide, smooth, and tubular without limits between the body and duct, whereas those of *Se. iyengari* are shorter with an easily observed limit between the body and duct. On the other hand, *Se. hivernus* exhibits many denticles on the cibarium (~10 in the original description; 3 to 8 in the present study; mean: 5.4; and standard error: 1.95 on one row (Fig. 1)), whereas *Se. iyengari* is described without any denticles (Sinton, 1933). In 1978, Lewis observed that "the cibarium of the female of *Se. iyengari* has central teeth smaller than the rest, and fore teeth are absent or ranging from one row of four to two rows of up to 20." We think he was mistaken. He included many closely related populations that likely correspond to closely related species under the same binominal name, *Se. iyengari*. Our position is based not only on the seven specimens included in the present study but also on hundreds of specimens from Thailand, Laos, Cambodia, Malaysia, and Vietnam (J. Depaquit, personal communication). The number of teeth of the specimens we observed ranged from 10 to 16, which is in agreement with the number recorded in the original description, 14. We noticed that the third antennal segment (A III = flagellomere 1) is shorter in our specimens (minimum 218 µm, maximum 239 µm, mean 230 µm, standard error 9.79 µm) compared with those described by Raynal & Gaschen (1935), which are 250 to 285 µm long. Consequently, we reinstate *Se. hivernus* as a valid species. In addition, we consider a range of the number of cibarial teeth (10 to 16) and cibarial denticles (variable 3 to ~10) to be more important for *Se. hivernus*.

The phylogenetic tree constructed based on the *CytB* gene revealed a strong link between morphological identification and molecular characterization (Fig. 2). Our study confirms that the *CytB* gene sequences are useful for sand fly species identification (Depaquit 2014). The *CytB* sequences showed that the *Ph. stantoni* sand flies collected from this study were 100%, 99%, and 99% identical to the *Ph. stantoni* sand flies collected from Chiang Mai, Thailand (KM409494), Malaysia (KM409495), and Vietnam (KM409497 and KM409498), respectively (Depaquit et al. 2015). Surprisingly, two highly supported clusters of *Se. barraudi* were clearly distinguished based on their *CytB* sequences without any apparent morphological evidence. We prefer the use of *Se. barraudi* s. l. because it could be a complex of species. Further taxonomic studies should be performed to explore this observation.

PCR was used to detect Trypanosomatidae parasites in the sand fly samples. One female *Ph. stantoni* sand fly sample was positive for *Trypanosoma* sp. The PCR amplicons of *ITS1* and *SSU rDNA* that were amplified from this study were 360 and 939 bp, respectively. The expected PCR amplicons amplified by LeF and LeR primers for *L. siamensis* is 371 bp, but our sample was only 360 bp for this primer set. We hypothesize that this amplicon could represent other trypansomatid species. However, the sequences are representative of Trypanosome parasites. The nucleotide sequences of the *ITS1* and *SSU rDNA* gene were submitted to GenBank and assigned the access numbers KJ467211 and KJ467217, respectively. Neither the *ITS1* nor *SSU rDNA* sequences were identical to those of any previously described *Trypanosoma* sequences available in the database. The trypanosome DNA found in this study could be a species that does not have sequence in GenBank or possibly a novel *Trypanosoma* species.

A phylogenetic ML tree was constructed using the *ITS1* and *SSU rDNA* regions. The *ITS1* gene of the *Trypanosoma* sp. found in the sand fly from this study revealed a unique cluster from other *Trypanosoma* species obtained in this database (Stevens et al. 1998, Kato et al. 2010, Nzelu et al. 2014), which are closely related to trypanosomes isolated from rodents (Fig. 3A). An ML tree constructed from the *SSU rDNA* gene found a close similarity to the *ITS1* gene (Fig. 3B). The suspected novel trypanosome species is closely related to *T. microti* and *T. kuseli* (accession numbers AJ009158 and AB175626, respectively), species previously isolated from rodents. Sequence comparisons between this unnamed species and *T. microti* and *T. kuseli* were 94% identical (Fig. 3C).

