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Detection of *Leishmania martiniquensis* DNA in various clinical samples by quantitative PCR

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Abstract. Leishmaniasis is a neglected tropical parasitic disease affecting a large number of countries in the world. Early diagnosis of *Leishmania* infections is essential for therapeutic reasons, as it can decrease morbidity and mortality. *L. siamensis* and *L. martiniquensis* are novel *Leishmania* species recently described in Thailand and Myanmar. The disease is usually found in immunocompromised patients, especially those who have AIDS. Currently, the diagnosis of *Leishmania* infection in Thailand relies on microscopy, microbial culture, and polymerase chain reaction (PCR). In this study, we established a quantitative PCR (qPCR) method for detection of *L. martiniquensis* DNA in various types of clinical specimens, including whole blood, buffy coat, saliva, and urine of *L. martiniquensis* infected patients. The results of the qPCR assay were positive in all saliva samples. The assay is therefore effective to detect *L. martiniquensis* DNA even in noninvasive specimens, and it could be used for the diagnosis, follow up, and survey of *L. martiniquensis* infections.

INTRODUCTION

Leishmania spp. are protozoans that cause leishmaniasis affecting humans and animals. The parasite is an obligate intracellular organism that infects vertebrates through the bite of infected female sand flies (Feasey *et al.*, 2010). Two forms of the parasite have been described: promastigotes, a motile (flagellated) form; and amastigotes, an intracellular form. Promastigotes are found in female sand flies, while amastigotes are found in the cells of vertebrate hosts, especially macrophages. Several genera of sand fly are vectors for leishmaniasis, including *Phlebotomus*, *Lutzomyia*, and *Sergentomyia* (Singh *et al.*, 2006; Chusri *et al.*, 2014; Kanjanopas *et al.*, 2014). Three clinical forms of leishmaniasis have been described, including cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (ML), and visceral leishmaniasis (VL) (Desjeux, 1996). Clinical presentation of leishmaniasis depends on the *Leishmania* species and the host's immune system (Mondal *et al.*, 2010). VL is the most severe form of leishmaniasis and is found most often in patients infected with *L. infantum* (Maharom *et al.*, 2008), *L. donovani* (Kongkaew *et al.*, 2007), and *L. siamensis* (Sukmee *et al.*, 2008). *L. siamensis* and *L. martiniquensis* are novel species recently described in Thailand and Myanmar (Sukmee *et al.*, 2008; Suankratay *et al.*, 2010; Bualert *et al.*, 2012; Chusri *et al.*,

2012; Phumee *et al*., 2013; Noppakun *et al*., 2014; Osatakul *et al*., 2014; Phumee *et al*., 2014). The infection has been described mostly in immuno-compromised patients, such as AIDS patients, and in a patient with systemic steroid therapy (Noppakun *et al*., 2014). Currently, diagnosis of *Leishmania* infection in Thailand is based on microscopic examination, parasite culture, and polymerase chain reaction (PCR) based on several target genes (Phumee *et al*., 2013; Hitakarun *et al*., 2014). However, microscopic examination for *Leishmania* parasite still requires a lot of skills and experience of trained personnel; in addition, cultivation is time consuming and only available in university hospitals (Shyam & Rai, 2002; Singh *et al*., 2006; Srivastava *et al*., 2011). Moreover, serological tests such as rK39, immunochromatography, direct agglutination tests (DATs), and enzyme-linked immunosorbent assay (ELISA), are not available for diagnosis of *L. siamensis* and *L. martiniquensis* infection. Thus, some patients die before receiving proper treatment because of delayed or missed diagnoses (Bualert *et al*., 2012; Harizanov *et al*., 2013). Early diagnosis of leishmaniasis is essential to decrease morbidity and mortality. Conventional PCR has been developed to diagnose *Leishmania* infection and has shown high sensitivity (Phumee *et al*., 2013). The technique has been used to detect *L. siamensis* DNA in various clinical specimens (Lemrani *et al*., 2009; Leite *et al*., 2010), including saliva of asymptomatic leishmaniasis patients (Phumee *et al*., 2013). Although conventional PCR has advantages for the diagnosis of leishmaniasis, it is time consuming and unable to quantitate the DNA, which may be essential to determine the efficacy of the treatment (Mortarino *et al*., 2004). Quantitative real-time PCR (qPCR) is an alternative rapid and accurate diagnosis technique that is used to solve the drawbacks of conventional PCR (Klein, 2002; Wortmann *et al*., 2005; Paiva-Cavalcanti *et al*., 2010). In this study, we established a qPCR method for the detection of *L. martiniquensis* DNA in various types of clinical specimens obtained from patients with different clinical presentations.

