Loop-mediated isothermal amplification for rapid molecular detection of *Enterocytozoon bieneusi* in faecal specimens

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A loop-mediated isothermal amplification (LAMP) assay was developed to detect *Enterocytozoon bieneusi* DNA for the first time from human faecal specimens. Four primers specific for *Enterocytozoon bieneusi* were designed corresponding to small subunit rRNA gene sequences and tested on 100 human faecal specimens. Thirty-nine of the faecal specimens (39%) were confirmed positive for *Enterocytozoon bieneusi* by LAMP compared with 33% by PCR and 32% by light microscopy. LAMP yielded 94% sensitivity and 88% specificity compared with microscopy (sensitivity 48%, specificity 76%). No significant differences in positive detection of *Enterocytozoon bieneusi* were found among the three methods (P=0.05). However, LAMP has shown a substantial agreement with PCR (κ=0.78) and fair agreement was demonstrated between microscopy and PCR (κ=0.25). In conclusion, the LAMP assay proved to be useful as a simplified, rapid, sensitive and specific alternative molecular screening tool in the diagnosis of *Enterocytozoon bieneusi* in faecal specimens.

INTRODUCTION

With a unique mechanism of host-cell infection, microsporidia have emerged as a group of human opportunistic pathogens most commonly infecting immunocompromised patients. The first human case of microsporidial infection was reported in 1959, and these parasites gained attention after 1985 when *Enterocytozoon bieneusi* was found in a human immunodeficiency virus-infected patient (Franzen & Müller, 2001). Since then, about 14 species of microsporidia have been reported infecting humans, of which *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* are responsible for most infections, with *Enterocytozoon bieneusi* being the most prevalent species detected in most studies performed (Matos et al., 2012).

Light microscopy is widely used for routine diagnosis to detect microsporidia in stool samples. Nonetheless, interpretation of the slides can be difficult due to varying staining quality and the small size of these pathogen spores (Didier et al., 2004). Moreover, the detection of microsporidium requires experienced microscopists and does not allow specific species identification, which is essential to determine suitable treatment. Albendazole is effective against most microsporidium species, particularly *Encephalitozoon* spp., infection, but has minimal efficacy against *Enterocytozoon bieneusi* (Botterel et al., 2002). Transmission electron microscopy, which can provide species-specific diagnosis in faecal specimens (Kumar et al., 2005), is expensive, time-consuming and insensitive for use in routine diagnosis. PCR methods have been successfully developed to detect microsporidial infections and are used mainly for further confirmation (Chabchoub et al., 2009). Even so, PCR assays are time-consuming, can be prone to contamination and in particular they require expensive equipment; thus, their routine implementation is not practicable for laboratories in developing countries (Parida et al., 2008; Verweij et al., 2007).

Abbreviations: GCK, Gram-chromotrope Kinyoun; LAMP, loop-mediated isothermal amplification.

The GenBank accession numbers for the SSU rRNA gene sequences of *Enterocytozoon bieneusi* reported in this paper are AF101198, KF271515.1, KF271513.1 and KF271512.1.
Recently, a novel nucleic acid amplification method, loop-mediated isothermal amplification (LAMP), has been introduced, which was originally developed by Notomi et al. (2000). This method is highly sensitive and specific compared with PCR, and can amplify up to $10^9$ copies of DNA in less than 1 h under isothermal conditions (Parida et al., 2008). Since its publication, many LAMP methods have been developed for identification of viruses, bacteria, parasites and fungi (Endo et al., 2004; Qiao et al., 2007; Qin et al., 2009). In this study, a LAMP assay was developed for detection of microsporidia in faecal samples. To the best of our knowledge, this is the first report on detection of Enterocytozoon bieneusi in stools by the LAMP method.

**METHODS**

**Sample source.** One hundred faecal samples from healthy, asymptomatic aborigines from various parts of Peninsular Malaysia, which were readily available in the Parasitology & Medical Entomology Department, Universiti Kebangsaan Malaysia Medical Centre (UKMMC), were used in this study. These samples were collected previously for research on intestinal parasitic infection, which was approved by the Ethics Committee of UKMMC, with written consent obtained from each participant.