Previous reports have stated that trypanosome parasites can be found in sand fly vectors. A long time ago, *Se. minuta* (Rondani) was incriminated in the transmission of *Trypanosoma polydactyly* (Gramiccia et al. 1989). McConnell and Correa (1964) suggested that most of the trypanosome infections in their study were in *Dampfomyia vespertilionis* (Fairchild & Hertig). Kato et al. (2010) reported a natural infection due to a species of *Trypanosoma* in the sand fly *Ph. kazeruni* Theodor & Mesghali in Pakistan. More recently, Nzelu et al. (2014) observed *Trypanosoma* DNA in *Se. africana* in Ghana. Finally, Ferreira et al. (2015) identified *Trypanosoma* spp. in Brazilian *Sciopemyia sordellii*, *Sc. servulolimai*, *Sc.* sp., and *Evandromyia infraspinosa*.

This is the first report of *Trypanosoma* sp. DNA detected in a *Ph. stantoni* from Thailand. We suspect that it belongs to a novel species of the genus *Trypanosoma*. Unfortunately, we have not yet isolated, cultivated, or described this putative novel species. Further investigations will be necessary to demonstrate the relationships among trypanosome parasites, sand fly vectors, and reservoir hosts.

Acknowledgments

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2. การนำผลงานวิจัยไปใช้ประโยชน์
   - เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป)
   - เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน)

   การนำผลงานวิจัยไปใช้ประโยชน์ (ดูคำจำกัดความ และตัวอย่างด้านหลังแบบฟอร์ม)

   ☐ ด้านนโยบาย

   กรมควบคุมโรค ได้เห็นความสำคัญของริ้นฝอยทราย และการระบาดของโรคลิชมาเนีย จึงได้วางนโยบายการควบคุมริ้นฝอยทรายโดยมีการให้ทุนสนับสนุนการศึกษาวิจัยโรคลิชมาเนียและริ้นฝอยทรายในพื้นที่ภาคใต้ แก่ สำนักงานควบคุมโรคที่ 11 ซึ่งจะได้มาศึกษาดูงานที่ห้องปฏิบัติการของ รศ. นพ. เผด็จ สิริยะเสถียร

ที่ สธ ๐๔๖๐.๒/๑๒๘๓

สำนักงานป้องกันควบคุมโรคที่ ๑๑
จังหวัดนครศรีธรรมราช
๑๘๔/๑๑๗ ต.โพธิ์เสด็จ อ.เมือง
จ.นครศรีธรรมราช ๘๐๐๐๐

๖ มิถุนายน ๒๕๖๐

เรื่อง ขออนุญาตเข้าพบที่ปรึกษาโครงการวิจัย

เรียน หัวหน้าภาควิชาปรสิตวิทยา คณะแพทยศาสตร์

ด้วยสำนักงานป้องกันควบคุมโรคที่ ๑๑ จังหวัดนครศรีธรรมราช ได้อนุมัติงบประมาณจากกรมควบคุมโรค ให้ดำเนินงานโครงการวิจัย เรื่อง "การศึกษาพาหะนำโรค และอัตราการติดเชื้อก่อโรคลิชมาเนียในพาหะนำโรคในแหล่งท่องเที่ยวเชิงนิเวศน์ในภาคใต้ ประเทศไทย" ของนายคณพศ ทองขาว ซึ่งในโครงการวิจัยดังกล่าว มี รศ.นพ.ดร. เผด็จ สิริยะเสถียร เป็นที่ปรึกษาโครงการวิจัย นั้น

ในการนี้ เพื่อให้การดำเนินงานวิจัยครั้งนี้ มีความถูกต้องและสมบูรณ์ยิ่งขึ้น สำนักงานป้องกันควบคุมโรคที่ ๑๑ จังหวัดนครศรีธรรมราช จึงขออนุญาตให้ทีมวิจัยเข้าพบที่ปรึกษาโครงการวิจัย เพื่อปรึกษาและขอคำแนะนำเกี่ยวกับการจำแนกชนิดริ้นฝอยทรายและการตรวจวิเคราะห์หาเชื้อก่อโรคลิชมาเนียในริ้นฝอยทราย ในระหว่างวันที่ ๑๗ – ๒๑ กรกฎาคม ๒๕๖๐ ณ ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

