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Parasitology

Development of a loop-mediated isothermal amplification assay for detection of *Trichomonas vaginalis*

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ABSTRACT

A loop-mediated isothermal amplification (LAMP) assay targeting the 2-kbp repeated DNA species-specific sequence was developed for detection of *Trichomonas vaginalis*, the causative agent of trichomoniasis. The analytical sensitivity and specificity of the LAMP assay were evaluated using pooled genital swab and urine specimens, respectively, spiked with *T. vaginalis* trophozoites. Genital secretion and urine did not inhibit the detection of the parasite. The sensitivity of the LAMP was 10–1000 times higher than the PCR performed. The detection limit of LAMP was 1 trichomonad for both spiked genital swab and urine specimens. Also, LAMP did not exhibit cross-reactivity with closely-related trichomonads, *Trichomonas tenax* and *Pentatrichomonas hominis*, and other enteric and urogenital microorganisms, *Entamoeba histolytica*, *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. This is the first report of a LAMP assay for the detection of *T. vaginalis* and has prospective application for rapid diagnosis and control of trichomoniasis.

1. Introduction

Trichomoniasis, caused by the flagellated protozoan parasite *Trichomonas vaginalis*, is the most common curable sexually transmitted infection (STI) with about 276.4 million cases worldwide (WHO, 2012). Women may present with symptoms of malodorous vaginal discharge, dyspareunia, dysuria, lower abdominal pain, and vulvovaginal irritation, while men may clinically manifest symptoms of urethral discharge, irritation, and dysuria (Coleman et al., 2013). Untreated *T. vaginalis* infection in women may lead to endometritis, infertility, cervical erosion, premature rupture of membranes, preterm deliveries, and low-birth-weight infants (Petrin et al., 1998). In men, complications of trichomoniasis include non-gonococcal urethritis, prostatitis, urethral strictures, epididymitis, and infertility (Petrin et al., 1998). Moreover, there is strong evidence of increased risk of human immunodeficiency virus (HIV) transmission and acquisition associated with *T. vaginalis* infection (Kissinger and Adamski, 2013). Yet, more than 50% of women and 75% of men infected with *T. vaginalis* are asymptomatic (Workowski and Berman, 2010). The control of STIs is grounded on prevention of further transmission of infection. The WHO (2007) recommends the syndromic management for the rapid diagnosis and treatment of STIs in developing countries that lack necessary resources for etiological laboratory diagnosis. This approach that follows an algorithm of signs and symptoms has low sensitivity and specificity for asymptomatic cases (Desai et al., 2003; Yin et al., 2008). Moreover, false diagnosis leads to unnecessary treatment, which could result in the emergence of drug-resistant STIs (Muzny and Schwebke, 2013). Thus, simple, rapid, and valid diagnostic tests must be developed to detect individuals with asymptomatic infections.

The most common laboratory diagnostic tests for *T. vaginalis* infections include wet-mount microscopy; culture; and, recently, nucleic acid amplification tests (NAATs) using genital swab, urine, or semen samples. Microscopy is rapid and inexpensive, but sensitivity is only 50–70% (Radonjic et al., 2006; Workowski and Berman, 2010). Culture is the gold standard for diagnosis of *T. vaginalis*, although its sensitivity is similarly low from 50% to 80% and requires at least a week of incubation and daily microscopy for optimal performance (Caliendo et al., 2005; Huppert et al., 2007). The development of NAATs showed improved sensitivity and specificity compared to microscopy and culture with sensitivity ranging from 64% to 100% (Huppert et al., 2007; Lawing et al., 2000; Schirm et al., 2007). However, expensive equipment and technical training make NAATs impractical for routine diagnosis in resource-limited settings.

Recently, a novel NAAT has been described, called loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000). Compared to PCR, LAMP uses a DNA polymerase with strand displacement activity and a set of 4–6 specifically designed primers, which amplify nucleic acids under isothermal conditions between 60 and 65 °C, and thus, incubation can be done in a heat block or water bath. Moreover, large amount of DNA product is generated in less than an hour and a positive reaction can be visualized with the naked eye without the

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need for gel electrophoresis (Tomita et al., 2008). Many LAMP-based assays have already been developed for the detection of infectious diseases (Mori and Notomi, 2009). Therefore, the LAMP method could become a simple, rapid, sensitive, specific, and cost-effective diagnostic tool in developing countries. In this study, we present the development of a LAMP assay for detection of *T. vaginalis* and the evaluation of its analytical sensitivity and specificity in spiked genital swab and urine specimens.