**Microscopic examination.** All faecal samples were screened for the presence of microsporidium spores by microscopy using the Gram-chromatrope Kinyoun (GCK) staining method as described by Salleh et al. (2011) and observed under $\times 100$ magnification by light microscopy.

**DNA extraction.** A positive Enterocytozoon bieneusi clinical faecal sample was extracted using a commercial kit (QiAamp DNA Stool Mini kit; Qiagen), following the manufacturer's instructions. The purity of extracted DNA was measured using a Nanodrop 2000c (Thermo Scientific), in which the absorbance ratio was 1.88 ($A_{260}/A_{350}$). The DNA was amplified by PCR (da Silva et al., 1996) using primers EBIEF1 (5'-GAACTTTGCCATCTCCCTAG-3') and EBIE1R1 (5'-CCATGCACCCACCCTCCGGATT-3') to produce a 607 bp DNA fragment of the small subunit rRNA gene of Enterocytozoon bieneusi. The amplified product was confirmed by sequencing. The gene matched the sequence of GenBank accession no. KF271515.1. Thus, this sample was used as the positive control in this study.

DNA from 100 faecal samples was also prepared using the same commercial kit in accordance with the manufacturer’s instructions. The purity of DNA was measured and was found to be satisfactory with an $A_{260}/A_{350}$ ratio within the range of 1.7–2.0 in all DNA samples.

**Conventional PCR.** PCR amplification was performed using primer pair EBIEF1/EBIE1R1 and HelixAmp Taq Polymerase (NanoHelix). The reaction was carried out in 25 μl reaction mixtures under the following conditions: 10 μM each primer, 10 mM DNTP mix, 10 × PCR buffer, 1.25 U Taq DNA polymerase, 5 μl DNA and distilled water. After initial denaturation of the DNA at 94 °C for 5 min, 35 cycles were run of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s, with a 10 min 72 °C extension (da Silva et al., 1996). The amplified product was analysed on a 2 % agarose gel together with a DNA ladder (Norgen Biotek). PCR-positive samples were sent for sequencing for further confirmation. The sequences were compared with Enterocytozoon bieneusi sequences available in GenBank using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

**LAMP.** LAMP primers were designed using LAMP Designer software version 1.10 (PREMIER Biosoft) on the small subunit rRNA sequence of Enterocytozoon bieneusi (GenBank accession no. AF101198). The four primers are shown in Table 1. The LAMP reaction was performed with a Loopamp DNA amplification kit (Eiken Chemical Co.). The reaction was carried out in a 25 μl volume containing 40 μM each FIP and BIP primer, 20 μM each F3 and B3 primer, 12.5 μl 2 × reaction mixture, 8 U Bst DNA polymerase, 2 μl DNA, distilled water and 1 μl fluorescent detection reagent for visual fluorescence detection. The mixture was incubated at 60 °C using a conventional heating block for 1 h.

**Detection of LAMP products.** Two detection methods were used to analyse the LAMP products: visual observation with fluorescent detection reagent and gel electrophoresis. By naked eye visualization, a change of colour from reddish orange to green was recorded and the fluorescence detection was carried out under UV light. The LAMP products were run on a 2 % agarose gel following restriction enzyme digestion with MaeI to generate the predicted fragments of 200 and 55 bp.

**Analytical sensitivity and specificity of LAMP.** To determine the analytical sensitivity of the LAMP assay, the positive faecal sample was concentrated by a modified water/ether sedimentation protocol as described by Müller et al. (1999). The pellet was diluted in 200 μl distilled water and serially diluted 10-fold. A part of each dilution was used for GCK staining, followed by modified quantitative spore counts (Goodgame et al., 1999). Ten fields were randomly selected to count the spores at $\times 100$ magnification. The step was repeated three times with another 10 different fields for each counting. The mean of the three counts was converted to spores per 20 μl. Dilutions of the homogenized faeces were produced to give different spore counts of 10, 7, 5 and 0 spores per 20 μl.