MATERIALS AND METHODS

Sample collection

Clinical samples were collected from 10 leishmaniasis patients suffering from different clinical types of the disease. The study was approved by the Institutional Review Board of the Faculty of Medicine Chulalongkorn University, Bangkok, Thailand Ethic (COA No. 725/2013).

DNA extraction

DNA was extracted from the clinical specimens using a DNA extraction kit (STRATEC Molecular; Berlin, Germany). Briefly, 1 mL of saliva or 30 mL of urine sample was spun at 5,000 ×*g* for 5 min, and the pellets were then used for further DNA extraction steps. Fifty microliters of blood or buffy coat were extracted using a blood kit (STRATEC Molecular; Berlin, Germany), following the manufacturer's instructions. All samples were eluted with 80 µL elution buffer and the concentration and purity of the extracted DNA were determined using a Nanodrop 2000c instrument (Thermo Scientific; Singapore). All DNA samples were kept at -80°C until used.

Primer design

Primers were designed for specific annealing to the ribosomal RNA gene of the internal transcribed spacer 1 (ITS1) region of *L. martiniquensis* (accession no KM677931). Primers used in this study were LeishF (forward), 5'-CGATATGCCTTTCCCACACAC -3'; and LeishR (reverse), 5'-CTGTATACGCG CGGCATTTG-3', with an expected PCR product size of 128 bp.

PCR conditions

The PCR mix contained 0.5 µL of each primer at 0.5 µM, 1.25 µL of 25 mM $MgCl_2$, 2.5 µL of 2 mM dNTP, 2.5 µL of 10X PCR buffer, 1 U of *Taq* DNA polymerase, and 50–100 ng of DNA template. The final volume was adjusted to 25 µL with ddH_2O. PCR amplification was performed in a PCR Mastercycler® pro (Eppendorf; Germany) using the following conditions: initial denaturation at 95ºC for 5 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec,

and extension at 72°C for 30 sec, with a final extension at 72°C for 10 min. The PCR amplicons were analyzed by electrophoresis, using a 1.5% agarose gel 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). Cultured *L. martiniquensis* promastigotes DNA were used as a positive control and ddH_2O and DNA extracted from ethylenediaminetetraacetic acid-blood of a healthy person was used as negative control.

Cloning and sequencing

Amplified PCR amplicons were ligated into pGEM-T Easy Vector (Promega; USA). The ligation reactions were transformed into DH5α competent cells and screened using the blue-white colony selection system. The suspected positive colonies were cultured and their DNA was extracted using Invisorb® Spin Plasmid Mini kit (STRATEC Molecular GmbH; Germany), following the manufacturer's instructions. Sequencing was performed by a commercial service in 1st BASE DNA sequencing system (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 GenBank using BLAST search (available at http://www.ncbi.nlm.gov/BLAST).

qPCR

All qPCRs were performed using the BIO-RAD, CFX96™ Optic Module. The total volume of 20 µL/reaction consisted of 10 µL Master Mix SYBR green (Thermo Scientific, Singapore), 0.2 µL of LeishF and LeishR primers, 0.25 µL of UDG (Thermo scientific, Singapore), 7.35 µL of ddH_2O, and 2 µL of DNA template. The amplification conditions comprised a two-step initial denaturation at 50°C for 2 min and 95°C for 10 min, respectively, followed by 40 cycles of 30 sec at 95°C, 30 sec at 61°C, 30 sec at 72°C, and one final melting cycle consisting of 5 sec at 65°C and 50 sec at 95°C. The changing of fluorescence was used to measure the concentration of plasmid, and the equation below was used to calculate the copy number of the plasmid. A standard curve of *L. martiniquensis* was set up and the copy number of the samples was determined (Whelan *et al.*, 2003).

$$\text{DNA (copy)} = \frac{6.02 \times 10^{23}\ \text{(copy/mol)} \times \text{DNA amount (g)}}{\text{DNA length (bp)} \times 660\ \text{(g/mol/dp)}}$$