2. Materials and methods

2.1. Reference culture and DNA extracts

T. vaginalis AC1 previously isolated from a female sex worker in Angeles City, Pampanga, Philippines was grown axenically in Diamond's BI-S-33 medium (Diamond et al., 1978; Rivera et al., 2009). This isolate was used as reference strain to develop the method and to spike clinical specimens for analytical sensitivity evaluation. Previously extracted DNA from *Trichomonas tenax* isolate, *Pentatrichomonas hominis* D34, *Entamoeba histolytica* HK-9, *Candida albicans* UPCC 2168, *Escherichia coli* UPCC 1195, *Pseudomonas aeruginosa* UPCC 1244, and *Staphylococcus aureus* UPCC 1143 were used to assess the analytical specificity of the LAMP assay.

2.2. Clinical specimens

A total of 16 genital swabs and 2 urine specimens were obtained from women attending the Reproductive Health and Wellness Center in Angeles City, Pampanga, Philippines. Informed consent was obtained from these individuals. This study was approved by the University of the Philippines-Manila Research Ethics Board. Only 1 type of sample came from each individual. Genital swab specimen was collected using a sterile cotton swab and dipped in 3 mL of phosphate-buffered saline (PBS). Approximately 10–15 mL of urine was collected using a sterile preservative-free container. The collected clinical specimens were immediately transported to the laboratory and kept at 4 °C until use. All specimens were examined for the presence of *T. vaginalis* by PCR as described in Section 2.4.

2.3. DNA extraction

Mid-log-phase axenic *T. vaginalis* cultures (10^7 trichomonads/mL) and 1 mL aliquot of clinical specimens were pelleted by centrifugation at 10,000 rpm at 25 °C for 2 min, respectively. The resulting pellet was washed thrice with PBS. DNA was extracted according to a previously described procedure (Caliendo et al., 2005). Briefly, the pellet was resuspended in 100 μL of TE buffer (0.01 mol/L Tris-HCl, 0.001 mol/L EDTA; pH 8.0) and boiled at 100 °C for 30 min. The suspension was stored at 4 °C until use.

2.4. Confirmatory PCR for T. vaginalis

DNA extracts were subjected to species-specific PCR for *T. vaginalis* (Mayta et al., 2000). Briefly, PCR was performed in a 20-μL reaction mixture consisting of 10.0 μL of 2X *Taq* Master Mix (Vivantis Technologies, Selangor, Malaysia), 0.4 μmol/L each of primers Tv1 and Tv2 (Table 1), 2.0 μL of DNA template, and 6.4 μL of nuclease-free water. Amplification conditions were set with initial denaturation step at 94 °C for 3 min, then 40 cycles consisting of 94 °C for 10 s, 58 °C for 45 s, and 72 °C for 15 s, with a final extension step at 72 °C for 5 min. Positive (*T. vaginalis* DNA) and negative (nuclease-free water) controls were included. PCR products were resolved in 1.5% agarose gel electrophoresis at 100 V pre-stained with 1.0 μL of 10,000X SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA), and visualized under UV transilluminator (UVP, Upland, CA, USA).

Table 1
PCR and LAMP oligonucleotide primer sequences used in this study.

Primer name	Nucleotide Sequence (5′ → 3′)	Target gene
Tv1	TAATGGCAGAATCTTTGGAG	18S rRNA
Tv2	GAACTTTAACCGAAGGACTTC	
TvK-FIP	CGAAGTGCTCGAATGCGATTGCATTGACCACACGGACAA	Repeated DNA
TvK-BIP	GGTGCAAGGCAGAGGTCATTATTGCCAATCCAAGGACG	of *T. vaginalis*
TvK-F3	ACTATGGCACGAGACACA	
TvK-B3	TTGAAGTGGACACAATCGTT	
TvK-LF	GCTGCTTGACCATCCGAA	
TvK-LB	GCCACTCTACGAGCAGTAC	

2.5. Design of LAMP primers

LAMP primers specific for *T. vaginalis* were designed based on the highly repeated 2-kbp DNA fragment of *T. vaginalis* (GenBank Accession No. L23861) using the software LAMP Designer version 1.02 (Premier Biosoft, Palo Alto, CA, USA). A set of 6 primers composed of forward and backward inner primers (TvK-FIP, TvK-BIP), outer primers (TvK-F3, TvK-B3), and loop primers (TvK-LF, TvK-LB) were selected (Table 1). All primers were assessed for specificity by BLAST search against nucleotide sequences in NCBI GenBank (http://www.blast.ncbi.nlm.nih.gov).

