Prevalence and risk factors associated with Leishmania infection in Trang Province, southern Thailand

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Abstract

Background Autochthonous cutaneous and visceral leishmaniasis caused by *Leishmania martiniquensis* and *Leishmania siamensis* have been considered an emerging infectious disease in Thailand. The disease burden is significantly underestimated, especially the prevalence of *Leishmania* infection among HIV-positive patients.

Methods A cross-sectional study was conducted to determine the prevalence and risk factors associated with *Leishmania* infection among patients with HIV/AIDS living in Trang Province, southern Thailand between 2015 and 2016. Antibodies against *Leishmania* infection were assayed using the direct agglutination test (DAT). DNA of *Leishmania* was detected by ITS1-PCR using the buffy coat. Species of *Leishmania* were also identified.

Results Of 724 participants, the prevalence of *Leishmania* infection was 25.1% (182/724) using either DAT and/or PCR assays. Seroprevalence of *Leishmania* infection was 18.5% (134/724), while *Leishmania* DNA detected by PCR method was 8.4% (61/724). Of these, 24.9% (180/724) were asymptomatic, whereas 0.3% (2/724) were symptomatic VL and VL/CL. At least five species were identified, that is, *L. siamensis*, *L. martiniquensis*, *L. donovani* complex, *L. lainsoni,* and *L. major*. Multivariate analysis showed that CD4+ levels <500 cells/μL, and living in stilt houses were independently associated with *Leishmania* infection. Those who were PCR positive for *Leishmania* DNA were significantly associated with detectable viral load. Whereas, non-injection drug use (NIDU) and CD4+ levels <500 cells/μL were potential risk factors of *Leishmania* seropositivity.

Conclusions A magnitude of prevalence of underreporting *Leishmania* infection among Thai patients with HIV was revealed in this study. Effective public health policy to prevent and control disease transmission is urgently needed.

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Protocol

Study design and population

Step 1.

A cross-sectional study of *Leishmania* infection was conducted between February 2015 and February 2016. Eligible participants were ≥18 years old, attending an HIV clinic, Trang Hospital, Trang

Province. They visited the clinic every six months for follow-up testing and to receive antiretroviral therapy (ART). They lived in ten districts of Trang Province, another nine provinces located in the south, and three provinces in other regions of Thailand. Clinical information of participants was collected from patients' medical records.

Ethics Statement

Step 2.

Written informed consents were obtained from all participants. All participants were aged more than 18 years old. All participants data analyzed were anonymized. The research protocol was approved by the Ethics Committee of the Royal Thai Army Medical Department and the Ethics Committee of Mahidol University, Thailand.

Blood collection

Step 3.

Eight milliliters of EDTA anti-coagulated blood samples were collected. The whole blood was centrifuged at 900 *g* for 10 minutes to separate plasma and buffy coat and then were kept at -20°until used.

Definition

Step 4.

- Seropositivity of *Leishmania* infection was defined as detection of antibodies in persons who have been exposed to the infection and being either symptomatic or asymptomatic.
- Asymptomatic *Leishmania* infection was defined as individuals who experienced no symptoms of VL but presented a positive test by DAT or PCR assays.
- Symptomatic VL was defined as individuals who had a history of fever lasting at least two weeks with splenomegaly. A single or combined clinical characteristics of the followings may be observed; hepatomegaly, weight loss, anemia, leucopenia, thrombocytopenia, and hypergammaglobulinemia. Detection of the parasites must be confirmed under microscopic examination or by PCR assay using any clinical samples i.e., bone marrow aspirates, lymph node, blood, and/or other biopsy samples.

Detection of Leishmania antibodies

Step 5.

Leishmania antibodies were assayed using commercial DAT kit (Biomedical Research, Amsterdam, Netherlands) according to the manufacturer's instruction. Positive plasma control was obtained from confirmed VL cases using the PCR method. For negative control, plasma from healthy individuals was used. The cut-off value of positive DAT titers was ≥1:100 following manufacturer recommendation.

Leishmania DNA detection

Step 6.

DNA was extracted from 200 µL of buffy coat sample using Gen UP™ gDNA Kit (Biotech, Germany). The nested-PCR was used to amplify the ITS1 region of the ribosomal DNA (rDNA) gene of *Leishmania*. In the primary PCR, primers LITSR and L5.8S were used to amplify 319-348 amplicons. The secondary primers, LITSR2 (CTG-GAT-CAT-TTT-CCG-ATG-ATT) and L5.8Sinner (GTT-ATG-TGA-GCC-GTT-ATC-C), newly designed primers, generated 230-280 amplicons depending on *Leishmania* species. PCR

reactions were performed using the MJ Mini™ thermal cycler (BioRad, USA) in volumes of 25 µL, containing 12.5 pmol of each primer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1x PCR buffer, 1 U of *Taq* DNA polymerase, and 4 µL of DNA template. DNA of *L. martiniquensis* promastigotes (MHOM/MQ/92/MAR1) was used as the positive control. The condition was started by predenaturation at 94°C for 3 minutes followed by 35 cycles: denaturation at 94°C for 1 minute, annealing temperature at 54°C for 30 seconds, and extension at 72°C for 30 seconds. Final extension was at 72°C for 5 minutes. PCR products were separated by electrophoresis in 1.5% agarose gel stained with SYBR® Safe (Invitrogen, USA). The results were visualized and documented by Molecular Imager® Gel Doc™ XR+ System with Imager Lab™ 3.0 (BioRad, USA).

Sequence analysis

Step 7.

Positive PCR products were sent to the U2Bio Co. Ltd., South Korea for sequencing. Chromatograms were validated using BioEdit version 7.0.1. The sequences were multiple-aligned with reference *Leishmania* strains retrieved from the GenBank. The phylogenetic tree was constructed by Neighbor Joining (NJ) method using program MEGA version 7.0. The reliability was tested by 1,000 bootstrap replications and the Tajima-Neiwas selected for the DNA substitution model of phylogenetic analysis.

Questionnaires

Step 8.

To determine the risk factors and outcome of *Leishmania* infection, standardized questionnaires were used. Enrolled subjects with HIV were face-to-face interviewed covering demographic data, socio-economic status, clinical symptoms, and risk associated behaviors.

Statistical analysis

Step 9.

The association between potential risk factors and *Leishmania* infection was assessed by univariate and multivariate logistic regression analysis. Odds ratios and 95% confidence intervals (CI) were calculated and *p* values <0.05 were considered statistically significant. All analyses were performed using STATA, version SE14 (Stata Corporation, College Station, Texas).