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

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Received 12 December 2014; received in revised form 15 January 2015; accepted 10 February 2015

**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 quantify 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 xg 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’-CGATATGCCCTTTCCCACAC-3’; and LeishR (reverse), 5’-CTGTATAGCCGCGGCATTGGTG-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 MgCl2, 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 ddH2O. PCR amplification was performed in a PCR Mastercycler® pro (Eppendorf; Germany) using the following conditions: initial denaturation at 95°C 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 ddH₂O 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 InvisorbSpin 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 ddH₂O, 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. 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).

DNA of *L. martiniquensis* was used to set up a standard curve with concentrations ranging from 5 × 10¹ to 5 × 10⁶ 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/mm³. 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;
Table 1. Mean and standard deviation (SD) of Cq series dilution values from plasmid DNA

<table>
<thead>
<tr>
<th>Standard</th>
<th>Cq value (copies/µL)</th>
<th>5 x 10^6</th>
<th>5 x 10^5</th>
<th>5 x 10^4</th>
<th>5 x 10^3</th>
<th>5 x 10^2</th>
<th>5 x 10^1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>12.81</td>
<td>16.64</td>
<td>20.29</td>
<td>24.77</td>
<td>28.18</td>
<td>32.40</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>13.01</td>
<td>16.26</td>
<td>19.75</td>
<td>24.18</td>
<td>28.09</td>
<td>32.59</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>13.44</td>
<td>17.02</td>
<td>20.43</td>
<td>24.68</td>
<td>28.94</td>
<td>32.60</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (Y)</th>
<th>Nationality</th>
<th>Clinical presentation/ host conditions</th>
<th>Mean ± SD of Cq value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Blood</td>
<td>Buffy coat</td>
</tr>
<tr>
<td>1</td>
<td>M</td>
<td>46</td>
<td>Thai</td>
<td>CL and VL/HIV infection and prednisolone one therapy</td>
<td>30.12±0.58</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>30</td>
<td>Thai</td>
<td>CL and VL/HIV infection</td>
<td>35.04±0.34</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>60</td>
<td>Burma</td>
<td>CL/prednisolone therapy</td>
<td>33.01±0.32</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>22</td>
<td>Burma</td>
<td>Asymptomatic/Normal</td>
<td>33.04±0.15</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>45</td>
<td>Thai</td>
<td>CL/HIV infection</td>
<td>38.42±0.18</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>34</td>
<td>Burma</td>
<td>CL/HIV infection</td>
<td>30.48±0.21</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>52</td>
<td>Thai</td>
<td>VL</td>
<td>28.56±0.29</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>28</td>
<td>Thai</td>
<td>Asymptomatic/HIV</td>
<td>29.97±0.16</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>39</td>
<td>Thai</td>
<td>CL and VL/HIV infection</td>
<td>26.71±0.23</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>52</td>
<td>Thai</td>
<td>CL and VL/HIV infection</td>
<td>34.14±0.64</td>
</tr>
</tbody>
</table>

Note: N/A = not available, Neg = negative by qPCR
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 $5 \times 10^4$ to $5 \times 10^6$ 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).
Acknowledgments. This study was supported by the Thailand Research Fund (TRF Senior Research Scholar: RTA5480006) and National Science and Technology Development Agency (Thailand), P-12-01458 and Research Chair Grant.

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