Detection of Anti-*Leishmania donovani* complex Antibodies of Dogs and Cats from Southern Thailand

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Abstract

Objective - To investigate the potential pet animal reservoir hosts of visceral leishmaniasis in Southern Thailand in areas where autochthonous human cases have been reported.

Materials and Methods - Between April and June 2010, the sera of 407 dogs and 237 cats from three provinces (Surathani, Nakhon si Thammarat and Phangnga) were analyzed for *Leishmania donovani* complex infection using the Direct Agglutination Test.

Results - Antibodies against the *L. donovani* complex were detected in two cats (0.84%) and no dogs. The two anti-sera positive cats, one from each of Surathani and Phangnga provinces, had antibody titers of 1:100 and 1:800, respectively, but were negative for *L. donovani* complex DNA by PCR assay.

Conclusion - Although the exposure number in the cats and dogs as reservoir hosts in this study was low, further surveillance for disease transmission in Thailand should be processed because visceral leishmaniasis is an emerging disease in the country and zoonotic disease which can cause death in humans, especially in untreated cases.

Keywords: *Leishmania donovani* complex, Reservoir host, Dog, Cat, Thailand

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การตรวจหาภูมิคุ้มกันต่อเชื้อในกลุ่ม *Leishmania donovani* complex ในสุนัขและแมวจากภาคใต้ของประเทศไทย

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บทคัดย่อ

วัตถุประสงค์ เพื่อสำรวจสัตว์เลี้ยงที่มีศักยภาพเป็นตัวกักเชื้อของโรค visceral leishmaniasis ในภาคใต้ของประเทศไทย ซึ่งเป็นพื้นที่ที่มีรายงานการเกิดโรคในคนที่ไม่มีประวัติเดินทางไปยังแหล่งระบาดของโรคมาก่อน

วัสดุ อุปกรณ์ และวิธีการ เก็บตัวอย่างเลือดสุนัข 407 ตัวอย่าง และแมว 237 ตัวอย่าง จากจังหวัดสุราษฎร์ธานี นครศรีธรรมราช และพังงา ระหว่างเดือนเมษายนถึงมิถุนายน 2553 นำเซรุ่มมาตรวจหาการติดเชื้อ *Leishmania donovani* complex ด้วยวิธี Direct Agglutination Test

ผลการศึกษา ผลจากการตรวจพบแอนติบอดีต่อเชื้อในกลุ่ม *L. donovani* complex ในแมวจำนวน 2 ตัว (ร้อยละ 0.84) แต่ตรวจไม่พบในสุนัข โดยแมว 2 ตัวที่ติดเชื้อมาจากจังหวัดสุราษฎร์ธานี และพังงา มีระดับ antibody titers ที่ 1:100 และ 1:800 ตามลำดับ อย่างไรก็ตามเมื่อทดสอบด้วยวิธี PCR ไม่สามารถตรวจหาสารพันธุกรรมของ *L. donovani* complex ได้

ข้อสรุป ในการศึกษาครั้งนี้ถึงแม้จะพบสัตว์เลี้ยงมีการติดเชื้อในปริมาณต่ำ แต่ควรมีการสำรวจเพิ่มเติมในพื้นที่ต่อไปในอนาคต เนื่องจากโรค visceral leishmaniasis ถือเป็นโรคอุบัติใหม่ในประเทศไทยและเป็นโรคสัตว์สู่คน ซึ่งในคนที่ติดเชื้ออาจเสียชีวิตได้หากไม่ได้รับการรักษา

