

Risk Factors of *Leishmania* Infection among HIV-Infected Patients in Trang Province, Southern Thailand: A Study on Three Prevalent Species

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Abstract. There are two main species of *Leishmania* reported in Thailand, that is, *Leishmania siamensis* and *Leishmania martiniquensis*. Moreover, leishmaniasis cases caused by *Leishmania donovani* complex were also reported. There is still a lack of information concerning risk factors of *Leishmania* infection in Thailand. This study aimed to identify the risk factors of *Leishmania* infection caused by these three species among HIV-infected patients. A cross-sectional study was conducted in HIV clinic at Trang Hospital, Thailand. Nested PCR and sequencing were performed to detect *Leishmania* DNA in blood and saliva samples and identify *Leishmania* species. A standardized questionnaire was used to interview individuals. A total of 526 patients were recruited in this study. Sixty-three (12.0%) were positive for *L. siamensis*, 24 (4.6%) were positive for *L. martiniquensis*, and 23 (4.4%) were positive for *L. donovani* complex. Risk factors of *L. siamensis* infection included using intravenous drug (adjusted odds ratio [AOR] 2.01, 95% CI: 1.01–4.02). Risk factors of *L. martiniquensis* infection included female gender (AOR 4.23, 95% CI: 1.52–11.75), using recreational drug (AOR 3.43, 95% CI: 1.00–11.74), and having comorbidities (AOR 4.94, 95% CI: 2.00–12.21). Risk factors of *L. donovani* complex infection included having opportunistic infection (AOR 4.22, 95% CI: 1.00–17.79), CD4 count 200–500 cells/mm³ (AOR 3.64, 95% CI: 1.14–6.86), and not using insect repellent (AOR 3.04, 95% CI: 1.08–8.58). This study identified the risk factors of *Leishmania* infection caused by three *Leishmania* species in Thailand. The data could be useful for disease prevention and control. Further studies on trends of *Leishmania* infection and preventive measures are recommended.

INTRODUCTION

Leishmania is a genus of the protozoan parasite, causes a disease called leishmaniasis, which ranges from chronic ulceration called cutaneous leishmaniasis (CL) to a fatal systemic disease called visceral leishmaniasis (VL).¹ Leishmaniasis is transmitted by a bite of an infected sandfly that introduces metacyclic promastigotes, a motile form of the parasite, via the host's skin.^{1–3} The parasites are then phagocytosed by the host's macrophages⁴ where the promastigote shifts to the amastigote form, an immotile obligative intracellular stage, which multiplies and further invades other cells.⁴

There are more than 20 *Leishmania* species which are able to infect humans.⁵ In Thailand, most studies identified two novel species, *Leishmania siamensis* and *Leishmania martiniquensis*, as the main causes of leishmaniasis.⁶ A recent study reported *Leishmania donovani* complex, a group of *Leishmania* comprising *L. donovani* and *Leishmania infantum*, infections among HIV-infected patients as well.⁷ There was also a report of VL in non-HIV-infected patients caused by *L. infantum* in Thailand as well.⁸ Cutaneous leishmaniasis in *L. martiniquensis* infection could be ranging from single or multiple nodules to generalized non-ulcerative nodules, ulcerative lesions, or fibrotic lesions, of which the generalized form is called disseminated CL.⁶ On the other hand, the disseminated CL form of *L. siamensis* infection, which manifested as thick skin nodules, is rare.⁶ For VL, the presentations included prolonged fever, hepatosplenomegaly, weight loss, anemia, leukopenia, thrombocytopenia, and hypergammaglobulinemia, which are similar among *L. martiniquensis*, *L. siamensis*, and *L. donovani* complex.^{6,9}

Emergences of both VL and CL have been continuously reported in Thailand, especially in the southern provinces of the country.^{6,10–15} In 2010, the first case of VL infection in an HIV-infected patient in Thailand was reported.¹⁶ In 2015, Thailand was first considered as the endemic area of leishmaniasis.^{5,12}

Risk factors of VL among HIV-infected patients in Thailand had already been addressed⁷; however, the differences of characteristics and risk factors of *L. siamensis*, *L. martiniquensis*, and *L. donovani* complex infections in HIV-infected patients are not yet identified. This study proposed to identify these differences among HIV-positive patients in Trang Province, an endemic area of leishmaniasis in southern Thailand. This information could be helpful in future diagnosis, determining disease progression, prevention of the disease, and control of vectors and reservoir hosts.