2.6. T. vaginalis LAMP assay

The LAMP assay was performed in a 25-μL reaction mixture containing 1.6 μmol/L each of TvK-FIP and TvK-BIP primers, 0.2 μmol/L each of TvK-F3 and TvK-B3 primers, 0.8 μmol/L each of TvK-LF and TvK-LB primers, 0.4 mmol/L each of dNTPs (Promega, Madison, WI, USA), 0.8 mol/L betaine (Sigma-Aldrich, St. Louis, MO, USA), 8.0 U *Bst* DNA polymerase, exonuclease minus (Lucigen, Middleton, WI, USA), 1X DNA polymerase Buffer B (20.0 mmol/L Tris HCl, 10.0 mmol/L KCl, 10.0 mmol/L $(NH_4)_2SO_4$, 8.0 mmol/L $MgSO_4$, 0.1% Triton X-100; pH 8.0), 2.0 μL of DNA template, and 9.2 μL of nuclease-free water (Notomi et al., 2000; Tomita et al., 2008). The LAMP reaction was incubated at 60 °C for 30–45 min in a heat block and terminated at 80 °C for 5 min. Positive (*T. vaginalis* DNA) and negative (nuclease-free water) controls were included.

2.7. Detection and visualization of LAMP products

The LAMP products were assessed by: i) gel electrophoresis, ii) assessment of turbidity, and iii) addition of SYBR Safe (Wastling et al., 2010). For gel electrophoresis, the LAMP products were resolved in 1.5% agarose gels as indicated in Section 2.4. A ladder-like band pattern showed a positive reaction. For turbidity, visual inspection after incubation under natural light was done. Turbidity or precipitate formation signified a positive reaction. For addition of SYBR Safe, 1.0 μL of 1000X SYBR Safe DNA Gel Stain was added to the LAMP mixture. An orange-to-pink color change under natural light or a dull to fluorescent signal under UV transilluminator indicated a positive reaction.

2.8. Analytical sensitivity of T. vaginalis LAMP in spiked-clinical specimens

The analytical sensitivity of the *T. vaginalis* LAMP was evaluated to establish the detection limit of the assay using spiked genital swab and urine specimens. A 10-fold serial dilution of *T. vaginalis* trophozoites from mid-log-phase axenic cultures were harvested and spiked into pooled genital swab and urine specimens, respectively, starting from 10^6 to 10^0 trichomonads/mL. DNA was extracted from each dilution by boiling at 100 °C for 30 min and kept at 4 °C until use (Caliendo et al., 2005). The results are representative of 8 independent replicate runs of each dilution series subjected to the LAMP assay over

a spread of 5 days (Burd, 2010). Each run included a positive control (*T. vaginalis* DNA) and a negative control (nuclease-free water). Also, sensitivity of LAMP was compared to PCR as described in Section 2.4.

2.9. Analytical specificity of T. vaginalis LAMP

The analytical specificity of the *T. vaginalis* LAMP was examined to determine cross-reactivity of the assay to DNA extracts of closely-related trichomonads, *T. tenax*, *P. hominis*, and other enteric and urogenital microorganisms, *E. histolytica*, *C. albicans*, *E. coli*, *P. aeruginosa*, and *S. aureus*. Positive (*T. vaginalis* DNA) and negative (nuclease-free water) controls were included. The results are representative of 3 independent replicate runs.

3. Results

3.1. Detection of T. vaginalis using LAMP and PCR

PCR of *T. vaginalis* AC1 isolate using species-specific primers gave the expected positive amplicon of 312-bp. No amplicon was observed when DNA extracts of *T. tenax*, *P. hominis*, *E. histolytica*, *C. albicans*, *E. coli*, *P. aeruginosa*, and *S. aureus* were assayed. Genital swab and urine specimens used for spiking were also checked for the presence of *T. vaginalis*. Of the 16 genital swab specimens, 15 were negative by PCR, and the 2 urine specimens were also negative. All negative samples were pooled and used for spiking in the analytical sensitivity evaluation.

LAMP amplification of boiled *T. vaginalis* trophozoites targeting the 2-kbp DNA fragment repeats yielded the characteristic multiple band patterns on gel electrophoresis at 45-min incubation time (Fig. 1).