จึงเรียนมาเพื่อโปรดพิจารณาอนุญาตด้วย จะเป็นพระคุณยิ่ง

ขอแสดงความนับถือ

(นางสาวศิริลักษณ์ ไทยเจริญ)
ผู้อำนวยการสำนักงานป้องกันควบคุมโรคที่ ๑๑
จังหวัดนครศรีธรรมราช

เรียน ผู้อำนวยการสำนักงานป้องกันควบคุมโรคที่ 11
ภาควิชาปรสิตวิทยา อนุญาตให้ทีมงานวิจัย
เข้าพบเพื่อปรึกษาโครงการวิจัยและขอคำแนะนำฯ
ตามวันและเวลาดังกล่าว
จึงเรียนมาเพื่อโปรดทราบ

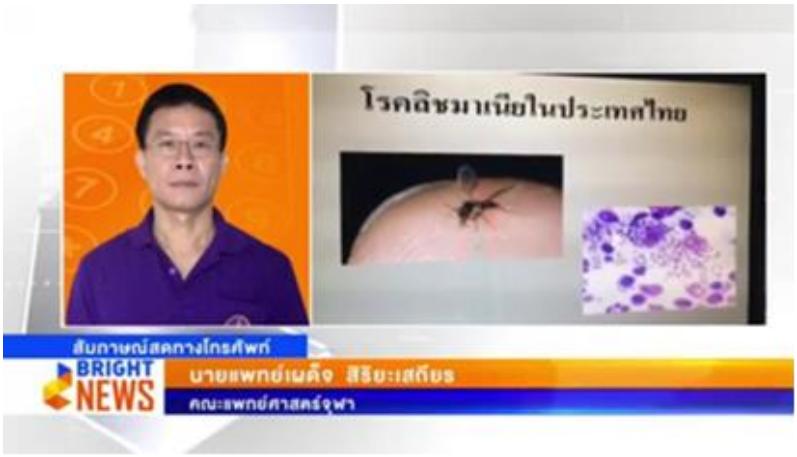
(รศ.นพ.ดร.เผด็จ สิริยะเสถียร)
หัวหน้าภาควิชาปรสิตวิทยา

กลุ่มพัฒนาวิชาการ
โทร. ๐ ๗๕๓๔ ๑๑๔๗
โทรสาร. ๐ ๗๕๓๔ ๒๓๒๘

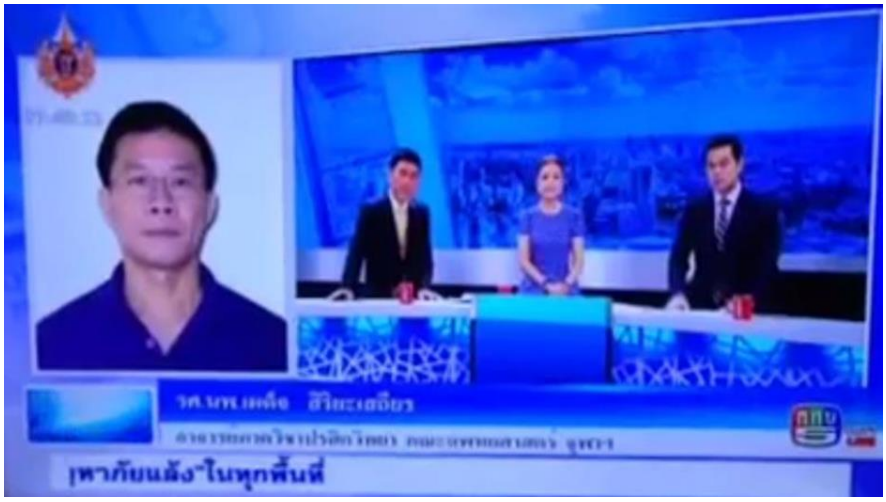
3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

   ให้ความรู้แก่ประชาเกี่ยวกับโรคลิชมาเนีย และโรคติดต่อนำโดยแมลงตามช่องทางต่างๆ รศ. นพ. เผด็จ สิริยะเสถียร ได้ให้ความรู้แก่ประชาชนในช่องทางต่างๆ ได้ แก่ ข่าวทางทีวี ช่อง 3, Bright TV20, ช่อง 5, ช่อง 9 และหนังสือพิมพ์เดลินิวส์ ในความรู้เรื่องโรคลิชมาเนีย ริ้นฝอยทราย และการป้องกัน