MATERIAL AND METHODS

Study design. This study is a continuity of the previously published study by Manomat et al.⁷ The current study was conducted in a cross-sectional design during February 2015 and February 2016 to identify the risk factors of *Leishmania* infection among HIV-infected patients who visited Trang Hospital, Trang Province, southern Thailand.

Study population and setting. Based on the study by Manomat et al.,⁷ this study included HIV-infected patients who were aged 18 years or older and went to the HIV clinic in Trang Hospital. Trang Hospital is a major public tertiary care center of Trang Province and was classified by the Ministry of Public Health as a regional hospital. Trang Province is a coastal province in southern Thailand. It is located 837 km from Bangkok, the capital of Thailand. The climate was tropical monsoon with an average temperature of 27.2°C for the year. The average amount of precipitation for the year was 2,385.1 mm. The highest precipitating month was September.

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The patients visited the HIV clinic every 6 months for follow-up test and received antiretroviral therapy. The patients lived in 10 districts of Trang Province, nine other provinces in southern Thailand, and three other provinces in other regions of Thailand. Information on the patients was retrieved from their medical records.

Ethical consideration. The research protocol was approved by the Ethical Committee of the Royal Thai Army Medical Department. The approval number was S017 h/56. Written consent was obtained before data collection. Personal information of the patients was disclosed and anonymized.

Samples collection and preparation. Blood samples of the patients were collected in an ethylenediaminetetraacetic acid (EDTA) anticoagulant tube. The amount of blood collected was 8 mL. After collection, whole blood was centrifuged at $900 \times g$ for 10 minutes for separation of plasma and buffy coat. The centrifuged samples were preserved at -20°C . One–two milliliters of saliva sample of each patient was collected by spitting into sterile collection vials. Samples were preserved at -20°C for 1–2 hours until used for DNA detection.

Definitions. *Leishmania* infection was defined as an individual who was positive for *Leishmania* DNA from nested PCR using buffy coat and saliva samples, and, then, sequenced for species identification. *Leishmania* non-infection can be defined as an individual who was positive by neither direct antiglobulin test (DAT) nor nested PCR. Previous *Leishmania* infection can be defined as an individual who was infected with *Leishmania* and completely cured in the past, without relation with current *Leishmania* infection. These individuals were tested positive for DAT, but negative for nested PCR.

Detection of *Leishmania* antibodies. This study used DAT for screening patients with positive *Leishmania* infection. DATkit (KIT Biomedical Research, Amsterdam, the Netherlands) was used with the assay for *Leishmania* antibodies. The instruction was provided by the manufacturer. Positive control was retrieved from plasma of confirmed VL cases by the PCR method. Negative control was retrieved from plasma of healthy individuals. Positive titers were detected at value $\geq 1:100$. The cutoff value was recommended by the manufacturer. Patients' samples which were positive for *Leishmania* antibodies were further tested with PCR for species identification.

Detection of *Leishmania* DNA. Two hundred microliters of the buffy coat and saliva collected from each participant were extracted using Gen UPTM gDNA Kit (biotechrabbit, Hennigsdorf, Germany). The final volume of 40 μL was eluted and preserved at -20°C . The nested PCR method was used for amplifying the internal transcribed spacer (ITS) 1 region on small subunit ribosomal DNA of the *Leishmania*. Primers LITSR and L5.8 were used for amplification of 319–348 amplicons during primary PCR process.¹⁷ For the secondary PCR process, two new primers were designed: LITSR2 and L5.8S inner; 25 μL of PCR master mix for both buffy coat and saliva specimens comprised 12.5 pmol of each primer, 0.2 mM of dNTP (Promega, Madison, WI), 1.5 mM of MgCl_2 , $1\times$ PCR buffer, and 1 U of Taq DNA polymerase (Promega, Madison, WI). Four and one microliters of DNA templates were used during primary and secondary reactions, respectively. Positive control was DNA of *L. martiniquensis* promastigotes. The overall processes consisted of pre-denaturation at 94°C for 3 minutes, 35 cycles of 94°C of denaturation for 1 minute, 54°C of annealing for 30 seconds, and 72°C of extension for 30 seconds. Final extension was performed at 72°C for 5 minutes. PCR products were separated by

electrophoresis. Electrophoresis results were visualized by Molecular Imager Gel Doc XR + System with Imager Lab™ 3.0 program (BioRad, Hercules, CA). The full detail of *Leishmania* DNA detection was described in study by Manomat et al.⁷