RESULTS

DNA of *L. martiniquensis* extracted from specimens of patient No. 9 was used for PCR and sequencing. The ITS1 region was amplified from the 4 types of clinical specimen: blood (B), buffy coat (BC), urine (U), and saliva (S). The product size of the PCR amplicons was 128 bp (Figure 1). Amplified PCR products were ligated into a pGEM-T Easy cloning vector as described previously. Sequences of the ITS1 region were compared to the reported sequences of *L. martiniquensis* (accession no KM677931) and were found to be 100% identical (Figure 2). Plasmid DNA containing the ITS1 region of *L. martiniquensis* was used to set up a standard curve with concentrations ranging from 5×10^1 to 5×10^6 copies/µL. The lowest detection limit of the qPCR method was 50 parasites/µL. The curve was linear in the range tested ($R^2 = 0.998$) (Figure 3). Figure 3 shows a representative result of three independent assays from Table 1, as a plot of quantification cycle (Cq) versus the logarithm starting quantity. Samples were collected from 10 leishmaniasis patients, eight of which were co-infected with HIV. Most of the patients were affected with CL and some were affected with both CL and VL (patients 1, 2, 9, 10). Patient 3 was affected with disseminated CL after systemic steroid therapy. Asymptomatic leishmaniasis patients in immune-competent (patient 4) and HIV-infected (patient 8) patients were also included in this study (Table 2). Details of patients 1–6 were described previously by Phumee *et al.* (2013) and Noppakun *et al.* (2014). Patient 8 is the wife of patient 1; she was also HIV-positive

but her CD+4 T-cell level was 617 cells/mm^3. The results of all specimens were positive, except for the urine of patient 10.

DISCUSSION

Leishmaniasis caused by novel *Leishmania* species, *L. siamensis* and *L. martiniquensis* have recently been described in Thailand and Myanmar (Phumee *et al.*, 2013; Noppakun *et al.*, 2014). Clinical presentations of *L. siamensis* infection include VL (Sukmee *et al.*, 2008; Suankratay *et al.*, 2010; Osatakul *et al.*, 2014), CL (Kattipathanapong *et al.*, 2012; Noppakun *et al.*, 2014; Phumee *et al.*, 2014), and a combination of VL and CL (Bualert *et al.*, 2012; Chusri *et al.*, 2012; Phumee *et al.*, 2013). The disease is usually found in immune-compromised patients, such as AIDS patients (Kongkaew *et al.*, 2007; Suankratay *et al.*, 2010) and those receiving systemic steroid therapy (Noppakun *et al.*, 2014). In Thailand, the disease is usually described in patients living in the southern region of the country (Thisyakorn *et al.*, 1999;

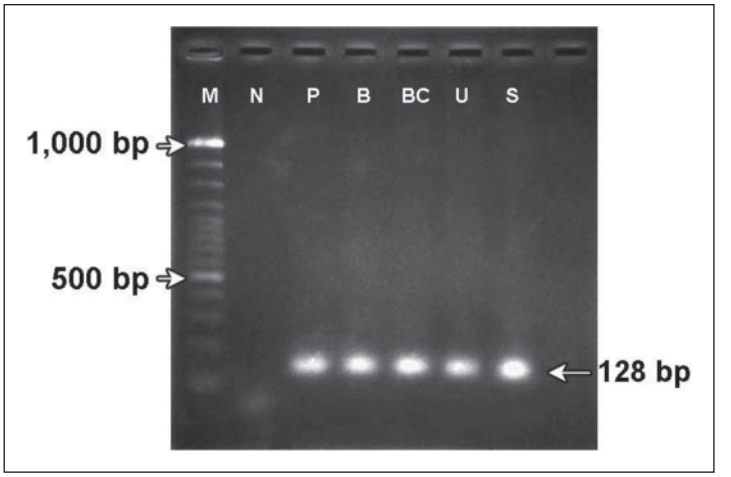

Figure 1. PCR amplifications of the ITS1 gene region of patient case No. 9. Lanes B, BC, U, and S: samples from culture, blood, buffy coat, urine, and saliva, respectively; Lane P: positive control (promastigotes of *L. martiniquensis)*; lane N: negative control (no DNA template); lane M: marker (100-bp size). All products were analyzed using a 1.5% agarose gel stained with ethidium bromide.

