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Development of loop-mediated isothermal amplification for rapid detection of *Entamoeba histolytica*

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ABSTRACT

Objective: To develop a loop-mediated isothermal amplification (LAMP) assay for the detection of *Entamoeba histolytica* (*E. histolytica*), the causative agent of amebiasis. **Methods:** The LAMP primer set was designed from *E. histolytica* hemolysin gene HLY6. Genomic DNA of *E. histolytica* trophozoites strain HK9 was used to optimize the LAMP mixture and conditions. Amplification of DNA in the LAMP mixture was monitored through visual inspection for turbidity of the LAMP mix as well as addition of fluorescent dye. **Results:** Positive LAMP reactions turned turbid while negative ones remained clear. Upon addition of a fluorescent dye, all positive reactions turned green while the negative control remained orange under ambient light. After elecrophoresis in 1.5% agarose gels, a ladder of multiple bands of different sizes can be observed in positive samples while no bands were detected in the negative control. The sensitivity of the assay was found to be 5 parasites per reaction which corresponds to approximately 15.8 ng/μL DNA. The specificity of the assay was verified by the absence of amplified products when DNA from other gastrointestinal parasites such as the morphologically similar but non-pathogenic species, *Entamoeba dispar*, and other diarrhea-causing organisms such as *Blastocystis hominis* and *Escherichia coli* were used. **Conclusions:** The LAMP assay we have developed enables the detection of *E. histolytica* with rapidity and ease, therefore rendering it is suitable for laboratory and field diagnosis of amebiasis.

1. Introduction

Amebiasis is an infection of human intestinal and extraintestinal organs by the protozoan parasite *Entamoeba histolytica* (*E. histolytica*)[1]. *E. histolytica* is responsible for an estimated 40 to 50 million cases of amebiasis and 100 000 deaths annually[2]. This invasive protozoan parasite can be detected by microscopic examination of stool samples from infected individuals. However, *E. histolytica* is morphologically similar to the closely related species, the non-invasive and non-pathogenic *Entamoeba dispar* (*E. dispar*). Differentiation of *E. histolytica* from *E. dispar* in stool is accomplished by culture and zymodeme analysis, recognition by species-specific monoclonal antibodies, and DNA hybridization[3], which are all expensive diagnostic methods of amebiasis, have different disadvantages[4] and may not be suitable when large numbers of samples have to be tested[5].

A number of varied isothermal nucleic acid amplification methods which require only a simple heating device have been developed to offer feasible platforms for rapid and sensitive detection of a target nucleic acid[6]. These

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include nucleic acid-based amplification or NASBA[7], ramification amplication[8] and loop-mediated isothermal amplification or LAMP[9]. LAMP, developed by Notomi and co-workers in 2000, is a novel and highly selective method of nucleic acid amplification that proves to be rapid, accurate, cost-effective diagnostic technique for infectious diseases. It has been applied as a diagnostic tool for several infectious diseases, including viral[10], bacterial[11], fungal[12] and parasitic diseases[13]. LAMP assay uses four to six primers that recognize six to eight regions of the target DNA, respectively, in conjunction with the enzyme *Bst* polymerase[14]. *Bst* polymerase, which is from *Bacillus stearothermophilus*, can synthesize a new strand of DNA while simultaneously displacing the complementary strand thereby enabling DNA amplification at a single temperature with a single enzyme[15]. Considering the advantages of LAMP, it could be a valuable tool for diagnosing amebiasis in developing countries particularly the Philippines. Therefore, the objective of the study was to develop *E. histolytica* LAMP, as a simple, rapid, specific and sensitive diagnostic tool for amebiasis.

2. Materials and methods

2.1. LAMP primer design, concentrations and reaction

The LAMP primer set was designed from *E. histolyica* hemolysin gene, HLY6 (GenBank accession number Z29969.1) by using the Primer Explorer software Version 4 (Eiken Chemical Co., Ltd., Japan; http://primerexplorer.jp/e/). The hemolysin gene HLY6 is encoded on the antisense strand of the large subunit ribosomal RNA (lsu rRNA) in the two inverted repeats of rDNA located on the 25 kbp palindromic circular episome of *E. histolytica*[16,17].The open reading frame of the HLY6 gene has recently been described as conserved and multi-copy (400 copies/cell) with hemolytic activity (Zindrou *et al* unpublished). The high copy number of HLY6 gene in *E. histolytica* genome makes it ideal for LAMP due to more regions of DNA to which the primers can hybridize[18]. In addition, primers specific for the HLY6 gene has already been used as tools for specific identification of *E. histolytica* in liver abscess and fecal samples[19].