Sequence analysis. Positive PCR products were sent to U2Bio Co. Ltd. (Seoul, South Korea) for sequencing. BioEdit version 7.0.1 (Ibis Therapeutics, Carlsbad, CA) was used for validation of chromatogram. The sequences were multiple-aligned with reference *Leishmania* strains from GenBank for species identification. The nested PCR results obtained from saliva samples were compared with those obtained from buffy coat samples. Phylogenetic tree was generated by neighbor-joining method using MEGA program version 7.0 (Pennsylvania State University, University Park, PA).

Questionnaire. A standardized questionnaire was constructed. The questionnaire included demographic data, socioeconomic status, clinical symptoms, and risk behaviors. The data were collected by face-to-face interview.

Statistical analysis. Risk factors of *L. siamensis*, *L. martiniquensis*, and *L. donovani* complex infections were analyzed by univariate analysis. A statistical significance was indicated when $P < 0.05$. Factors which were statistically significant in previous studies, statistically significant by univariate analysis, or $P < 0.25$ by univariate analysis were recruited for multivariate analysis using “Enter” function. Odds ratio and 95% CI were calculated for both univariate and multivariate analyses. Factors which had $P < 0.05$ by multivariate analysis were considered to be associate factors of *L. siamensis*, *L. martiniquensis*, and *L. donovani* complex. The program used for statistical analysis was STATA, version SE14 (Stata Corporation, College Station, TX).

RESULTS

A total of 724 patients with HIV infection were enrolled. From these, 643 (88.8%) patients consented and answered the questionnaire. This study excluded 91 (12.6%) people because of discordance between DAT (positive for *Leishmania* antibodies) and PCR (negative for species identification) results because these individuals were clarified as having previous infection. One case of *Leishmania major* infection and four cases of *Leishmania lainsoni* infection were identified, but excluded from the analysis because of limited number of cases and not being the common species found in Thailand. Twenty-one patients positive for *Leishmania* spp. were excluded because of species unidentification. As a result, 526 (72.7%) patients were eligible for further analysis in this study.

Baseline characteristics of population. From 526 patients in this study, most were male (52.5%) and worked as agricultural workers (31.2%), and their age ranged from 40 to 50 years (46.0%). The mean age was 43.85 ± 0.37 years. Clinical characteristics were as follows: 93.9% did not have opportunistic infections, 44.1% had lived with HIV for 5–10 years, 78.1% had viral loads fewer than 50 copies/mL, 70.7% had CD4 count greater than 500 cells/mL, and 75.5% did not have comorbidities. Risk factor characteristics included a majority of patients who had pets (50.2%), did not live near forests (79.3%), did not use either intravenous drug (82.1%) or recreational drug (87.5%), and did not use insect repellent (77.8%), but used bed net (63.5%). The data is displayed in Supplemental Table 1.

Identification of *Leishmania* species. A total of 110 (20.9%) patients were infected with each of the three selected

Leishmania species. Most patients were PCR positive from either buffy coat or saliva. However, some patients had overlapping positive PCR results from buffy coat and saliva. From nested PCR and sequencing results, patients infected with *L. siamensis* were 63 (12.0%). Of these, 50 (9.5%) were PCR positive from buffy coat, 24 (4.6%) were PCR positive from saliva, and 11 (2.1%) were PCR positive from both buffy coat and saliva. Twenty-four (4.6%) patients were infected with *L. martiniquensis*, of which 17 (3.2%) were PCR positive from buffy coat, 10 (1.9%) were PCR positive from saliva, and three (0.6%) were PCR positive from both buffy coat and saliva. There were 23 (4.4%) patients who were infected with *L. donovani*

complex of which 21 (4.0%) were positive for buffy coat PCR and two (0.4%) were positive for saliva PCR. No crossing between positive *L. donovani* complex infection from buffy coat and saliva PCRs were found.