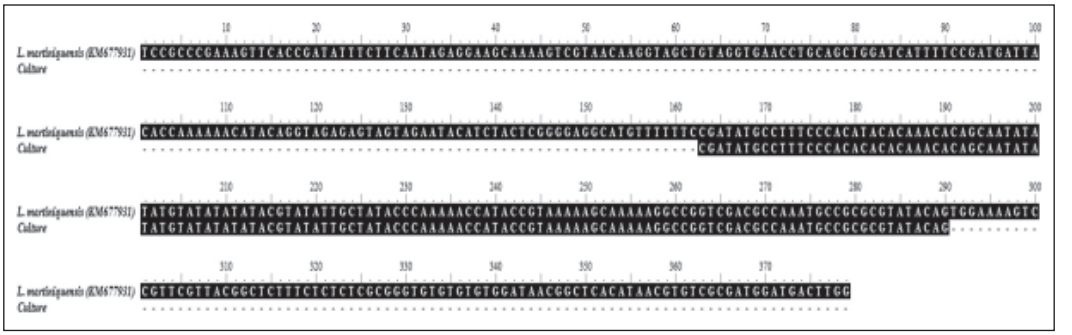

Figure 2. Comparison between the sequence amplified from cultures of *L. martiniquensis* and the sequence of the ITS1 region from *L. martiniquensis* previously reported in GenBank (accession no. KM677931).

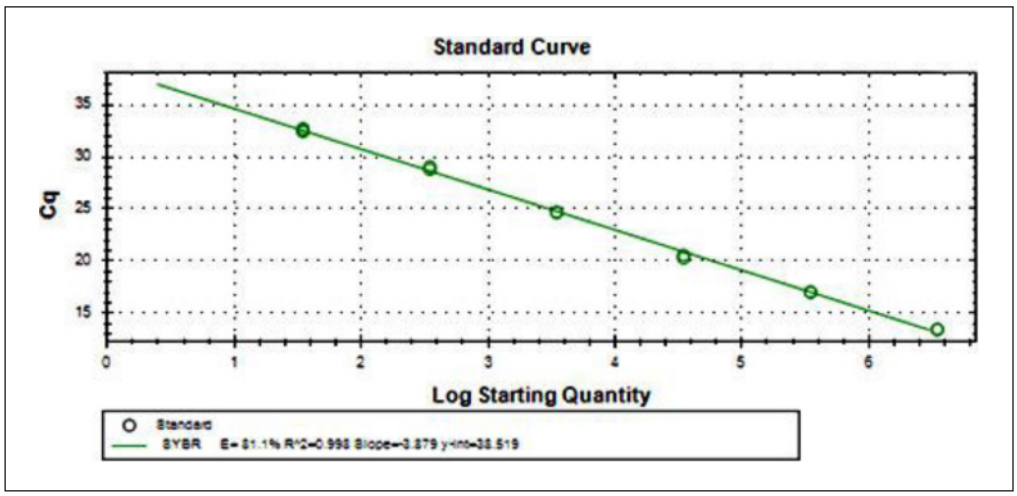

Figure 3. Standard curve of qPCR, constructed with serial duplicate 10-fold dilutions of plasmid DNA of *L. martiniquensis*, and superseded of three independent standards (mean and SD of triplicate independent experiments; see Table 1). The curve was obtained by plotting Cq values (quantification cycle) against the logarithmic starting quantity of parasite DNA (5×10^1 to 5×10^6 copies/µL).

Table 1. Mean and standard deviation (SD) of Cq series dilution values from plasmid DNA

Standard	Cq value (copies/µL)					
	5×10^6	5×10^5	5×10^4	5×10^3	5×10^2	5×10^1
1	12.81	16.64	20.29	24.77	28.18	32.40
2	13.01	16.26	19.75	24.18	28.09	32.59
3	13.44	17.02	20.43	24.68	28.94	32.60
Mean ± SD	13.09 ± 0.32	16.64 ± 0.38	20.15 ± 0.36	24.54 ± 0.32	28.40 ± 0.46	32.53 ± 0.11