The two outer primers were Eh-2F3 (5′-GCA CTA TAC TTG AAC GGA TTG-3′) and Eh-2B3 (5′- GTT TGA CAA GAT GTT GAG TGA-3′). The two inner primers were Eh-2FIP (5′-TCG CCC TAT ACT CAA ATA TGA CAA GAC TTT GGT GGA AGA TTC ACG-3′) and Eh-2BIP (5′-ATC TAG TAG CTG GTT CCA CCT GAA CAC CTA ATC ATT ATC TTT ACC AAT C-3′). Two additional loop primers were nominated as Eh-2F2 (5′-ACT TTG GTG GAA GAT TCA CG-3′) and Eh-2B2 (5′-CAC CTA ATC ATT ATC TTT ACC AAT C-3′).

Genomic DNA of *E. histolytica* trophozoites strain HK9 were extracted using phenol-choloroform-isoamyl alcohol[20]. The final, optimized LAMP reaction was conducted in a 25 μL reaction mixture with the inner primers at 0.48 μM, the outer primers at 0.2 μM and the loop primers at 0.24 μM. The reaction mixture also contained 2.5 μL of 10× *Bst* DNA polymerase reaction buffer [1×containing 20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 10 mM KCl, 0.1% Triton×-100] 2 μL of an 8 U/μL concentration of *Bst* DNA polymerase (New England Biolabs, Inc., MA), 1.5 mM $MgSO_4$, 0.8 M of betaine (Sigma-Aldrich, St. Louis, MO), and 3 μL of genomic *E. histolytica* template DNA. PCR-grade water was used as a negative control to ensure no amplification from the reaction mixture alone. Amplification was performed at 63 ℃ for 60 min, and then the mixture was heated at 90 ℃ for 1 min to terminate the reaction.

2.2. Detection of LAMP product

Amplification of DNA in the LAMP mixture can be monitored through visual inspection for turbidity of the LAMP mix. The amplification for *E. histolytica* was also observed as a change in color under ambient light after the addition of 2 μL SYBR green (1:10 dilution of a 10 000 ×stock solution). Upon addition of the flourescent dye, the samples were observed for any change in color under UV illumination and with a dark and white background.

LAMP products were also electrophoresed in a 1.5% Tris-acetic acid-EDTA agarose gel at 100 V and stained with ethidium bromide (5 μg/mL) for visualization under UV light.

2.3. Sensitivity and specificity of LAMP

The sensitivity of the LAMP assay was tested on DNA extracted from 10-fold dilutions of cultured *E. histolytica* trophozoites as well as *E. histolytica* cells spiked with feces. Briefly, cultured ameba trophozoites were counted using Neubauer counting chamber. A 10-fold serial dilution was then obtained. For testing the sensitivity using spiked samples, a volume of 100 μL of each diluted sample was used to spike 100 μL parasite-free stools. Each spiked sample was then subjected to genomic DNA extraction using ZR Fecal DNA MiniPrep (Zymo Research, Irvine, CA, USA). A conventional nested PCR[21] was used as a standard method

of comparison, since nested PCR has been implicated to be a highly sensitive method to detect *E. histolytica* parasites from clinical samples such as urine and liver abscess of patients[22, 23]. DNA concentrations were determined using NanoDrop 2000C spectrophotometer (ThermoScientific). The specificity of the newly designed LAMP assay was determined by using the genomic DNA of three common pathogens that can cause similar clinical symptoms of amebiasis: the morphologically similar but non-pathogenic species, *E. dispar*, and *Blastocystis hominis* and *Escherichia coli*.

3. Results

Positive LAMP reactions were turbid while the negative control samples were clear when examined at room temperature under ambient light (Figure 1). Direct visual inspection after addition of SYBR green 1 showed that positive reactions turned green while the negative control remained orange. Under UV illumination, positive reactions turned bright green while negative reactions remained dull orange (Figure 2). Agarose gel electrophoresis of positive samples showed a ladder of multiple bands while no bands were detected in the negative control. It was observed that the LAMP assay amplified DNA in all *E. histolytica* DNA dilutions used. It was also determined that there was no difference in the intensity of the bands with regard to the *E. histolytica* DNA concentration in the mixture (Figure 3).