The genomic DNA was extracted from the 200 μl solution of each dilution using a QiAamp DNA Stool Mini kit following the manufacturer’s instructions. All extraction products were subjected to PCR and LAMP assays to determine and compare the sensitivity.

Genomic DNA extracted from Acanthamoeba spp., Toxoplasma gondii, Entamoeba dispar, Entamoeba histolytica, hookworm, Trichuris trichiura, Ascaris lumbricoides and Giardia intestinalis was used to determine the specificity of the LAMP assay.

**Clinical sensitivity and specificity of LAMP.** The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were evaluated as described by Nagdev et al. (2011) using PCR as the reference test.

**Statistical analysis.** The diagnostic agreement between microscopy, PCR and LAMP assays was calculated using the $\kappa$ statistic and expressed as a $\kappa$ value. A $\chi^2$ test was used to determine the significance of the difference between these methods.

### Table 1. Sequence of four LAMP primers (based on GenBank accession no. AF101198)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
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<tbody>
<tr>
<td>F3</td>
<td>TCAGGATGATGGTAGGTA</td>
</tr>
<tr>
<td>B3</td>
<td>CGACGGATCCAAAGTCAC</td>
</tr>
<tr>
<td>BIP</td>
<td>TTGGATGGATGGATGGATGGGCAACCTCCCGATTCTCT</td>
</tr>
<tr>
<td>FIP</td>
<td>GGAGGACCCCTCTACCCCTCCTCCTGTAAGGGGAGGTGAT</td>
</tr>
</tbody>
</table>
RESULTS

Screening of the faecal samples by light microscopy showed microsporidian spores in 32 samples (32%). The microsporidian spores appeared as pinkish blue with a belt-like stripe, known as the polar tube, in the middle of the spores (Salleh et al., 2011). All samples were then subjected to PCR and LAMP assays. Out of 100 stool samples, 33 were positive for Enterocytozoon bieneusi by PCR (33%), whereas LAMP detected 39 samples as positive (39%).

Positive Enterocytozoon bieneusi amplification through PCR showed bands at approximately 607 bp (data not shown). All LAMP reactions were visualized under normal and UV light before being subjected to gel electrophoresis. Under normal light, the positive amplified LAMP products changed from reddish orange to a green colour, whereas negative LAMP results remained the same. Under UV light, all positive reactions turned bright green (Fig. 1). Moreover, when positive reactions were subjected to gel electrophoresis, a ladder-like pattern of multiple bands was observed (Fig. 2). The specificity of the amplification was further confirmed by restriction enzyme digestion of the LAMP products. As shown in Fig. 3, the results of digestion products were in good agreement as the sizes of the fragments generated were ~190 and 60 bp.

To evaluate the sensitivities of the PCR and LAMP methods, samples with known spore concentrations were used. The results showed that LAMP could detect Enterocytozoon bieneusi at concentrations as low as seven spores per 20 μl, while the sensitivity of the PCR was also equivalent to seven spores per 20 μl. This LAMP assay was also tested using several other genomic DNAs. No amplification was observed with DNA samples from Acanthamoeba spp., Toxoplasma gondii, Trichuris trichiura, Ascaris lumbricoides, hookworm, Entamoeba histolytica, Entamoeba dispar and G. intestinalis (Fig. 4).

The sensitivity and specificity of the LAMP assay and light microscopy in this study are shown in Table 2. Comparing the results for LAMP and PCR, 33 samples were positive for Enterocytozoon bieneusi by PCR and 31 samples were positive by the LAMP assay (94% sensitivity). Out of 67 PCR-negative samples, eight were positive by LAMP (88% specificity). In contrast, microsporidian spores were detected by microscopy in 16 out of the 33 PCR-positive samples (48% sensitivity), while 16 of the 67 specimens that were negative by PCR were detected as positive by microscopy (76% specificity). Based on the sequencing results, the two samples that were PCR-positive but LAMP-negative were matched to the published Enterocytozoon bieneusi sequences in the database (99% identical to GenBank accession nos KF271513.1 and KF271512.1).