Factors associated with the infection of each *Leishmania* species. Univariate and multivariate analyses for risk factors of *L. siamensis* infection are shown in Table 1. *Leishmania siamensis* infection was associated with using intravenous drug (adjusted odds ratio [AOR] 2.01, 95% CI: 1.01–4.02) after having adjusted for confounding factors with gender, age, having pets, CD4 counts, intravenous drug, recreational drug, using insect repellent, using bed net, and having comorbidities.

TABLE 1
Univariate and multivariate analyses for risk factors of *L. siamensis* infection among HIV patients in Trang Province, Thailand ($n = 479$)

Characteristic	Outcomes		Univariate analysis			Multivariate analysis		
	Uninfected with <i>L. siamensis</i> , n (%)	Infected with <i>L. siamensis</i> , n (%)	Crude OR	95% CI	P -value	Adjusted OR	95% CI	P -value
Gender								
Male	221 (86.3)	35 (13.7)	1.00	–	–	1.00	–	–
Female	195 (87.4)	28 (12.6)	0.91	0.53–1.54	0.719	0.88	0.50–1.56	0.668
Age (years)								
< 40	142 (89.9)	16 (10.1)	1.00	–	–	1.00	–	–
40–50	190 (85.6)	32 (14.4)	1.49	0.79–2.83	0.217	1.35	0.69–2.65	0.381
> 50	84 (84.8)	15 (15.2)	1.58	0.75–3.37	0.232	1.44	0.66–3.14	0.365
Occupation								
Unemployed	40 (87.0)	6 (13.0)	1.00	–	–	–	–	–
Agricultural worker	132 (89.8)	15 (10.2)	0.76	0.28–2.08	0.590	–	–	–
Government officer	30 (81.1)	7 (18.9)	1.56	0.47–5.11	0.466	–	–	–
Owning business	82 (87.2)	12 (12.8)	0.98	0.34–2.79	0.963	–	–	–
Laborer	91 (85.1)	16 (14.9)	1.17	0.43–3.22	0.758	–	–	–
Others	41 (85.4)	7 (14.6)	1.14	0.35–3.68	0.829	–	–	–
Opportunistic infection								
Yes	23 (82.1)	5 (17.9)	1.47	0.54–4.03	0.450	–	–	–
No	393 (87.1)	58 (12.9)	1.00	–	–	–	–	–
Have pets								
Yes	210 (89.0)	26 (11.0)	0.69	0.40–1.18	0.175	0.69	0.40–1.21	0.198
No	206 (84.8)	37 (15.2)	1.00	–	–	1.00	–	–
Forest located near residence								
Yes	84 (87.5)	12 (12.5)	0.93	0.47–1.82	0.833	–	–	–
No	332 (86.7)	51 (13.3)	1.00	–	–	–	–	–
Years having HIV								
< 5	129 (89.0)	16 (11.0)	1.00	–	–	–	–	–
5–10	182 (85.9)	30 (14.2)	1.33	0.70–2.54	0.389	–	–	–
> 10	105 (86.1)	17 (13.9)	1.31	0.63–2.71	0.474	–	–	–
Viral load (copies/mL)								
< 50	319 (86.0)	52 (14.0)	1.00	–	–	–	–	–
50 and above	97 (89.8)	11 (10.2)	0.70	0.35–1.39	0.302	–	–	–
CD4 count (cells/mm ³)								
> 500	298 (86.9)	45 (13.1)	1.00	–	–	1.00	–	–
200–500	80 (87.0)	12 (13.0)	0.99	0.50–1.97	0.985	1.13	0.56–2.31	0.734
< 200	38 (86.4)	6 (13.6)	1.05	0.42–2.61	0.924	1.24	0.48–3.20	0.650
Intravenous drug use								
Yes	72 (82.7)	15 (17.2)	1.49	0.79–2.81	0.215	2.01	1.01–4.02	0.047*
No	344 (87.8)	48 (12.2)	1.00	–	–	1.00	–	–
Recreational drug use								
Yes	54 (90.0)	6 (10.0)	0.71	0.29–1.72	0.442	0.52	0.19–1.37	0.183
No	362 (86.4)	57 (13.6)	1.00	–	–	1.00	–	–
Using insect repellent								
Yes	85 (82.5)	18 (17.5)	1.00	–	–	1.00	–	–
No	331 (88.0)	45 (12.0)	0.64	0.35–1.17	0.145	0.56	0.30–1.05	0.070
Using bed net								
Yes	263 (86.2)	42 (13.8)	1.00	–	–	1.00	–	–
No	153 (87.9)	21 (12.1)	0.86	0.67–2.04	0.596	0.81	0.45–1.44	0.468
Having comorbidities								
Yes	90 (81.8)	20 (18.2)	1.68	0.94–3.01	0.078	1.66	0.89–3.08	0.110
No	326 (88.4)	43 (11.7)	1.00	–	–	1.00	–	–

L. siamensis = *Leishmania siamensis*; OR = odds ratio.

* Significant value at 95% CI.

