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Original papers

Loop-mediated isothermal amplification (LAMP) assay for the rapid detection of the sexually-transmitted parasite, *Trichomonas vaginalis*

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ABSTRACT. A loop-mediated isothermal amplification (LAMP) assay was developed to detect the sexually-transmitted parasite, *Trichomonas vaginalis* in vaginal swabs. The presence of *T. vaginalis* was detected from 121 female sex workers attending a social hygiene clinic in Balibago, Angeles City, Pampanga, Philippines using culture, polymerase chain reaction (PCR), and the developed LAMP assay. The high analytical sensitivity of LAMP detected a higher prevalence of *T. vaginalis* (42.06%) compared to culture (8.26%) and PCR (7.44%). Additionally, this assay did not cross-react with DNAs of other trichomonads that can infect humans such as *Trichomonas tenax* and *Pentatrichomonas hominis* as well as the pathogens, *Candida albicans* and *Staphylococcus aureus*. The LAMP assay developed had a limit of detection (0.036 ng/μl) lower than that of PCR using the primers TvK3 and TvK7 (0.36 ng/μl). Prevalence of *T. vaginalis* in female sex workers in this area of the Philippines may be higher than previously estimated. Discordant results of PCR and LAMP may be due to different reactions to different kinds of inhibitors in the vaginal swabs.

Key words: loop-mediated isothermal amplification (LAMP), limit of detection, PCR, culture, *Trichomonas vaginalis*

Introduction

Trichomonas vaginalis is a protozoan parasite that infects the urogenital system of humans. It is found worldwide but receives less attention compared to other agents of sexually transmitted infections (STIs) such as *Neisseria gonorrhoeae* and *Chlamydia trachomatis* [1–3]. The parasite is transferred through coitus, which causes trichomoniasis. Clinical manifestations of the disease include vaginal discharge, pruritus, and irritation [2]. Generally, the methods used in detecting this parasite in vaginal swabs are wet mount microscopy, culture, and nucleic acid amplification test (NAAT)-based kits [2]. Other types of assays can still be developed to fully understand the prevalence and mode of transmission of this parasite. Thus, one possible technique for its detection is the loop-mediated isothermal amplification (LAMP). This method relies on amplification of target DNA sequences using 2 to 3 specific primer pairs at a single temperature using the enzyme *Bst* polymerase [4–5]. Hence, it offers simple and rapid diagnosis. This method has already been developed for the rapid detection of several species of other protozoan parasites particularly *Trypanosoma* species [6–9], *Toxoplasma* species [10], *Cryptosporidium parvum* [11], *Plasmodium* species [12], *Entamoeba histolytica* [13–14], *Giardia duodenalis* [15], and *Tritrichomonas foetus* [16]. In line with this, the purpose of this study was to design a set of LAMP primers that can detect *T. vaginalis* DNA in vaginal swab samples.