คำสำคัญ: ลิชมาเนีย โดโนวานิ คอมเพล็กซ์ ตัวเก็บเชื้อ สุนัข แมว ประเทศไทย

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Introduction

Visceral leishmaniasis (VL) is caused by haemoflagellate protozoa in the *Leishmania donovani* complex (*L. donovani and L. infantum*). *L. infantum* affects both the viscera and skin of dogs [1]. The sandflies from the genus *Phlebotomus* in the Old World or *Lutzomyia* in the New World are the vectors responsible for their transmission. Major epidemic areas occur in tropical and sub-tropical zones, with over 90% of worldwide cases being found in Brazil, Bangladesh, India and Sudan [2]. There have been limited reports of cutaneous leishmaniasis (CL) and VL in humans in Southeast Asia, in Singapore and East Timor [3-4], but Thailand is a non-endemic region for leishmaniasis [5-6]. CL and VL were reported in five Thai workers who returned from Middle East countries, with the first case being reported in 1986 [7]. However, the first autochthonous case was reported in a three-year-old girl who lived in Surathani province [8]. Subsequentially, in 2005 in a 40-year-old man who worked as a construction worker in Nan province and Bangkok [9], and in a 55-year-old non-HIV-infected Thai man who had been working at his rubber plantation in Phangnga province, Southern Thailand, were reported [10]. From 2005 to date, there have been ten further reports of autochthonous cases in humans in Thailand, where most of them lived in Southern Thailand [11]. The sandflies *Sergentomyia gemmea, S. barraudi, S. indica* and *Plebotomus stantoni* have been collected from the areas where the patients lived, but all were found to be negative for *Leishmania* DNA using specific PCR [9-10]. There are reports of *Phlebotomus argentipes* in Thailand but the potential for this species to act as a vector in *Leishmania* transmission is still not clear [12-13]. Generally, asymptomatic dogs are accepted as being the main reservoir host of *L. infantum* infections for humans and other susceptible animals [1-2, 14-15], while cats are alternative reservoir hosts for VL rather than accidental hosts [16-17]. There have been a few reports studying infected animals in Thailand, but these have been restricted to cats and cows, as determined by anti-*Leishmania* anti-sera positive using a direct agglutination test (DAT), with low anti-sera titers ranging from 1:100 - 1:400 [9-10]. Although the study number and infection frequency was low, the potential for transmission to humans remains unclear because all of the tested animals lived in the same villages as the patients. In the past five years, VL has been reported in HIV-infected patients, which most of them lived in Southern Thailand [11,18], which suggests a potential link between immune compromised individuals and susceptibility to disease progression following infection, and the potential co-occurrence of both vector and reservoir in Southern Thailand.

Moreover, *Leishmania siamensis* is a newly found protozoan isolate in Thai patients who live in Southern Thailand [11,19], with a currently unknown infection ability across the population. Thus, the objective of this study was to investigate the potential animal reservoir hosts of VL in Thailand, and in particular in pet animals, to understand the situation of the disease in the country.

Materials and Methods

Selection of study area

The present survey was carried out in the three provinces of Southern Thailand where human cases of VL have been sporadically reported [8,10]. The study was conducted in nine districts, comprised of Phrasaeng, Chaiburi and Phanom in Surathani province, Phrom Khiri, Phipun and Chawang in Nakhon si Thammarat province, and Thapput, Takua Thung and Thai Mueang in Phangnga province (Figure 1). These districts are connected with each other. The climate of Southern Thailand is tropical with heavy rainfall between June and January.