ช่อง 3 รายการเที่ยงเปิดประเด็น “ระวังริ้นฝอยทรายกัด เสี่ยงป่วยลิชมาเนีย” วันที่ 19-02-58

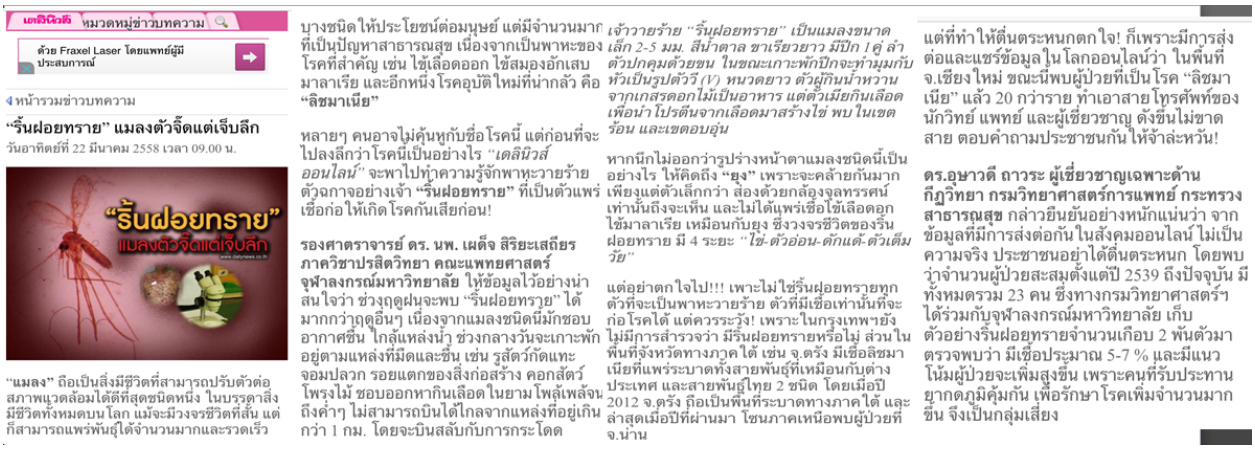


ช่อง Bright TV 20 รายการ Bright NEWS “โรคอุบัติใหม่-ลิชมาเนีย” วันที่ 20-02-58



ช่อง 5

ช่อง 9 รายการ บอก 9 เล่าสิบ “ลิชมาเนีย สายพันธุ์ไทย โรคอุบัติใหม่ วันที่ 30-03-2558



หนังสือพิมพ์เดลินิวส์ ในความรู้เรื่องโรคลิชมาเนีย ริ้นฝอยทราย และการป้องกัน

- เป็นวิทยากรในการจัดอบรมพัฒนาศักยภาพบุคลากรในการเฝ้าระวังและเก็บตัวอย่างแมลงและสัตว์ขาข้อปล้อง ของกรมควบคุมโรค กระทรวงสาธารณสุข ระหว่างวันที่ 6-8 พฤษภาคม 2558 ณ ศูนย์ฝึกอบรมโรคติดต่อนำโดยแมลง อ.พระพุทธบาท จ. สระบุรี วัตถุประสงค์เพื่อให้ผู้เข้ารับการอบรมสามารถพัฒนาความรู้ระบบการเฝ้าระวังและการให้คำแนะนำในการป้องกันควบคุมริ้นฝอยทราย และแมลงอื่นที่เป็นปัญหาทางสาธารณสุขได้