Table 2. Detection results of the qPCR

Patient	Sex	Age (Y)	Nationality	Clinical presentation/ host conditions	Mean ± SD of Cq value			
					Blood	Buffy coat	Saliva	Urine
1	M	46	Thai	CL and VL/HIV infection and prednisolone one therapy	30.12±0.58	28.26±0.09	28.8±0.06	N/A
2	M	30	Thai	CL and VL/HIV infection	35.04±0.34	32.79±0.23	31.74±0.08	N/A
3	M	60	Burma	CL/prednisolone therapy	33.01±0.32	31.06±0.19	33.09±0.04	35.09±0.59
4	F	22	Burma	Asymptomatic/Normal	33.04±0.15	31.69±0.11	33.25±0.08	35.23±0.18
5	M	45	Thai	CL/HIV infection	38.42±0.18	36.99±0.08	30.67±0.54	N/A
6	M	34	Burma	CL/HIV infection	30.48±0.21	28.50±0.07	28.27±0.09	33.74±0.45
7	M	52	Thai	VL	28.56±0.29	27.02±0.15	20.3±0.15	N/A
8	F	28	Thai	Asymptomatic/HIV	29.97±0.16	28.73±0.13	30.54±0.18	N/A
9	M	39	Thai	CL and VL/HIV infection	26.71±0.23	25.6±0.15	32.12±0.11	34.77±0.21
10	M	52	Thai	CL and VL /HIV infection	34.14±0.64	32.06±0.04	31.53±0.18	Neg

Note : N/A = not available, Neg = negative by qPCR

Chappuis *et al*., 2007; Sukmee *et al*., 2008). Diagnosis of *Leishmania* infection in local hospitals is based on microscopic examination. Cultivation of the parasites and PCR are only available in some university hospitals. Microscopic examination requires experienced personnel to discriminate between *Leishmania* and other pathogens such as *Histoplasma* and *Penicillium*. Serological tests have been developed to replace traditional method for diagnosing of VL in the field (Singh *et al*., 2006; Mondal *et al*., 2010). For example, the rK39 strip test is the best choices for efficient test (Chappuis *et al*., 2006; Canavate *et al*., 2011; Maia *et al*., 2012), because they are simple to use, cheap, rapid, and high sensitivity and specificity (Maia *et al*., 2012). rK39 strip test are limited because of potential cross-reactivity with other infections such as *Trypanosome* spp; moreover, it cannot diagnosis of relapsed cases because the antibody is still presented for a long time (Chappuis *et al*., 2007). However, the commercial available rK39 was not routinly used in Thailand because it has never been evaluated for sensitivity and specificity for *Leishmania* infections. PCR methods have been developed for diagnosis of *Leishmania* infection (Leelayoova *et al*., 2013; Phumee *et al*., 2013; Hitakarun *et al*., 2014; Pereira *et al*., 2014) and have shown high sensitivity (Mondal *et al*., 2010). Comparation to qPCR, PCR cannot detect DNA copy number (Paiva-Cavalcanti *et al*., 2010). Also, conventional PCR procedure is time-consuming and less practical in research since it still requires additional steps for staining and visualization.

In this study, we designed new primer set for qPCR to specifically amplify the ITS1 region of *L. martiniquensis*. The primer set was used in PCR and was shown to amplify the desired region of the parasite's DNA, as confirmed by sequencing (Figure 1 and 2). The standard curve for qPCR was determined using various concentrations of *L. martiniquensis* DNA, ranging from 5×10^1 to 5×10^6 copy/µL, and the sensitivity of detection was 50 copies/µL or 50 parasites/µL.

qPCR and PCR techniques have been applied for the diagnosis of invasive and noninvasive forms of VL. Both qPCR and PCR (Gomes *et al*., 2008) can solve the false-negative (small amount of parasite) that occurs from traditional and serological tests (Paiva-Cavalcanti *et al*., 2010). In this study, our qPCR method detected *L. martiniquensis* DNA in all blood, buffy coat, and saliva samples. A new qPCR method was recently used to detect the parasite genome in the blood of patients suffering from VL (Pereira *et al*., 2014). Saliva is a good source for *L. martiniquensis* DNA, and a report from Phumee *et al.* demonstrated that *L. martiniquensis* DNA was detected in saliva of leishmaniasis patients, including an asymptomatic patient (Phumee *et al*., 2013). Our study confirms that qPCR also can detect *L. martiniquensis* DNA in all saliva samples.