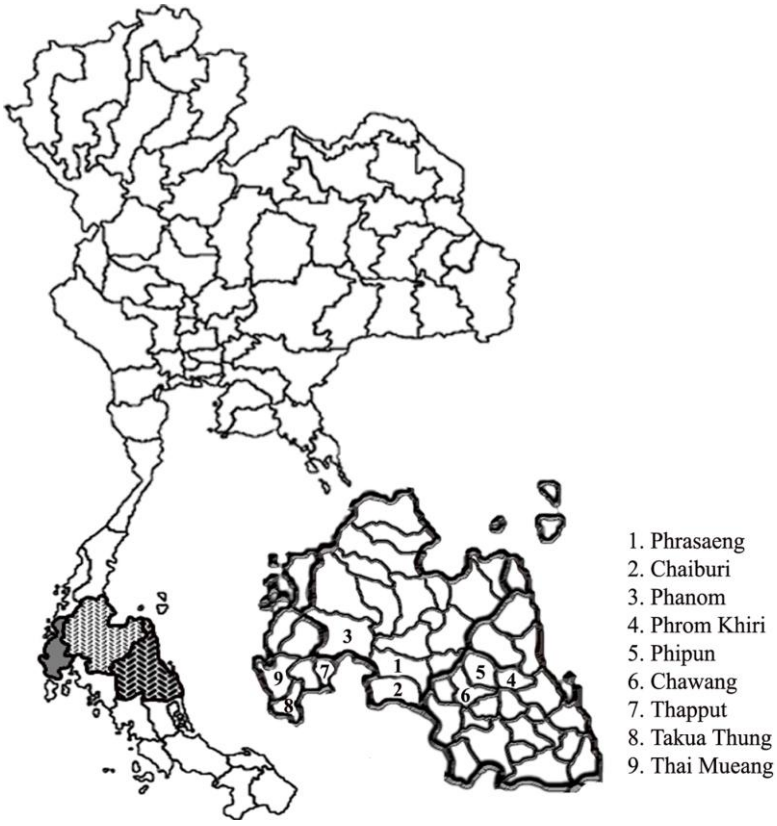

Figure 1. Map of the study areas (nine districts) in Surathani, Nakhon si Thammarat and Phangnga provinces.

Sample size

A total of 407 and 237 pet dogs and cats, respectively, were evaluated for their infection status in this study. All of these pet animals lived with their guardians (owners) in rubber, palm, or mangosteen and rambutan plantation areas, and were selected for taking blood samples with a stratified random sampling procedure. The research protocols were strictly followed the National Research Council manual of experimental animal ethics.

Sample and data collection

Three-mL blood samples were collected from pet dogs and cats, during March - May 2010. After collection, half of the blood volume was transferred to a 3.2% (w/v) sodium citrate containing tube (FUSHINO®) for DNA extraction and microfilaria detection. The remaining volume was transferred to a serum tube (FUSHINO®) and centrifuged at 100 x g for 5 min and then the serum supernatant harvested. Blood and serum samples were placed into chilled iceboxes and transferred to the laboratory room. All samples were kept in -20°C until use. Relevant information on the species, age and sex of the animal along with the location and guardian's details were recorded. Physical examination of each animal was performed to check the general condition, including the mucous membrane and any evidence for infestation by ectoparasites.

Serological test for infection by the L. donovani complex

Serum samples were checked for the potential exposure to *L. donovani* by the DAT assay using the commercial DAT KIT (Biomedical Research, the Netherlands) following the manufacturer's procedure. Briefly, 100 µL of a 1:100 serum dilution in 0.9% (w/v) normal saline supplemented with 0.2 M β-mercaptoethanol (DAT-diluent) was transferred into the first well of V-shaped microtiter plate (nunc™, Denmark), and subsequently sequentially two-fold serial diluted in DAT-diluent (50 µL:50 µL) using a multi-channel pipette into subsequent wells, so as to give a total of seven serum dilutions from 1:100 to 1:6,400. Negative and positive controls were also run at the same time. Plates were incubated at the room temperature (~30°C) for 1 hour and then 50 µL of *L. donovani* complex antigen for the DAT assay was added into each well, shaken gently and incubated overnight at room temperature. In the case of antibodies to *Leishmania* were present, the reaction between antigen and antibody formed a pale blue film over the well and this was a positive result. The end-titer was ascribed to the last serum dilution that still gave a clear visual agglutination as compared to the negative control.