Materials and Methods

Microbial cultures. Axenic cultivation of *T.*

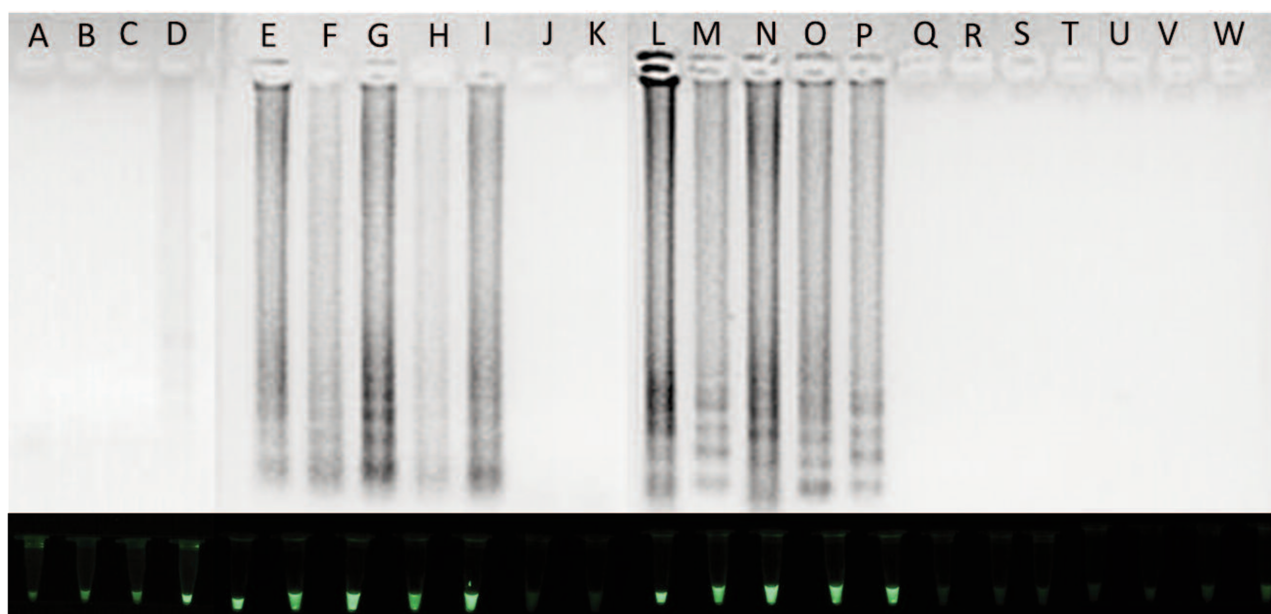

Fig. 1. Reaction time results and analytical sensitivity and specificity of the developed LAMP assay. The amplification temperature was standardized at 63°C incubated for: A – 15 min, B – 30 min, C – 60 min, D – 90 min. LAMP analytical sensitivity is shown at *T. vaginalis* DNA concentrations of: E – 360 ng/µl, F – 36 ng/µl, G – 3.6 ng/µl, H – 0.36 ng/µl, I – 0.036ng/µl, J – 0.0036 ng/µl, K – 0.00036 ng/µl. LAMP analytical sensitivity is shown at *T. vaginalis* cell counts of: L – 10^6 cells/ml, M – 10^5 cells/ml, N – 10^4 cells/ml, O – 10^3 cells/ml, P – 10^2 cells/ml, Q – 10 cells/ml, R – 1 cell/ml, S – 0.1 cells/ml. LAMP analytical specificity using the DNA of: T – *Pentatrichomonas hominis*, U – *Trichomonas tenax*, V – *Candida albicans*, W – *Staphylococcus aureus*.

vaginalis was grown in BI-S-33 medium [17], modified TYI-S-33 medium wherein the casein digest and yeast extract were substituted with BBL® Biosate peptone. Cultures of *Trichomonas tenax*, *Pentatrichomonas hominis*, *Candida albicans*, and *Staphylococcus aureus* were also cultivated for specificity assays. *T. tenax* and *P. hominis* were both grown in biphasic medium supplemented with horse serum (Life Technologies, CA, USA) [18] while *C. albicans* and *S. aureus* were grown in tryptic soy broth (Difco Laboratories, MD, USA) and were incubated at 37°C for 18–24 hours. DNA of the trichomonads was extracted after 3 to 4 days of incubation. On the other hand, DNA extraction of *C. albicans* and *S. aureus* were done immediately after 24h incubation.

Sample collection. Vaginal swab samples were taken from 121 female sex workers undergoing weekly check-ups at the Balibago Social Hygiene Clinic (BSHC) in Angeles City, Pampanga, Philippines. Informed consent was obtained from all subjects and this study was approved by the Ethics Review Committee of the Office of the City Health Officer, Angeles City, Pampanga. Two swabs were taken from each individual: one was placed in BI-S-33 complete medium for culture while the other was placed in phosphate-buffered saline (PBS) intended for DNA extraction. BI-S-33 medium samples were immediately placed at 37°C incubator and were microscopically observed for 5 days to check for the presence of growth. The PBS samples were stored at -4°C until DNA extraction.

DNA extraction. The Chelex DNA extraction method as described by Ong and Rivera [19] was

Table 1. Primers designed for LAMP detection of *T. vaginalis*

Primer name	Primer sequence (5'-3')
F3	ACCCTGGTAGTTCCTACCTT
B3	GGTAAGTTTCCCCGTGTTGA
FIP (F1c+F2)	TCCCCCAGAGCCCAAGAACTATTTTTACGATGCCGACAGGAGTT
BIP (B1c+B2)	CTACGACCGCAAGGCTGAAACTTTTTATTAAGCCACAGGCTCCAC