Risk factors of *L. martiniquensis* infection included female gender (AOR 4.23, 95% CI: 1.52–11.75), using recreational drug (AOR 3.43, 95% CI: 1.00–11.74), and having comorbidities (AOR 4.94, 95% CI: 2.00–12.21). Adjusted confounding factors were gender, age, viral load, recreational drug use, and having comorbidities (Table 2).

Table 3 shows risk factors of *L. donovani* complex infection which included having opportunistic infection (AOR 4.22, 95% CI: 1.00–17.79), CD4 count 200–500 cells/mm³ (AOR 3.64, 95% CI: 1.14–6.86), and not using insect repellent (AOR 3.04, 95% CI: 1.08–8.58) following adjustment for confounders with age, occupation, opportunistic infection, having pets, years

having HIV, CD4 count, recreational drug use, and using insect repellent.

DISCUSSION

This study identified the risk factors of *L. siamensis*, *L. martiniquensis*, and *L. donovani* complex infections among HIV-infected patients who visited Trang Hospital, Trang, southern Thailand. This study included DNA detection from both blood and saliva samples. Although there was a study which had stated associated factors of *Leishmania* infection,⁷ there has never been a study which demonstrated associated factors of each individual species. Unlike other endemic areas

TABLE 2
Univariate and multivariate analyses for risk factors of *L. martiniquensis* infection among HIV patients in Trang Province, Thailand (*n* = 440)

Characteristic	Outcomes		Univariate analysis			Multivariate analysis		
	Uninfected with <i>L. martiniquensis</i> , <i>n</i> (%)	Infected with <i>L. martiniquensis</i> , <i>n</i> (%)	Crude OR	95% CI	<i>P</i> -value	Adjusted OR	95% CI	<i>P</i> -value
Gender								
Male	221 (96.9)	7 (3.1)	1.00	–	–	1.00	–	–
Female	195 (92.0)	17 (8.0)	2.75	1.12–6.78	0.028*	4.23	1.52–11.75	0.006*
Age (years)								
< 40	142 (95.9)	6 (4.1)	1.00	–	–	1.00	–	–
40–50	190 (95.5)	9 (4.5)	1.12	0.39–3.22	0.832	0.75	0.24–2.31	0.610
> 50	84 (90.3)	9 (9.7)	2.54	0.87–7.38	0.088	2.16	0.69–6.73	0.184
Occupation								
Unemployed	40 (97.7)	1 (2.4)	1.00	–	–	–	–	–
Agricultural worker	132 (89.8)	10 (7.0)	3.03	0.38–24.40	0.298	–	–	–
Government officer	30 (100.0)	0 (0.0)	–	–	–	–	–	–
Owning business	82 (94.3)	5 (5.8)	2.44	0.28–21.58	0.423	–	–	–
Laborer	91 (94.8)	5 (5.2)	2.20	0.25–19.42	0.479	–	–	–
Others	41 (93.2)	3 (6.8)	2.93	0.29–29.33	0.361	–	–	–
Opportunistic infection								
Yes	23 (95.8)	1 (4.2)	0.74	0.96–5.75	0.776	–	–	–
No	393 (94.5)	23 (5.5)	1.00	–	–	–	–	–
Have pets								
Yes	210 (94.2)	13 (5.8)	1.16	0.51–2.65	0.175	–	–	–
No	206 (94.9)	11 (5.1)	1.00	–	–	–	–	–
Forest located near residence								
Yes	84 (92.3)	7 (7.7)	1.63	0.65–4.05	0.295	–	–	–
No	332 (95.1)	17 (4.8)	1.00	–	–	–	–	–
Years having HIV								
< 5	129 (94.2)	8 (5.8)	1.00	–	–	–	–	–
5–10	182 (94.3)	11 (5.7)	0.97	0.38–2.49	0.957	–	–	–
> 10	105 (95.5)	5 (4.6)	0.77	0.24–2.42	0.652	–	–	–
Viral load (copies/mL)								
< 50	318 (93.5)	22 (6.5)	1.00	–	–	1.00	–	–
50 and above	98 (98.0)	2 (2.0)	0.29	0.07–1.28	0.102	0.25	0.05–1.14	0.073
CD4 count (cells/mm ³)								
> 500	298 (94.6)	17 (5.4)	1.00	–	–	–	–	–
200–500	80 (95.2)	4 (4.7)	0.88	0.29–2.68	0.985	–	–	–
< 200	38 (92.7)	3 (7.3)	1.38	0.39–4.94	0.924	–	–	–
Intravenous drug use								
Yes	72 (94.7)	4 (5.3)	0.96	0.32–2.88	0.936	–	–	–
No	344 (94.5)	20 (5.5)	1.00	–	–	–	–	–
Recreational drug use								
Yes	54 (91.5)	5 (8.5)	1.76	0.63–4.92	0.278	3.43	1.00–11.74	0.049*
No	362 (95.0)	19 (5.0)	1.00	–	–	1.00	–	–
Using insect repellent								
Yes	85 (94.4)	5 (5.6)	1.00	–	–	–	–	–
No	331 (94.6)	19 (5.3)	1.02	0.37–2.82	0.962	–	–	–
Using bed net								
Yes	263 (94.6)	15 (5.4)	1.00	–	–	–	–	–
No	153 (94.4)	9 (5.6)	1.03	0.44–2.41	0.943	–	–	–
Having comorbidities								
Yes	90 (87.4)	13 (13.6)	4.28	1.86–9.87	0.001*	4.94	2.00–12.21	0.001*
No	326 (96.7)	11 (3.3)	1.00	–	–	1.00	–	–