ที่ สธ ๐๔๒๓.๕ / ๑๓๒๔ กรมควบคุมโรค
ถนนติวานนท์ จังหวัดนนทบุรี ๑๑๐๐๐

๑๖ เมษายน ๒๕๕๘

เรื่อง ขอความอนุเคราะห์วิทยากร

เรียน คณบดี คณะแพทย์ศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

สิ่งที่ส่งมาด้วย กำหนดการฝึกอบรมฯ จำนวน ๑ ชุด

ด้วยกรมควบคุมโรค ได้จัดอบรมพัฒนาศักยภาพบุคลากรในการเฝ้าระวังและเก็บตัวอย่างแมลงและสัตว์ขาข้อปล้อง ระหว่างวันที่ ๖-๘ พฤษภาคม ๒๕๕๘ ณ ศูนย์ฝึกอบรมโรคติดต่อนำโดยแมลง อำเภอพระพุทธบาท จังหวัดสระบุรี วัตถุประสงค์เพื่อให้ผู้เข้ารับการอบรมสามารถพัฒนาความรู้ระบบการเฝ้าระวัง และการให้คำแนะนำในการป้องกันควบคุมยุง แมลง และสัตว์ขาข้อปล้องอื่นที่เป็นปัญหาทางสาธารณสุขได้

ในการนี้ กรมควบคุมโรค ขอความอนุเคราะห์ รศ.ดร.นพ.เผด็จ สิริยะเสถียร หัวหน้าหน่วยกีฏวิทยาทางการแพทย์ ภาควิชาปรสิตวิทยา คณะแพทย์ศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย เป็นวิทยากรในหัวข้อ ริ้นฝอยทราย: ชีววิทยา ความสำคัญทางการแพทย์และการจัดการ และหัวข้อ แมลงวัน แมลงสาบ: ชีววิทยาความสำคัญทางการแพทย์ และการจัดการ ในวันที่ ๖ พฤษภาคม ๒๕๕๘ เวลา ๑๐.๓๐ – ๑๒.๐๐น. ณ ศูนย์ฝึกอบรมโรคติดต่อนำโดยแมลง อำเภอพระพุทธบาท จังหวัดสระบุรี ตามกำหนดการฝึกอบรมดังสิ่งที่ส่งมาด้วย

จึงเรียนมาเพื่อโปรดพิจารณาให้ความอนุเคราะห์ต่อไปด้วย จะเป็นพระคุณ

ขอแสดงความนับถือ

(นายโอภาส การย์กวินพงศ์)
รองอธิบดี ปฏิบัติราชการแทน
อธิบดีกรมควบคุมโรค

สำนักโรคติดต่อนำโดยแมลง
โทร. ๐ ๒๕๙๐ ๓๑๔๔
โทรสาร ๐ ๒๕๙๑ ๘๔๒๒

- เป็นวิทยากรการอบรมเชิงปฏิบัติการเครือข่ายห้องปฏิบัติการโรคติดเชื้ออุบัติใหม่ (EID LAB NETWORK) เมื่อวันที่ 20-22 กุมภาพันธ์ 2560 ณ โรงแรมโรแมนติค รีสอร์ท แอนด์ สปา จ. นครราชสีมา

ที่ สธ ๐๖๑๘.๐๑.๑/ ๑๗๕๕

สถาบันวิจัยวิทยาศาสตร์สาธารณสุข
กรมวิทยาศาสตร์การแพทย์
กระทรวงสาธารณสุข
ถนนติวานนท์ จังหวัดนนทบุรี ๑๑๐๐๐

๕ กุมภาพันธ์ ๒๕๖๐

เรื่อง ขอเชิญบุคลากรในสังกัดเป็นวิทยากร

เรียน คณบดีคณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

สิ่งที่ส่งมาด้วย ๑. กำหนดการอบรม จำนวน ๒ แผ่น
๒. แบบตอบรับการวิทยากร จำนวน ๑ แผ่น

ด้วยสถาบันวิจัยวิทยาศาสตร์สาธารณสุข โดยกลุ่มวินิจฉัยโรคกลาง ได้รับอนุมัติให้ดำเนินการจัดอบรมเชิงปฏิบัติการเครือข่ายห้องปฏิบัติการโรคติดเชื้ออุบัติใหม่ (EID Lab Network) ระหว่างวันที่ ๒๐ – ๒๒ กุมภาพันธ์ ๒๕๖๐ ณ โรงแรมโรแมนติค รีสอร์ท แอนด์ สปา จังหวัดนครราชสีมา โดยมีวัตถุประสงค์เพื่อเตรียมความพร้อมและวางแผนสนับสนุนการตอบโต้การระบาดของโรคติดเชื้ออุบัติใหม่และเชื้ออันตราย รวมทั้งแลกเปลี่ยนประสบการณ์ในการดำเนินการระหว่างห้องปฏิบัติการในส่วนกลางและส่วนภูมิภาค รายละเอียดดังกำหนดการแนบมาพร้อมนี้