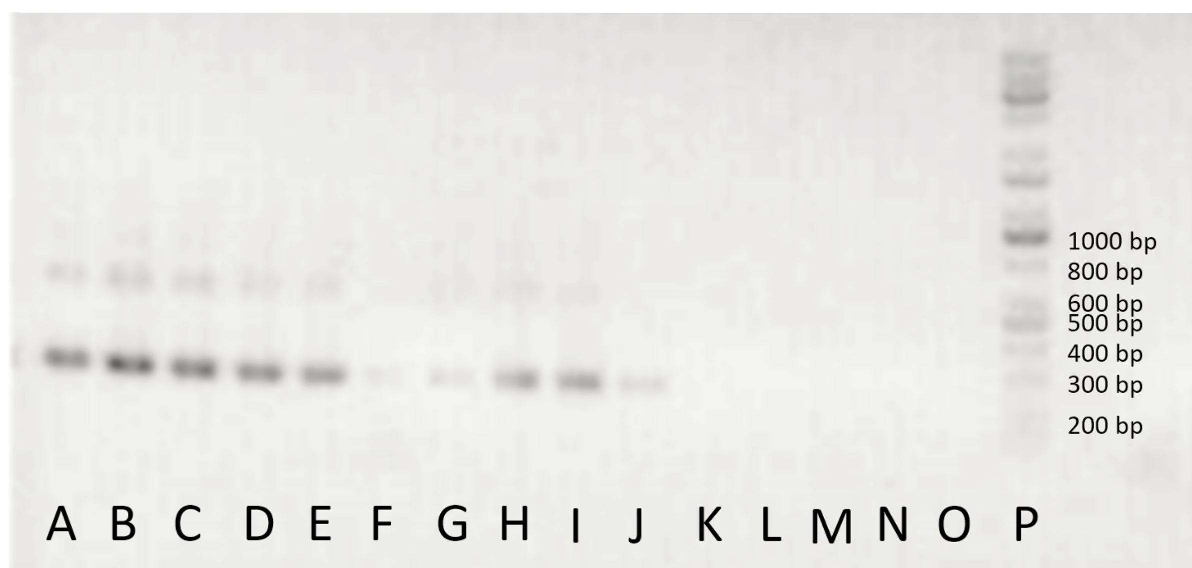

Fig. 2. Analytical sensitivity and specificity of direct DNA extraction from vaginal swabs (Chelex-100) and PCR using the primers TvK3 and TvK7. PCR analytical sensitivity is shown at DNAs extracted from *T. vaginalis* cell counts of: A – 10^6 cells/ml, B – 10^5 cells/ml, C – 10^4 cells/ml, D – 10^3 cells/ml, E – 10^2 cells/ml, F – 10 cells/ml. PCR analytical sensitivity is shown at *T. vaginalis* DNA concentrations of: G – 360 ng/µl, H – 36 ng/µl, I – 3.6 ng/µl, J – 0.36 ng/µl, K – 0.036 ng/µl, L – 0.0036 ng/µl, M – 0.00036 ng/µl, N – TE buffer (blank), O – 0 cells/ml (culture medium only), P – DNA ladder.

performed for all cultures and swab samples used in this study. *T. vaginalis* cultures were harvested and pooled to 10^6 cells per ml after 3–4 days. These samples were washed 2 to 3 times with PBS before serially diluting into 10^6, 10^5, 10^4, 10^3, 10^2, 10, 1, and 0.1 cells per ml. Cells were re-suspended in 100 µl of sterile distilled water before addition of 200 µl of 5% Chelex-100 (Sigma). The re-suspended mix was incubated at 56°C for 30 min and was mixed briefly afterwards. These were then placed in a boiling water bath for 8 min and centrifuged at 13,000 rpm for 2 min. The supernatant containing the DNA was collected and stored at -4°C until use. The same protocol was used on 1.5 ml of vaginal swab samples re-suspended in PBS.

The concentration of DNA extract from 10^6 cells per ml of *T. vaginalis* was determined using NanoDrop 2000c (Thermo Scientific). This was serially diluted to produce the following concentrations: 360, 36, 3.6, 0.36, 0.036, 0.0036, and 0.00036 ng/µl.

Primer design and LAMP reaction mix. The primers used were designed from the 18S rRNA region of the NDMR 100 18S SSU-rRNA strain of *T. vaginalis* (GenBank reference no. AY338475) using Primer explorer v.4.0 (https://primerex - plorer.jp). These were checked for specificity through alignment with different *T. vaginalis* 18S rRNA strain sequences and other closely-related trichomonad species. Moreover, basic local alignment search tool (BLAST) was used to further check primer specificity. Table 1 shows the LAMP primers designed and used in this study.

All reactions were performed using the following reagent mix: 4 mM $MgSO_4$, 1.064 M betaine, 1.6 µM FIP, 1.6 µM BIP, 0.2 µM F3, 0.2 µM B3, 0.4 mM of dNTPs, 1X Thermopol buffer [20mM Tris-HCl, 10mM $(NH_4)_2SO_4$, 10mM KCl, 2mM $MgSO_4$, 0.1% Triton® X-100 pH 8.8], 8U of *Bst* polymerase large fragment (Biotechnologies, New England), and 2 µl of DNA extract. The reagents were divided into 4 reaction mixes: Primer A (FIP, BIP, F3, B3, and dNTPs), Buffer B (1X Thermopol buffer, $MgSO_4$, and betaine), *Bst* polymerase, and nucleotide-free distilled water. Reagents were mixed except for the polymerase which was thawed at 4°C and stored in a cold rack before mixing. Results were visualized after the addition of 1µl of 1000x SYBR Safe (Invitrogen). Fluorescent green reactions were considered positive while those that remained orange were considered negative. Samples that did not fluoresce were run in an agarose gel to confirm the presence of ladder-like DNA band patterns for positive results.