L. martiniquensis = *Leishmania martiniquensis*; OR = odds ratio.

*Significant value at 95% CI.

TABLE 3

Univariate and multivariate analysis for risk factors of *L. donovani* complex infection among HIV patients in Trang Province, Thailand ($n = 439$)

Characteristic	Outcomes		Univariate analysis			Multivariate analysis		
	Uninfected with <i>L. donovani</i> complex, n (%)	Infected with <i>L. donovani</i> complex, n (%)	Crude OR	95% CI	<i>P</i> -value	Adjusted OR	95% CI	<i>P</i> -value
Gender								
Male	221 (94.4)	13 (5.6)	1.00	–	–	–	–	–
Female	195 (95.1)	10 (4.9)	0.87	0.37–2.03	0.751	–	–	–
Age (years)								
< 40	142 (93.4)	10 (6.6)	1.00	–	–	1.00	–	–
40–50	190 (94.5)	11 (5.5)	0.82	0.34–1.99	0.664	0.55	0.19–1.54	0.545
> 50	84 (97.7)	2 (2.3)	0.34	0.07–1.58	0.168	0.35	0.07–1.75	0.348
Occupation								
Unemployed	40 (97.6)	1 (2.4)	1.00	–	–	1.00	–	–
Agricultural worker	132 (95.0)	7 (5.0)	2.12	0.25–17.76	0.488	1.38	0.14–13.48	0.783
Government officer	30 (100.0)	0 (0.0)	–	–	–	–	–	–
Owning business	82 (93.2)	6 (6.8)	2.93	0.34–25.14	0.328	2.79	0.29–27.19	0.376
Laborer	91 (91.9)	8 (8.1)	3.52	0.43–29.06	0.243	3.34	0.36–31.56	0.291
Others	41 (97.6)	1 (2.4)	0.98	0.06–16.14	0.986	0.89	0.05–16.81	0.935
Opportunistic infection								
Yes	23 (88.5)	3 (11.5)	2.56	0.71–9.26	0.151	4.22	1.00–17.79	0.050*
No	393 (95.2)	20 (4.8)	1.00	–	–	1.00	–	–
Have pets								
Yes	210 (94.2)	15 (6.7)	1.84	0.76–4.43	0.175	1.54	0.59–4.06	0.381
No	206 (96.3)	8 (3.7)	1.00	–	–	1.00	–	–
Forest located near residence								
Yes	84 (93.3)	6 (6.8)	1.39	0.53–3.65	0.497	–	–	–
No	332 (95.1)	17 (4.8)	1.00	–	–	–	–	–
Years having HIV								
< 5	129 (96.3)	5 (3.7)	1.00	–	–	1.00	–	–
5–10	182 (95.3)	9 (4.7)	1.28	0.42–3.90	0.669	1.79	0.53–6.09	0.349
> 10	105 (92.1)	9 (7.9)	2.21	0.79–6.80	0.166	2.58	0.75–8.86	0.132
Viral load (copies/mL)								
< 50	319 (94.7)	18 (5.3)	1.00	–	–	–	–	–
50 and above	97 (95.1)	5 (4.5)	0.91	0.33–2.52	0.862	–	–	–
CD4 count (cells/mm ³)								
> 500	298 (96.1)	12 (3.8)	1.00	–	–	1.00	–	–
200–500	80 (89.9)	9 (10.1)	2.79	1.14–6.86	0.025*	3.64	1.29–10.28	0.015*
< 200	38 (95.0)	2 (5.0)	1.38	0.28–6.06	0.732	1.97	0.37–10.50	0.425
Intravenous drug use								
Yes	72 (96.0)	3 (4.0)	0.72	0.21–2.48	0.598	–	–	–