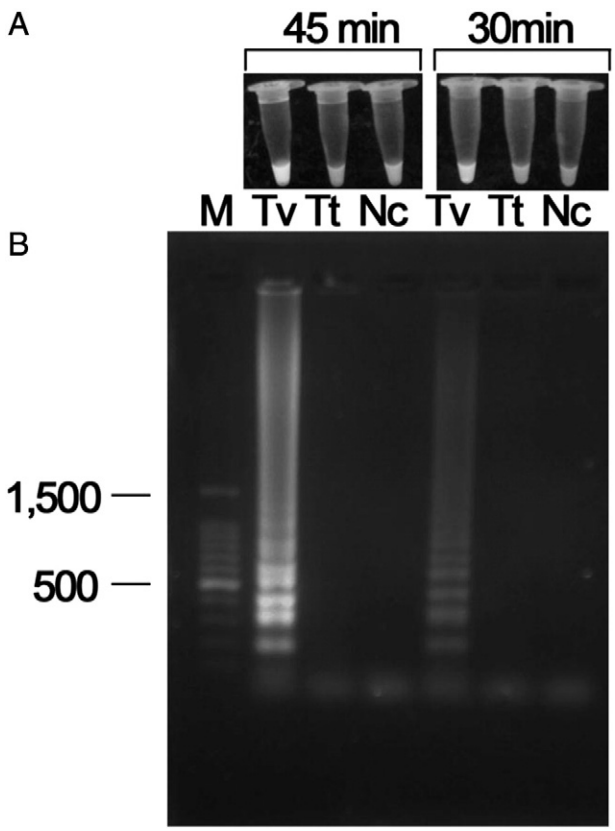

Fig. 1. Optimization of the LAMP reaction incubated at 60 °C for 30 and 45 min, respectively, and detected by (A) addition of 1000X SYBR Safe DNA Gel Stain (Invitrogen) and (B) 1.5% agarose gel electrophoresis stained with 10,000X dilution of SYBR Safe. Tv, *T. vaginalis* positive control; Tt, *T. tenax*, control; Nc, nuclease-free water negative control; M, 100-bp DNA ladder (TaKaRa Bio, Shiga, Japan).

The LAMP mixture showed turbidity and presence of precipitate. Addition of SYBR Safe to LAMP products, either under natural light or UV light, clearly showed positive results (Fig. 1).

3.2. Analytical sensitivity of T. vaginalis LAMP

In spiked genital swab samples, results indicate that the detection limit of the LAMP method was 10^0 trichomonads/mL or 1 trichomonad either by gel electrophoresis, turbidity, or addition of SYBR Safe, while the detection limit of PCR was 10^3 trichomonads/mL or 1000 trichomonads (Fig. 2). The results of 8 independent replicates for every dilution concentration were the same.

In spiked urine samples, the LAMP method detected up to 10^0 trichomonads/mL or 1 trichomonad either by gel electrophoresis, turbidity, or addition of SYBR Safe, while the detection limit for PCR was 10^1 trichomonads/mL or 10 trichomonads (Fig. 2). The LAMP results observed were the same in 8 independent replicates from 10^6 to 10^1 trichomonads/mL dilution except for 10^0 trichomonads/mL in which only 5 of 8 replicates or 62.5% were positive.

3.3. Analytical specificity of T. vaginalis LAMP

T. vaginalis LAMP exhibited no cross-reactivity with DNA extracts of *T. tenax*, *P. hominis*, *E. histolytica*, *C. albicans*, *E. coli*, *P. aeruginosa*, and *S. aureus*. LAMP products were only observed using *T. vaginalis* DNA, either by gel electrophoresis, turbidity, or addition of SYBR Safe (Fig. 3). Results of all 3 independent replicate runs were the same.