Furthermore, qPCR can detect *L. martiniquensis* DNA in some urine specimens from patients. We got a positive result for patient 3, 4, 6, 9 and a negative result for patient 10. The negative result can be explained in 2 ways: i) *L. martiniquensis* DNA is very low in this case, or ii) the quality of the urine sample was low (Vu *et al*., 1999). Noninvasive samples, such as saliva (Phumee *et al*., 2013) and urine (Fisa *et al*., 2008), are a good source for detection of *Leishmania* spp. DNA. Many articles describe the benefits of qPCR, such as high sensitivity (Paiva-Cavalcanti *et al*., 2010; Weirather *et al*., 2011; Ramos *et al*., 2012), and reproducibility. qPCR may be used instead of PCR in diagnostic routines because qPCR is very similar to PCR in its speed, quantitative, and amplification ability, especially small amount of parasites (Cota *et al*., 2011), but qPCR remain high cost and require specialist Moreover, patients with HIV can be followed up and the prognosis of disease can be determined (Bossolasco *et al*., 2003; Antinori *et al*., 2009). The clinical management of relapsed patients can be based on the level of *Leishmania* DNA (Bossolasco *et al*., 2003).

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REFERENCES

Antinori, S., Calattini, S., Piolini, R., Longhi, E., Bestetti, G. & Cascio, A. *et al.* (2009). Is real-time polymerase chain reaction (PCR) more useful than a conventional PCR for the clinical management of leishmaniasis?. *American Journal of Tropical Medicine and Hygiene* **81**(1): 46-51.

Bossolasco, S., Gaiera, G., Olchini, D., Gulletta, M., Martello, L. & Bestetti, A. *et al.* (2003). Real-time PCR assay for clinical management of human immunodeficiency virus-infected patients with visceral leishmaniasis. *Journal of Clinical Microbiology* **41**(11): 5080-5084.

Bualert, L., Charungkiattikul, W., Thongsuksai, P., Mungthin, M., Siripattanapipong, S. & Khositnithikul, R. *et al.* (2012). Autochthonous disseminated dermal and visceral leishmaniasis in an AIDS patient, southern Thailand, caused by *Leishmania siamensis*. *American Journal of Tropical Medicine and Hygiene* **86**(5): 821-824.

Cañavate, C., Herrero, M., Nieto, J., Cruz, I., Chicharro, C. & Aparicio, P. *et al.* (2011). Evaluation of two rK39 dipstick tests, direct agglutination test, and indirect fluorescent antibody test for diagnosis of visceral leishmaniasis in a new epidemic site in Highland Ethiopia. *American Journal of Tropical Medicin and Hygiene* **84**(1): 102-106.

Chappuis, F., Rijal, S., Soto, A., Menten, J. & Boelaert, M. (2006). A meta-analysis of the diagnostic performance of the direct agglutination test and rK39 dipstick for visceral leishmaniasis. *British Medical Journal* **333**(7571): 723.

Chappuis, F., Sundar, S., Hailu, A., Ghalib, H., Rijal, S. & Peeling, R.W. *et al.* (2007). Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nature Reviews Microbiology* **5**(11): 873-882.

Chusri, S., Hortiwakul, T., Silpapojakul, K. & Siriyasatien, P. (2012). Consecutive cutaneous and visceral leishmaniasis manifestations involving a novel *Leishmania* species in two HIV patients in Thailand. *American Journal of Tropical Medicine and Hygiene* **87**(1): 76-80.

Chusri, S., Thammapalo, S., Chusri, S., Thammapalo, S., Silpapojakul, K. & Siriyasatien, P. (2014). Animal reservoirs and potential vectors of *Leishmania siamensis* in southern Thailand. *Southeast Asian Journal of Tropical Medicine And Public Health* **45**(1): 13-19.

Cota, G.F., de Sousa, M.R. & Rabello, A. (2011). Predictors of visceral leishmaniasis relapse in HIV-infected patients: a systematic review. *PLoS Neglected Tropical Diseases* **5**(6): e1153.

Cota, G.F., de Sousa, M.R., Demarqui, F.N. & Rabello, A. (2012). The diagnostic accuracy of serologic and molecular methods for detecting visceral leishmaniasis in HIV infected patients: meta-analysis. *PLoS Neglected Tropical Diseases* **6**(5): 1-11.

Desjeux, P. (1996). Leishmaniasis and *Leishmania*/HIV co-infection. *Clinics in Dermatology* **14**: 417-423.

Feasey, N., Wansbrough-Jones, M., Mabey, D.C. & Solomon, A.W. (2010). Neglected tropical diseases. *British Medical Bulletin* **93**: 179-200.

Fisa, R., Riera, C., López-Chejade, P., Molina, I., Gállego, M. & Falcó, V. *et al.* (2008). *Leishmania infantum* DNA detection in urine from patients with visceral leishmaniasis and after treatment control. *American Journal of Tropical Medicine and Hygiene* **78**(5): 741-744.