No	344 (94.5)	20 (5.5)	1.00	–	–	–	–	–
Recreational drug use								
Yes	54 (98.2)	1 (1.8)	0.30	0.04–2.31	0.250	0.24	0.03–1.97	0.184
No	362 (94.3)	22 (5.7)	1.00	–	–	1.00	–	–
Using insect repellent								
Yes	85 (90.4)	9 (9.6)	1.00	–	–	1.00	–	–
No	331 (95.9)	14 (4.1)	0.40	0.17–0.95	0.039*	3.04	1.08–8.58	0.036*
Using bed net								
Yes	263 (94.9)	14 (5.1)	1.00	–	–	–	–	–
No	153 (94.4)	9 (5.6)	1.11	0.47–2.61	0.820	–	–	–
Having comorbidities								
Yes	90 (93.8)	6 (6.3)	1.28	0.49–3.34	0.616	–	–	–
No	326 (95.0)	17 (5.0)	1.00	–	–	–	–	–

L. donovani = *Leishmania donovani*; OR = odds ratio.

* Significant value at 95% CI.

where only single *Leishmania* species was dominantly found,^{18–21} Thailand, as an endemic area, harbored multiple species detection.^{6,22} Therefore, it is crucial to address varying associated factors contributed to infection of different *Leishmania* species.

This study reported an association between *L. siamensis* and intravenous drug use. There were sparse and unproven theories of such association, but not unprecedented.^{23–26} Previous studies also showed that *Leishmania* parasites could be transmitted via blood transfusion from healthy seropositive donors to recipients.^{27–30} A study from Madrid, Spain, found that the parasites can be found in clotted blood lodged in the

syringes shared by various users.²³ As *Leishmania*'s life cycle included the amastigote form, which inoculates in hosts' macrophage circulating in blood vessels by sharing intravenous syringes, the artificial life cycle of *Leishmania* is formed.³¹ Through this mode, the remaining amastigote-infected macrophages in the shared syringes were transmitted from infected individuals to others.³² Support of this theory was statistically reported as intravenous drug use accounted to be one of the risks of HIV-associated leishmaniasis in Spain.²⁶ Because the study in Seville, Spain, reported that the species infected among intravenous drug users was *L. infantum*,³³ this study hypothesized that there

were some degrees of genetic and biologic resemblance between *L. infantum* and *L. siamensis* which affected their greater transmission from infected individuals to others via the route of injected syringes more than other species.