ในการนี้ สถาบันวิจัยวิทยาศาสตร์สาธารณสุข จึงมีความประสงค์ขอเชิญ รองศาสตราจารย์ ดร. นายแพทย์เผด็จ สิริยะเสถียร บุคลากรสังกัดภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย เป็นวิทยากรบรรยายในหัวข้อ โรคติดต่อที่นำโดยแมลง ภาวะโลกร้อนซ่อนโรค...อุบัติใหม่ ในวันที่ ๒๓ กุมภาพันธ์ ๒๕๖๐ เวลา ๐๙.๐๐ – ๑๐.๐๐ น. ณ ห้องประชุมโรงแรมโรแมนติค รีสอร์ท แอนด์ สปา จังหวัดนครราชสีมา ทั้งนี้ โปรดทำหนังสือแจ้งตอบรับการเป็นวิทยากรให้สถาบัน ฯ ทราบด้วย **ภายในวันที่ ๑๕ กุมภาพันธ์ ๒๕๖๐**

จึงเรียนมาเพื่อโปรดพิจารณาอนุญาต และแจ้งผู้เกี่ยวข้องเป็นวิทยากรในการอบรมตามวันเวลา และสถานที่ดังกล่าวด้วย จะเป็นพระคุณ

ขอแสดงความนับถือ

นายสมชาย แสงกิจพร
ผู้อำนวยการสถาบันวิจัยวิทยาศาสตร์สาธารณสุข

เรียน คณบดี
ภาควิชาปรสิตวิทยา อนุญาตให้
รศ.ดร.นพ.เผด็จ สิริยะเสถียร เป็นวิทยากร
ตามวัน และเวลาดังกล่าว
จึงเรียนมาเพื่อโปรดทราบ

(รศ.ดร.นพ.เผด็จ สิริยะเสถียร)
หัวหน้าภาควิชาปรสิตวิทยา
กลุ่มวินิจฉัยโรคกลาง
โทร. ๐ ๒๘๕๑ ๐๐๐๐-๑๑ ต่อ ๗๗๓๐๒
โทรสาร ๐ ๒๕๘๑ ๕๔๔๙
สำเนาส่ง รองศาสตราจารย์ ดร. นายแพทย์เผด็จ สิริยะเสถียร

- แต่งหนังสือ

  เผด็จ สิริยะเสถียร โรคลิชมาเนียและริ้นฝอยทรายแมลงพาหะในประเทศไทย พิมพ์ครั้งที่ 1. กรุงเทพฯ: บริษัทหนังสือดีวันจำกัด, 2559. จำนวนหน้าทั้งหมด 116 หน้า เผยแพร่ในห้องสมุดของคณะแพทย์ต่างๆ

การนำเสนอผลงานในที่ประชุมวิชาการ

- เรื่อง First Detection of *Trypanosoma* species DNA in a *Phlebotomine* sand fly and molecular identification of sand flies collected from southern Thailand ในการประชุม IXth International Symposium of *Phlebotomine* Sand Flies (ISOPS IX 2016) หน่วยงานที่จัด International Society of *Phlebotomine* Sand Flies (ISOPS) สถานที่จัดเมือง Reims ประเทศ France ระหว่างวันที่ 27มิถุนายน 2559-1 กรกฎาคม 2559
- เรื่อง First isolation of unknown *Leishmania* parasites from a *Idiophlebotomus longiforceps* sand fly collected in an endemic area of leishmaniasis of Thailand ในการประชุม 6th World Congress on Leishmaniasis 2017 หน่วยงานที่จัด The WHO Collaborating Centre for Leishmaniasis at the Instituto de Salud Carlos III, Madrid and the Drugs for Neglected Diseases initiative, Geneva สถานที่จัดเมือง Toledo ประเทศ สเปน ระหว่างวันที่ 16 พฤษภาคม 2560-20 พฤษภาคม 2560