Gomes, Y.M., Paiva Cavalcanti, M., Lira, R.A., Abath, F.G. & Alves, L.C. (2008). Diagnosis of canine visceral leishmaniasis: biotechnological advances. *The Veterinary Journal* **175**(1): 45-52.

Harizanov, R.N., Kaftandjiev, I.T., Jordanova, D.P., Marinova, I.B. & Tsvetkova, N.D. (2013). Clinical features, diagnostic tools, and treatment regimens for visceral leishmaniasis in Bulgaria. *Pathogens and Global Health* **107**(5): 260-266.

Hitakarun, A., Tan-Ariya, P., Siripattanapipong, S., Mungthin, M., Piyaraj, P. & Naaglor, T. *et al.* (2014). Comparison of PCR methods for detection of *Leishmania siamensis* infection. *Parasites & Vectors* **7**(1): 458.

Kanjanopas, K., Siripattanapipong, S., Ninsaeng, U., Hitakarun, A., Jitkaew, S., Kaewtaphaya, P. *et al.* (2013). *Sergentomyia (Neophlebotomus) gemmea*, a potential vector of *Leishmania siamensis* in southern Thailand. *BMC Infectious Diseases* **13**: 333.

Kattipathanapong, P., Akaraphanth, R., Krudsood, S., Riganti, M. & Viriyavejakul, P. (2012). The first reported case of autochthonous cutaneous leishmaniasis in Thailand. *Southeast Asian Journal of Topical Medicine and Public Health* **43**(1): 17-20.

Klein, D. (2002). Quantification using real-time PCR technology: applications and limitations. *Trends in Molecular Medicine* **8**(6): 257-260.

Kongkaew, W., Siriarayaporn, P., Leelayoova, S., Supparatpinyo, K., Areechokchai, D. & Duang-ngern, P. *et al.* (2007). Autochthonous visceral leishmaniasis: a report of a second case in Thailand. *Southeast Asian Journal of Topical Medicine and Public Health* **38**: 8-12.

Leelayoova, S., Siripattanapipong, S., Hitakarun, A., Kato, H., Tan-ariya, P. & Siriyasatien, P. *et al.* (2013). Multilocus characterization and phylogenetic analysis of *Leishmania siamensis* isolated from autochthonous visceral leishmaniasis cases, southern Thailand. *BMC Microbiology* **13**(1): 60.

Leite, R.S., Ferreira Sde, A., Ituassu, L.T., de Melo, M.N. & de Andrade, A.S. (2010). PCR diagnosis of visceral leishmaniasis in asymptomatic dogs using conjunctival swab samples. *Veterinary Parasitology* **170**(3-4): 201-206.

Lemrani, M., Hamdi, S., Laamrani, A. & Hassar, M. (2009). PCR detection of *Leishmania* in skin biopsies. *Journal of Infection in Developing Countries* **3**(2): 115-122.

Maharom, P., Siripattanapipong, S., Mungthin, M., Naaglor, T., Sukkawee, R. & Pudkorn, R. *et al.* (2008). Casereport: visceral leishmaniasis caused by *Leishmania infantum* in Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health* **39**(6): 988-990.

Maia, Z., Lírio, M., Mistro, S., Mendes, C.M., Mehta, S.R. & Badaro, R. (2012). Comparative study of rK39 *Leishmania* antigen for serodiagnosis of visceral leishmaniasis: systematic review with meta-analysis. *PLoS Neglected Tropical Diseases* **6**(1): e1484.

Mondal, S., Bhattacharya, P. & Ali, N. (2010). Current diagnosis and treatment of visceral leishmaniasis. *Expert Review of Anti-infective Therapy* **8**(8): 919-944.

Mortarino, M., Franceschi, A., Mancianti, F., Bazzocchi, C., Genchi, C. & Bandi, C. (2004). Quantitative PCR in the diagnosis of *Leishmania*. *Parassitologia* **46**(1-2): 163-167.

Noppakun, N., Kraivichian, K. & Siriyasatien, P. (2014). Disseminated Dermal Leishmaniasis caused by *Leishmania siamensis* in a Systemic Steroid Therapy Patient. *American Journal of Tropical Medicine and Hygiene* **91**(5): 869-870.