Using DNA extracted from serially-diluted pure cultures of *E. histolytica* cells as well the corresponding dilution series spiked with feces, the sensitivity of the assay was found to be 5 parasites per reaction which corresponds to approximately 15.8 ng/μL DNA. Thus, the sensitivity of the LAMP was lower compared to that of conventional nested PCR which was determined by the current study to detect to as low as 2 ng/μL DNA sample (data not shown) The specificity of the assay was verified by the absence of amplified products when DNA from other gastrointestinal parasites such as the morphologically similar but non-pathogenic species, *E. dispar*, and other diarrhea-causing organisms such as *Blastocystis hominis* and *Escherichia coli* were used (Figure 4).

Figure 1. Differentiation of the LAMP reaction by visually observing the mixture for increase in turbidity.
Left, without template (negative); right, with template DNA (positive). LAMP positive reaction produced an increase in turbidity of the mixture while the negative sample remained clear.

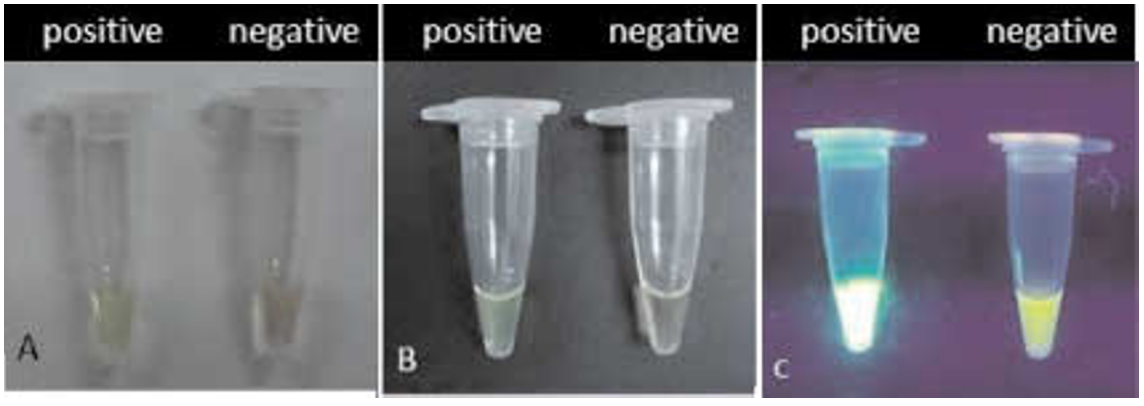

Figure 2. Visual detection of LAMP products after the addition of SYBR green 1 against a white (A) and dark (B) background and under UV illumination (C).
Positive reactions turned green under ambient light and fluoresced bright green under UV illumination, while the negative control remained dull orange under ambient light and did not emit strong green fluorescence under UV light.

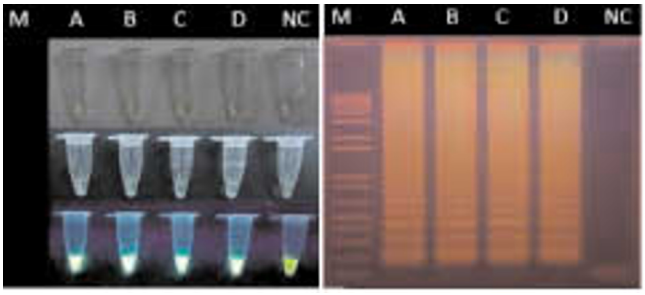

Figure 3. Visual inspection under both ambient light and UV illumination after addition of SYBR green, and corresponding agarose gel electrophoresis profile for each LAMP reaction.
Lane M, marker; lanes A, B, C, and D correspond to 260 ng/μL, 135 ng/μL,68.2 ng/μL and 9.4 ng/μL *E. histolytica* DNA template, respectively. NC, negative control. Upon addition of the fluorescent reagent, positive samples produced green color while negative samples remained orange when examined under ambient light. Under UV illumination, positive samples fluoresced bright green while the negative sample was dull orange. Agarose gel analysis showed LAMP products as a ladder of multiple bands. No bands were detected in the negative control sample. It can be observed that there is no difference in the intensity of the bands with regard to *E. histolytica* DNA concentration in the LAMP mix.