4. Discussion

In this study, application of the LAMP method for detection of *T. vaginalis* demonstrated advantages over conventional PCR. The LAMP assay amplifies DNA with high efficiency under isothermal conditions as compared to PCR (Nagamine et al., 2001). Thus, LAMP does not require sophisticated equipment such as thermal cycler and can be performed in a regular heat block or water bath (Notomi et al., 2000). The LAMP reaction is capable of generating 10^9 copies of target genes in less than an hour and can be accelerated to less than half the original reaction time using additional loop primers, ideal for rapid point-of-care tests (Mori and Notomi, 2009). Aside from an isothermal enzyme activity, the DNA polymerase can tolerate PCR inhibitors found in sputum, blood, stool, and urine. Thus, simple sample preparation is needed for DNA amplification (Poon et al., 2006). In this study, spiked clinical specimens were directly boiled for 30 min as previously done by Caliendo et al. (2005), which lessens turnaround time and complicated DNA extraction procedures. Moreover, the large amount of amplified DNA products by LAMP can be observed visually by turbidity due to accumulation of magnesium pyrophosphate by-product and addition of a nucleic acid stain such as SYBR dye (Mori et al., 2001). The LAMP method is a rapid and cost-effective tool that has previously been applied in the detection of a number of infectious diseases such as malaria and HIV but has so far never been applied for detection of *T. vaginalis* (Curtis et al., 2009; Poon et al., 2006).

The sensitivity of the LAMP assay for *T.* vaginalis was 10–1000 times more sensitive than the conventional PCR performed. The detection limit of LAMP was determined to be 10^0 trichomonads/mL or 1 trichomonad for both spiked genital swab and urine specimens (Fig. 2). PCR showed amplification up to 10^3 trichomonads/mL or 1000 trichomonads for swab samples, while amplification was successful up to 10^1 trichomonads/mL or 10 trichomonads for urine samples. The higher sensitivity of LAMP for detection of trichomonads may be explained by the tolerance of *Bst* DNA polymerase to NAATs inhibitors. Toye et al. (1998) reported that PCR inhibition was rare with urine samples and infrequent with endocervical swabs but frequent with urethral swabs. However, various specimen preparation methods

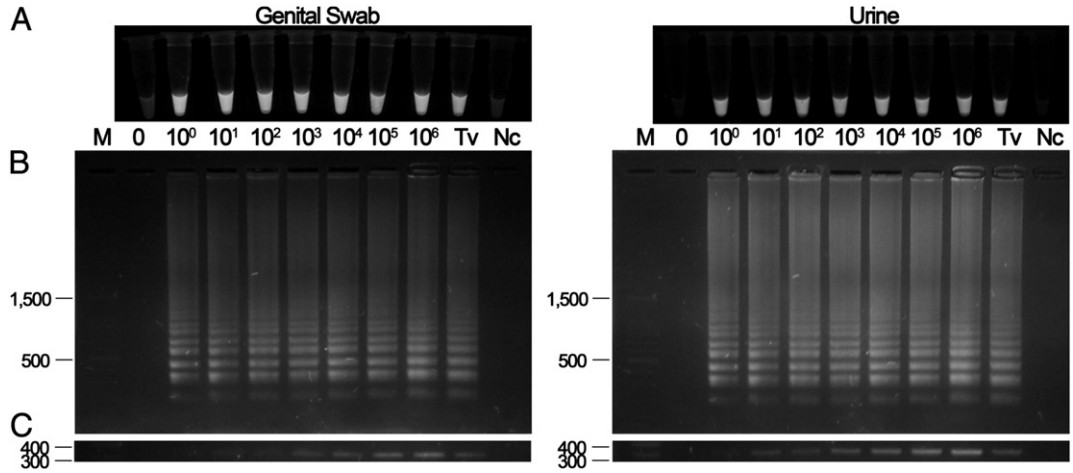

Fig. 2. Analytical sensitivity of the *T. vaginalis* LAMP was evaluated using serially-diluted spiked genital swab and urine specimens as detected by (A) addition of SYBR Safe DNA Gel Stain (Invitrogen) and (B) 1.5% agarose gel electrophoresis stained with 10,000X dilution of SYBR Safe. Tv, *T. vaginalis* positive control; Nc, nuclease-free water negative control; M, 100-bp DNA ladder (TaKaRa Bio). Also, spiked clinical samples were subjected to (C) conventional PCR showing the 10–1000-fold higher sensitivity of LAMP.