Leishmania martiniquensis infection was more prevalent and associated with female gender in this study, which contrasted to other studies.^{34–36} However, some studies reported females being equally to or having higher prevalent of males with leishmaniasis.^{37,38} A study in Kabul, Afghanistan, identified female gender as an associated factor of *Leishmania tropica* infection.³⁹ Although an earlier experimental study showed that there was no significant difference between female and male genders toward sandfly attractiveness,⁴⁰ the exposure of females and males to sandfly was still inconclusive by epidemiological study because there were limited sources. A previous study showed that the associated factors of *Leishmania* infection in Thailand might include living in stilt houses, an architectural feature common in southern Thailand where floods and monsoons occurred frequently.⁷ Some females were more likely to be bound to house works and, thus, have more chances of exposure to sandflies.⁴¹

There were no reports of a direct association between recreational drug use and *Leishmania* infection. This study assumed that there was an indirect relationship between recreational drug use and *Leishmania* infection via increasing risk of HIV infection. Recreational drug use is highly associated with risks of HIV infection in various modes such as interaction between recreational drugs and anti-HIV agents⁴² and increasing risky sexual behaviors.^{43,44} As a result, leishmaniasis, as an opportunistic infection associated with HIV infection because of impaired immunity,^{45–48} was indirectly associated with recreational drug use. Moreover, this study indicated the association between existence of comorbid illnesses and *L. martiniquensis* in HIV-infected patients. Antiretroviral therapy used for HIV treatment was usually linked to increased risks of comorbidities such as diabetes mellitus and dyslipidemia.^{49–51} In various ways, such comorbidities interfere with immune responses and resulted in susceptibilities of opportunistic infections, including *Leishmania* infection.^{52–54}

In this study, *L. donovani* complex included *L. donovani* and *L. infantum*. These two species were grouped together as *L. donovani* complex because of their proximities that the differentiation of the ITS1 region was difficult.⁵⁵ *Leishmania donovani* complex infections could be considered an emerging infection in Thailand because there had been very limited report of the infection.⁵⁶ However, there was a report that cats were reservoir host of *L. donovani* complex in southern Thailand.⁵⁷ As a result, domestic infection was not impossible.

Associated factors of *L. donovani* complex infections included having opportunistic infections and CD4 between 200 and 500 cells/mm³ in this study. Patients with lower CD4 might have acquired opportunistic infections easier than those with high CD4.⁵⁸ Association between CD4 200–500 cells/mm³ and *L. donovani* complex infection could be explained with data from a previous study, stating that *L. donovani* complex was associated with VL relapse in HIV-infected patients with CD4 greater than 200 cells/mm³, which is clinically latent, because of virulence of the species.⁵⁹ Visceral leishmaniasis was also found to deteriorate immunological recovery of HIV-infected patients which increased risks of acquiring opportunistic infections.⁶⁰ Thus, this study suspected correlation between *L. donovani* complex infections which accompanied

other opportunistic infections even in latent HIV-infected patients because of species' virulence. This study did not find a relationship between *L. donovani* complex infections and CD4 level lower than 200 cells/mm³ because of the limited number of cases positive with *L. donovani* complex infections. The small number of cases could be subjected to the high efficacy of HIV treatment which, in turn, reduced the incidence of leishmaniasis in patients with a low CD4 level.⁶¹

It was found that patients with no insect repellent usage were more likely to be infected with *L. donovani* complex significantly. Previous studies reported a lower incidence of leishmaniasis in individuals who used insect repellent, insecticidal control, or permethrin-impregnated cloth because of reduced risks of sandfly bite in both human and animal studies.^{62–65}

This study contained some limitations. First, there were only three species which were studied. Other isolated species including *L. major* and *L. lainsoni* were omitted from this study because of their limited number of cases. Unidentified species were also excluded from the analysis. It is suggested that further studies on the prevalence of these species in other regions of Thailand should be processed. Second, although this study separately analyzed associated factors of infection by each of three species, some factors could not solely be explained by epidemiological studies. As stated in the statement, this study suspected that there were some degrees of genetic and biologic factors of both the parasite and host, which influenced infection of specific species more than others. Some of these factors, such as survival of the parasites in discarded syringes and clotted blood and virulence of each species in immunocompromised hosts, needed thorough investigations in further studies to establish theories of their infections relating to genetic and biologic factors.

This study identified the risk factors of infection of three *Leishmania* species prevalent in Thailand among HIV-infected patients. Several factors identified in this study should be dealt with thoroughly, such as elimination of illegal intravenous and recreational drugs use, effective control and monitoring of comorbid illnesses in HIV-infected individuals, and prevention of opportunistic infections in HIV-infected patients and vector controls by either insecticidal or repellent methods in endemic areas. It is recommended that further studies on leishmaniasis in other regions of Thailand due to different environmental factors should be processed.

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