PCR confirmation of *L. donovani* infection and PCR detection of infection with *Tryponosoma evansi*

L. donovani anti-sera positive samples from the above DAT assay were also determined for infection by *L. donovani*, as well as for another haemoflagellate, *Trypanosoma evansi*, by the polymerase chain reaction (PCR). DNA extraction was done by the standard Phenol-Chloroform-Ethyl alcohol method [20]. The PCR assay was performed using the specific primers LITSR and L5.8S for *L. donovani* [21] with an expected product size of 300-350 bp, and with TBR1 and TBR2 for *T. evansi* [22] with an expected product size of 164 bp. PCR reactions for *T. evansi* were performed with 50 µL and thermal cycled at 94°C for 1 min, followed by 30 cycles of 94°C for 30 s, 60°C for 60 s and 72°C for 30 s, and then a final 72°C for 2 min. Amplification products were resolved by 1.2% (w/v) agarose-TBE gel electrophoresis and visualized by UV transillumination after being stained with ethidium bromide.

Serological evaluation of *L. donovani* co-infection with Feline Leukemia Virus/Feline Immunodeficiency Virus (FeLV/FIV)

The sera from the *L. donovani* complex antisera-positive cats were also checked for infection with Feline Leukemia Virus/Feline Immunodeficiency Virus (FeLV/FIV) and filarial nematodes (heartworm) using the SNAP Feline Triple Test (IDEXX Laboratories), following the manufacturer's procedure.

Microscopic evaluation of microfilariae

To check for filarial nematode infection, in terms of the blood born microfilariae stage, the blood samples were drawn into microcapillary hematocrit tubes, centrifuged at 9,600 x g for 5 min and then checked for the presence of microfilaria under a light microscope (100 x magnification). Buffy coat smears were prepared from the positive samples, Giemsa-stained and examined under the light microscope (1000 x magnification) for microfilaria identification.

Results

Serological test and PCR confirmation for infection with the *L. donovani* complex

Sera from all 407 dogs evaluated for *L. donovani* complex anti-sera by the DAT assay were negative, but sera from two of the 237 (0.84%) cats gave positive results. The titers of anti-*Leishmania* antibodies in these two cats, one from Phanom district, Surathani province and the other from Thapput district, Phangnga province, were relatively low at 1:100 and 1:800, respectively (Table 1). The positive cat from Surathani province was a 2-year-old male living

with the owner in a rubber plantation area, whilst the cat from Phangnga province was a 5-year-old female living with the owner in a palm plantation area. Both cats did not show any sign of illness and they were free from visible signs of infestation with ectoparasites.

However, when the serum DNA samples from the two cats that were found to be *L. donovani* anti-sera positive by the DAT assay were tested for *L. donovani* DNA by PCR they were both found to be negative (data not shown).

Table 1. Number of dogs and cats that were *L. donovani* complex anti-sera positive in three provinces from Southern Thailand.

Province	Infected (total no / sample size; (%))	
	Dogs	Cats
Surathani	0/127 (0%)	1/73 (1.36%)
Nakhon si Thammarat	0/144 (0%)	0/58 (0%)
Phangnga	0/136 (0%)	1/106 (0.94%)
Total	0/407 (0%)	2/237 (0.84%)

Co-infection of animals with *L. donovani* complex and *T. evansi* or FeLV/FIV

The two *L. donovani* anti-sera positive samples were also checked for infection with *T. evansi* to confirm the lack of cross-reactivity of the serological test. The PCR assays for both cases were negative for *T. evansi* DNA, whilst the sera of two cats were also negative for FeLV antigen and anti-FIV antibodies in the SNAP Feline Triple Test.

Prevalence of filarial infections

Blood examination revealed that 61 of the 407 dogs (15.0%) and 2 of the 237 cats (0.84%) were positive for filarial infections. *Dirofilaria immitis* was predominant over *Brugia* spp. in dogs with 14.0% (57/407) of the dogs and 0.42% (1/237) cats being infected with *D. immitis,* compared to 1.22% (5/407) and 0.42% (1/237) *Brugia* spp. infected dogs and cats, respectively. Mixed infection with *D. immitis* and *Brugia* was found in one dog only, but in no cats. Surathani province had the highest prevalence of *D. immitis* infection in dogs at 22.8% (29/127) and *Brugia* spp. infection was only found in this province (Table 2). However, the two *L. donovani* anti-sera positive cats were not infected with these filarial nematodes.