may be done to reduce inhibition. As performed in the study, boiling was not sufficient to remove PCR inhibitors from genital swab specimens. LAMP showed no decrease in signal strength up to 10^0 trichomonads/mL and typical ladder-like band in agarose gel stained with SYBR Safe that can be accounted by the high amount of DNA product yield of LAMP compared to PCR (Mori et al., 2001). Both genital secretions and urine are complex body fluids and have popularly been used for nucleic acid-based detection of *T. vaginalis* with sensitivities varying from 81% to 100% for genital swabs and 64% to 100% for urine specimens (Huppert et al., 2007; Lawing et al., 2000; Schwebke and Burgess, 2004). In this study, all replicate runs for all dilutions of the spiked genital swab specimens yielded 100% positive, while the 10^0 cell/mL dilution of the spiked urine specimen yielded 62.5% positive. No inhibition of LAMP reaction occurred in spiked urine samples. However, the low positive rate for the 10^0 trichomonads/mL dilution could be accounted by possible errors during the preparation of the reaction mixture. Since this LAMP method utilized a wild-type enzyme, undesired activity from DNA polymerase during setup may result to irreproducible amplification. Recently, Tanner et al. (2012) utilized a warm start strand-displacing DNA polymerase that is inactive below 50 °C and stable at room temperature. Nevertheless, *T. vaginalis* LAMP performed with high analytical sensitivity in less than 90 min from sample preparation to LAMP product detection using spiked genital swab and urine specimens.

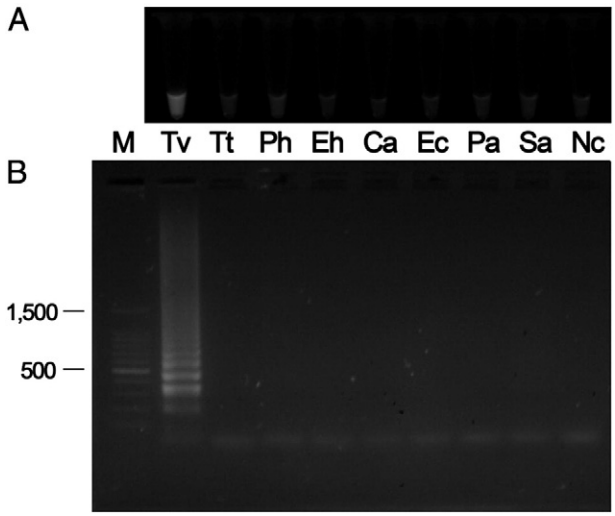

Fig. 3. Analytical specificity of the *T. vaginalis* LAMP was evaluated using DNA extracts of: Tv, *T. vaginalis*; Tt, *T. tenax*; Ph, *P. hominis*; Eh, *E. histolytica*; Ca, *C. albicans*; Ec, *E. coli*; Pa, *P. aeruginosa*; Sa, *S. aureus* as detected by (A) addition of SYBR Safe DNA Gel Stain (Invitrogen) and (B) 1.5% agarose gel electrophoresis stained with 1:10,000X dilution of SYBR Safe. Nc, nuclease-free water negative control; M, 100-bp DNA ladder (TaKaRa Bio).

The specificity of the *T. vaginalis* LAMP clearly showed no cross-reactivity to closely related trichomonad species found in humans like *T. tenax* and *P. hominis* and other microorganisms like *E. histolytica*, *C. albicans*, *E. coli*, *P. aeruginosa*, and *S. aureus* (Fig. 3). Therefore, the 2-kbp repeated DNA fragment of *T. vaginalis* as chosen target for LAMP performed with high specificity similar to previously reported PCR and real-time PCR assays (Caliendo et al., 2005; Lawing et al., 2000; Queza and Rivera, 2013; Schirm et al., 2007). The 6 primers recognizing 8 different sites in the target sequence make LAMP highly specific (Notomi et al., 2000; Tomita et al., 2008).

However, the LAMP method has its own disadvantages. Although the LAMP assay was very sensitive and specific, the high amount of DNA product produced by the reaction is prone to contamination due to opening of reaction tubes that may cause false-positive results. Most LAMP methods utilized addition of nucleic acid stains (i.e., SYBR Green I and Pico Green) post-incubation for detection of products, but these stains inhibit LAMP amplification. Thus, closed-tube monitoring of reaction by a real-time turbimeter (Mori et al., 2001), colorimetric detection by pre-addition of hydroxylnaphthol blue dye (Goto et al., 2009), or addition of wax barrier for SYBR Green I (Tao et al., 2011) could prevent contamination caused by opening of tubes for end-point LAMP result visualization.

In summary, the *T. vaginalis* LAMP assay developed in this study is simple, rapid, sensitive, and specific method for the detection of trichomoniasis. However, this is a proof-of-concept study, and further experiments must be conducted to validate the clinical diagnostic performance of the *T. vaginalis* LAMP assay. This efficient and cost-effective NAAT has a great potential application for accurate diagnosis and large-scale surveillance, screening, and control of trichomoniasis in point-of-care and resource-limited settings.

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