Osatakul, S., Mungthin, M., Siripattanapipong, S., Hitakarun, A., Kositnitikul, R., Naaglor, T. & Leelayoova, S. (2014). Recurrences of visceral leishmaniasis caused by *Leishmania siamensis* after treatment with amphotericin B in a seronegative child. *American Journal of Tropical Medicine and Hygiene* **90**(1): 40-42.

Paiva-Cavalcanti, M., Regis-da-Silva, C.G. & Gomes, Y.M. (2010). Comparison of real-time PCR and conventional PCR for detection of *Leishmania (Leishmania) infantum* infection: a mini-review. *Journal of Venomous Animals and Toxins including Tropical Diseases* **16**(4): 537-542.

Pereira, M.R., Rocha-Silva, F., Graciele-Melo, C., Lafuente, C.R., Magalhães, T. & Caligiorne, R.B. (2014). Comparison between conventional and real-time PCR assays for diagnosis of visceral leishmaniasis. *BioMed Research International* **2014**: 639310.

Phumee, A., Kraivichian, K., Chusri, S., Noppakun, N., Vibhagool, A. & Sanprasert, V. *et al.* (2013). Detection of *Leishmania siamensis* DNA in saliva by polymerase chain reaction. *American Journal of Tropical Medicine and Hygiene* **89**(5): 899-905.

Phumee, A., Chusri, S., Kraivichian, K., Wititsuwannakul, J., Hortiwakul, T. & Thavara, U. *et al.* (2014). Multiple Cutaneous Nodules in an HIV-Infected Patient. *PLoS Neglected Tropical Diseases* **8**(12): e3291.

Ramos, R.A., Ramos, C.A., Jusi, M.M., de Araújo, F.R., Machado, R.Z., Faustino, M.A. & Alves, L.C. (2012). Polymerase chain reaction and real-time PCR for diagnosing of *Leishmania infantum* chagasi in dogs. *Revista Brasileira de Parasitologia Veterinaria* **21**(3): 192-195.

Shyam, S. & Rai, M. (2002). Laboratory diagnosis of visceral leishmaniasis. *Clinical and Diagnostic Laboratory Immunology* **9**(5): 951-958.

Singh, R.K., Pandey, H.P. & Sundar, S. (2006). Visceral leishmaniasis (kala-azar): challenges ahead. *Indian Journal of Medical Research* **123**: 331-344.

Srivastava, P., Dayama, A., Mehrotra, S. & Sundar, S. (2011). Diagnosis of visceral leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **105**: 1-6.

Suankratay, C., Suwanpimolkul, G., Wilde, H. & Siriyasatien, P. (2010). Autochthonous visceral leishmaniasis in a human immunodeficiency virus (HIV)-infected patient: the first in thailand and review of the literature. *American Journal of Tropical Medicine and Hygiene* **82**(1): 4-8.

Sukmee, T., Siripattanapipong, S., Mungthin, M., Worapong, J., Rangsin, R. & Samung, Y. *et al.* (2008). A suspected new species of *Leishmania*, the causative agent of visceral leishmaniasis in a Thai patient. *International Journal for Parasitology* **38**(6): 617-622.

Thisyakorn, U., Jongwutiwes, S., Vanichsetakul, P. & Lertsapcharoen, P. (1999). Visceral leishmaniasis: the first indigenous case report in Thailand. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **93**(1): 23-24.

Weirather, J.L., Jeronimo, S.M., Gautam, S., Sundar, S., Kang, M. & Kurtz, M.A. *et al.* (2011). Serial quantitative PCR assay for detection, species discrimination, and quantification of *Leishmania* spp. in human samples. *Journal of Clinical Microbiology* **49**(11): 3892-3904.

Whelan, J.A., Russel, N.B. & Whelan, M.A. (2003). A method for the absolute quantification of cDNA using real time PCR. *Journal of Immunological Methods* **278**: 261-269.

Wortmann, G., Hochberg, L., Houng, H.H., Sweeney, C., Zapor, M. & Aronson, N. *et al.* (2005). Rapid identification of *Leishmania* complexes by a real-time PCR assay. *American Journal of Tropical Medicine and Hygiene* **73**(6): 999-1004.

Vu, N.T., Chaturvedi, A.K. & Canfield, D.V. (1999). Genotyping for DQA1 and PM loci in urine using PCR-based amplification: effects of sample volume, storage temperature, preservatives, and aging on DNA extraction and typing. *Forensic Science International* **102**(1): 23-34.