![Fig. 1. Amplified LAMP products visualized under UV light. Samples 2, 4–6 and the positive control (+ve) turned bright green, indicating a positive reaction. –ve, Negative control.](http://jmm.microbiologyresearch.org)

![Fig. 2. Electrophoresis of LAMP products amplified from Enterocytozoon bieneusi DNA. Lanes: M, 100 bp DNA ladder; –ve, negative control (distilled water); 1–7, DNA samples; +ve, positive control.](http://jmm.microbiologyresearch.org)

![Fig. 3. Restriction analysis of Enterocytozoon bieneusi LAMP products amplified from faecal specimens. Lanes: 1, positive control; 2, one sample positive for Enterocytozoon bieneusi from the previous LAMP. MaeII digestions of Enterocytozoon bieneusi products were at the expected sizes of approximately 190 and 60 bp.](http://jmm.microbiologyresearch.org)
Additionally, a $\chi^2$ test and $\kappa$ statistics were computed to determine the diagnostic agreement between PCR, LAMP and light microscopy. As shown in Table 2, these three methods were considered not to be significantly different ($P>0.05$). However, there was substantial agreement between LAMP and PCR, while some agreement was demonstrated between light microscopy and PCR.

**DISCUSSION**

Three different detection methods (PCR, LAMP and microscopy) were evaluated to determine and compare their sensitivity and specificity in detecting *Enterocytozoon bieneusi* in stool samples. LAMP was successfully developed and exhibited amplification of *Enterocytozoon bieneusi* DNA within 1 h at 60 °C, resulting in a 39 % positivity rate. Based on the results, the lowest detection limit shown by both assays was seven spores per 20 μl. Although the minimum detection limit was equivalent, the LAMP assay was much more feasible to perform compared with the PCR assay. This LAMP assay required only 1 h of amplification at one constant temperature, whereas PCR required approximately 2 h to complete all the cycles. The LAMP assay also showed no amplification and no cross-reactivity with different DNA samples from parasites. The increase in sensitivity and specificity shown by LAMP compared with the PCR assay was due to the four primers used in this amplification and also to the DNA strand-displacement activity of the *Bst* enzyme, which can produce large amounts of DNA in isothermal conditions (Nagamine *et al.*, 2002). The amplification efficiency of LAMP is extremely large because the assay requires less time to complete compared with a PCR protocol (Fakruddin, 2011).

The LAMP assay yielded a sensitivity and specificity of 94 % and 88 %, respectively, while microscopy yielded a lower sensitivity and specificity (48 % and 76 %). Moreover, a substantial agreement was found between the PCR and LAMP assays, suggesting LAMP as a potential alternative technique to microscopy that may be applied in routine diagnostic laboratories to improve the sensitivity and specificity of diagnosis. Even though the sensitivity and specificity of the microsporidial LAMP were high, several false-positive and false-negative results were also obtained using this assay. Thus, analysis using sequencing and restriction enzymes would provide a great help in distinguishing between these results and contamination (Nagdev *et al.*, 2011). Therefore, further analysis between these LAMP-negative but PCR-positive samples was done by sequencing. By performing BLAST, identification of these samples was matched with the closest *Enterocytozoon bieneusi* sequences in the database, thus indicating false-negative amplification. The samples that were LAMP-positive but negative by PCR may be due to the presence of inhibitors in the samples, which are known to have a stronger inhibitory effect on the polymerases used in PCR than on the *Bst* enzyme used in LAMP. Nonetheless, further study needs to be undertaken by incorporating internal amplification controls to determine whether there is any inhibition occurring in the PCR and LAMP assays that may be responsible for these results (Nagdev *et al.*, 2011).