Table 2. Filarial infections of dogs and cats, as detected by microcapillary haematocrit centrifugation method and Giemsa-staining.

Province	Dogs (Infected (%))[1]			Cats (Infected (%))[1]	
	D. immitis	*Brugia* spp.	Mixed	*D. immitis*	*Brugia* spp.
Surathani	28 (22.0%)	4 (3.14%)	1 (0.74%)	1 (1.37%)	1
Nakhon si Thammarat	15 (10.4%)	-	-	-	-
Phangnga	13 (9.56%)	-	-	-	-
Total	56 (13.8%)	4 (0.98%)	1 (0.24%)	1 (0.42%)	1 (0.42%)

[1]Number of infected dogs or cats and the % of the total sampled cats or dogs that are infected. Mixed (*D. immitis* plus *Brugia* spp.) infections were not detected in the cats.

- = none detected

Discussion

Infection with *L. donovani* complex was only found in a low proportion of the sampled cats (0.84%), and in no dogs, in this survey of the three Southern provinces of Thailand. This infection rate is lower than those reported previously in endemic areas [16,23], consistent with that Thailand is not currently an endemic area for VL although it is considered to be an emerging disease in the country. Moreover, the exact *Leishmania* infection status of these two DAT anti-sera positive cats is unclear since they were negative by PCR. Generally, most infected cats do not show clinical signs of the infection which is compatible with their low antibody production. However, the negative PCR results, if correct, suggest either a very low parasitemia or a past infection. In contrast, the over-production of antibodies that occurs in dogs correlates with that they show clear symptoms from the infection [16]. From the present and previously reported results, cats are positive for VL infection in Southern Thailand, albeit at varying infection frequencies in the population between this and previous reports, and so they potentially could act as an important reservoir host for VL in Thailand.

The two *L. donovani* complex anti-sera positive cats, as determined by the DAT immunological assay, were from (one each) Phanom district, Surathani province and Thapput district, Phangnga province. These two districts are connected to each other and most of this area is occupied by rubber and palm plantations and it also has three national parks which support a high humidity in this area. This may then provide suitable environmental conditions for

the sandflies *P. argentipes*, which have been reported in Phrom Khiri district, Nakhon si Thammarat province [24], and *P. stantoni*, which has been reported in Takua Thung district, Phangnga province [10], to act as vectors for VL.

The *L. donovani* complex anti-sera positive cats were not infected with either *T. evansi* or filarial nematodes, or with FeLV/FIV viruses. This confirms the positive results for the *L. donovani* complex anti-sera by the DAT assay, but not the PCR assay, were not false positive results from cross immunity with *T. evansi* or filarial worm infections. We checked the cross reactivity because *Trypanosoma* spp. infection could possibly react with DAT [9,11]. There was no association between the infection with the immunosuppressive viruses (FeLV/FIV) and feline leishmaniasis, which is in accord with that previously reported [16]. In contrast to human, VL should be considered as a co-infection in HIV-infected patients in the countries where VL is endemic [25-26].

Conclusions

From the results of the present study, it seems cats could be an important reservoir host of VL in Southern Thailand. The positive cats did not show any signs of illness, which means that they can act as good reservoir hosts. The disease transmission from cat to cat or even cat to human via sandflies could theoretically occur. Given that most (Islamic) residents in Southern Thailand rear cats in their houses, it is possible that they will be infected from these asymptomatic cats. Accordingly, disease prevention education should be introduced to the people who reside in these areas. For example, sleeping under fine mesh nets, spraying effective insecticides around the houses and controlling the animal reservoirs should be considered as approaches to reduce the occurrence of VL in humans in the future.

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