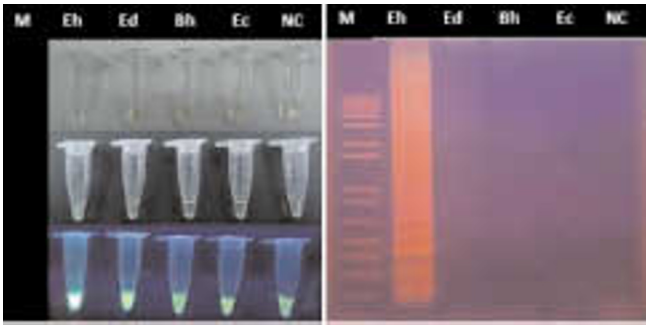

Figure 4. Specificity of the LAMP assay for the detection of *E. histolytica*.
Lane M, marker; lanes Eh, Ed, Bh, and Ec, correspond to lamp reactions using DNA from *E. histolytica*, *E. dispar*, *Blastocystis hominis* and *Escherichia coli*, respectively. NC, negative control. No amplified products were detected when DNA from other diarrhea-causing microorganisms were used such as *E. dispar*, *Blastocystis hominis* and *Escherichia coli* were used, thus ensuring the specificity of the assay.

4. Discussion

Nucleic acid amplification is one of the most useful tools in application-oriented fields such as clinical medicine, particularly in the molecular diagnosis of infectious diseases. Although conventional DNA amplification using PCR can provide fast results, it is not widely used partly because it requires considerable skill and expensive equipment[24], has insufficient specificity, and rather low amplification efficiency[25]. LAMP is a novel technique developed by Notomi and co-workers in 2000 that has been claimed to be cheaper and more efficient than PCR. The process can amplify a few copies of DNA to 10^9 in less than an hour under isothermal conditions and with greater specificity[9]. In LAMP, the amplification reaction relies on recognition of six independent regions of the target DNA by the use of four primers during the initial stage, and four further sites recognized by two additional primers during the next stages, thus ensuring the specificity of the assay. The principle of LAMP is autocycling strand displacement DNA synthesis in the presence of *Bst* DNA polymerase with high strand displacement activity under isothermal conditions between 60 and 65℃ within 60 min[26].

The successful development and evaluation of LAMP procedures has been reported for many clinical applications, including viral and bacterial infections[24,27,28], and for diagnosis of protozoan diseases[29,30]. In the present study, we developed LAMP for the rapid and cost-effective identification of *E. histolytica*. Optimization of the LAMP reaction was done using DNA from pure cultures of *E. histolytica* maintained in the laboratory. It was observed that a positive LAMP reaction corresponds to an increase in turbidity of the reaction mixture. This can be explained by the production of a large amount of pyrophosphate ion, which reacts with magnesium ions in the reaction to form magnesium pyrophosphate, a white precipitate by-product[25]. Judging the absence or presence of this white precipitate allows easy and rapid visual identification that the target DNA was amplified by LAMP. Thus, visual assessment is more appropriate for bedside monitoring due to its ease and rapidity, as well as allows a reduction in operation time and reduces contamination risks[28]. Further establishment of a sample's LAMP reaction can be attained through the addition of fluorescent dye, SYBR green 1. SYBR green 1 is one of the most sensitive general nucleic acid fluorescence dyes available[31]. Compared to calcein, which is now often used as a fluorescent dye in closed-tube LAMP analyses, SYBR green 1 can be excited by both UV and visible light[32]. The color of this dye changes from orange to bright green fluorescence when conjugated into DNA and excited by UV thus accounting to the change in color of the positive LAMP reactions.

Upon agarose gel electrophoresis, positive samples exhibit a ladder of multiple bands of different sizes. This amplification pattern is characteristic of the LAMP reaction and indicates that stem-loop DNAs with inverted repeats of the target sequence were produced[9]. Since LAMP amplifies DNA under isothermal conditions, simple heater such as water bath or block heater are sufficient for the reaction process. There is no need for special device used for PCR such as thermal cyclers therefore making LAMP suitable for the diagnosis of an infectious disease in field situations. Due to its ease of use in the developing countries particularly the Philippines, the development of a LAMP assay specific and sensitive for *E. histolytica* may facilitate the identification of the protozoan parasite with utmost rapidity and efficiency. This feat may be of great advantage to the clinical community particularly in the laboratory and field diagnosis of amebiasis.

Conflict of interest statement

We declare that we have no conflict of interest.

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