**Table 2.** Diagnostic performance of the LAMP assay and microscopy for the detection of *Enterocytozoon bieneusi* from human faecal specimens using PCR as the reference test

<table>
<thead>
<tr>
<th>Method and result</th>
<th>PCR (+ve)</th>
<th>PCR (−ve)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>Statistical analysis ($\chi^2$ test)</th>
<th>$\kappa$ value (95% confidence interval)</th>
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</thead>
<tbody>
<tr>
<td>Microscopy (+ve)</td>
<td>16</td>
<td>16</td>
<td>48</td>
<td>76</td>
<td>50</td>
<td>75</td>
<td>$P=0.530$</td>
<td>Microscopy and PCR: 0.25 $\kappa$ (0.049–0.447)</td>
</tr>
<tr>
<td>Microscopy (−ve)</td>
<td>17</td>
<td>51</td>
<td>94</td>
<td>88</td>
<td>79</td>
<td>97</td>
<td></td>
<td>LAMP and PCR: 0.78 $\kappa$ (0.658–0.910)</td>
</tr>
<tr>
<td>LAMP (+ve)</td>
<td>31</td>
<td>8</td>
<td>94</td>
<td>88</td>
<td>79</td>
<td>97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAMP (−ve)</td>
<td>2</td>
<td>59</td>
<td></td>
<td></td>
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</table>
Using microscopy, researchers can detect the spores but not the species. The presence of other small organisms, for example, bacterial and fungal spores, can lead to false-positive results (Rayan et al., 2013; Salleh et al., 2011). Therefore, a molecular technique is needed to further confirm the infections. In contrast, LAMP also demonstrated high sensitivity and specificity in several protozoan studies. Successful amplification of Toxoplasma gondii, Leishmania and Plasmodium falciparum showed a sensitivity of 87.5, 90.7 and 95.8 %, respectively, and a specificity of 100 for all (Lau et al., 2010; Ghayour Najafabadi et al., 2014; Khan et al., 2012). The LAMP assay has also been successfully developed to detect other parasites in different types of clinical samples, such as human saliva, buffy coats, urine and blood (Ghayour Najafabadi et al., 2014; Lau et al., 2011; Khan et al., 2012).

The combination of isothermal conditions and the Bst polymerase in the LAMP assay showed resistance to inhibitors that usually hinder the later stages of conventional PCR (Khan et al., 2012; Mori et al., 2001). LAMP reagents and Bst polymerase are also relatively stable even if kept at 25 and 37 °C (Thekiso et al., 2009), and only a conventional heating block is required to perform this assay. The requirement for an electrical supply for the heating block can be changed to an alternative power source such as a battery to suit its application (Khan et al., 2012). A water bath could also be used; however, it is recommended that the tubes be wrapped in parafilm to avoid contamination (Khan et al., 2012) as LAMP assays can sometimes lead to false-positive amplification due to the very high specificity and sensitivity (Zhou et al., 2014). These results indicate the possibility of the application of LAMP assays in field conditions and resource-poor laboratories. There are also a few limitations for LAMP, by which the selected primers should be designed appropriately and the target DNA should be less than 500 bp (Lau et al., 2011).

In conclusion, the developed LAMP assay is a promising technique for application in diagnostic laboratories for the detection of microsporidia in clinical specimens. Species identification using the LAMP assay is important for suitable treatment, given that albendazole is less effective in treating Enterocytozoon bieneusi infection compared with other microsporidial species. Nitazoxanide therapy was proven to be clinically effective against Enterocytozoon bieneusi infection by Bicart-Sée et al. (2000). Therefore, this drug has been suggested for use as an alternative treatment for Enterocytozoon bieneusi infection. The simplicity, rapidity and flexibility of the reagents and application make this technique favourable to be performed as an alternative in developing countries with limited access to advanced technology and expertise. The high sensitivity and specificity of the LAMP assay shown in this study are suitable for the rapid screening of Enterocytozoon bieneusi diagnosis as an alternative to PCR.

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