Reaction time. Optimum LAMP reaction time was determined using DNA extracts from 10^6 cells per ml of *T. vaginalis* as positive control and

Table 2. *T. vaginalis* detection using culture, PCR, and LAMP on 121 vaginal swab samples from the Balibago Social Hygiene Clinic, Angeles City, Pampanga, Philippines

Method of Detection			N	Percent (%)
Culture	PCR	LAMP		
+	+	+	6	4.96
+	+	–	2	6.61
+	–	+	1	0.83
+	–	–	1	0.83
–	+	+	1	0.83
–	+	–	0	0.00
–	–	+	45	36.36
–	–	–	65	53.72

nuclease-free distilled water as negative control. Reaction mixtures were incubated at 63°C for 15, 30, 60, and 90 min followed by 80°C for 2 min for the deactivation of the *Bst* polymerase.

Analytical sensitivity and specificity. *T. vaginalis* DNA extracts from serially-diluted DNA (360, 36, 3.6, 0.36, 0.036, 0.0036, and 0.00036 ng/μl) and serially-diluted cells of *T. vaginalis* (10^6, 10^5, 10^4, 10^3, 10^2, 10, 1, and 0.1 cell per ml) were tested with the LAMP assay to determine its analytical sensitivity. Results were compared with PCR using the primers Tvk3 and Tvk7 – which also specifically amplify the 18S SSU-rRNA segment of *T. vaginalis* – similar to the protocol of Crucitti et al. [20]. Analytical specificity was determined by testing the LAMP assay with DNA of *P. hominis*, *T. tenax*, *S. aureus*, and *C. albicans*.

Field test of the developed LAMP assay. The optimized LAMP assay was also tested on the DNAs extracted from vaginal swabs collected from the BSHC. Results were compared to culture and PCR methods.

Results

Analytical specificity and sensitivity. The LAMP assay developed had an optimum incubation time of 90 min at 63˚C. Its limit of detection was 100 cells/ml of *T. vaginalis* when used on 1:10 serial dilutions of 10^6 cells/ml of *T. vaginalis*. On the other hand, its limit of detection was 0.036 ng/μl *T. vaginalis* DNA when used on 1:10 serial dilutions of 360 ng/μl *T. vaginalis* DNA. The LAMP assay did not cross-react with DNA extracts from *P. hominis*, *T. tenax*, *C. albicans*, and *S. aureus* (Fig. 1). Similar to LAMP, PCR using primers TvK3 and TvK7 also had a limit of detection of 100 *T. vaginalis* cells/ml when used on 1:10 serial dilutions of 10^6 cells/ml of *T. vaginalis*. However, its limit of detection was 0.36 ng/μl when used on 1:10 serial dilutions of 360 ng/μl of *T. vaginalis* DNA (Fig. 2).

Field test of the developed LAMP assay. Out of the 121 samples collected, *T. vaginalis* was detected on 53 (42.06%) samples employing the LAMP assay, 10 (8.26%) using the culture method and 9 (7.44%) using PCR. Eight samples were positive for all 3 methods. Moreover, 44 samples were only positive for LAMP while 2 samples were only positive for culture and PCR but not LAMP (Table 2).

Discussion

The developed LAMP assay for detecting *T. vaginalis* in vaginal swabs is a rapid and easy-to-perform method with high analytical sensitivity and specificity. The Chelex DNA extraction protocol followed by LAMP assay can be performed within a few hours. This is faster compared to culture and PCR. *T. vaginalis*-positive cultures were observed after 3–5 days. On the other hand, PCR can also be performed with the same amount of time as LAMP but additional time is required to view results in an agarose gel. The LAMP assay requires only addition of a visualizing agent (e.g., SYBR Safe) to quickly obtain results. Moreover, the LAMP assay only requires 2 sets of temperatures (63°C for 90 min followed by 80°C for 2 min) that can be performed in a simple water bath unlike PCR, which requires a thermal cycler. Hatano et al. [21] have even shown that a LAMP assay for detecting *Bacillus anthracis* can be performed with a simple pocket heater.

The prevalence of *T. vaginalis* in vaginal swabs from female sex workers in BSHC was noted as 42.06% using LAMP, 8.26% using culture, and 7.42% using PCR. Similarly, *T. vaginalis* prevalence has been recorded to be as high as 37.93% of the sample population in women attending sexually-transmitted disease (STD) clinics in India [22], and 43.2% in female sex workers in China [23]. Prevalence higher than 40% has also been observed in high risk groups such as pregnant inmates [24], female detainees of a juvenile detention center, [25] and men attending STD clinics. [26–27] In contrast, Ong and Rivera in 2010 [19] reported only a prevalence of 9.56% in sex workers undergoing weekly medical check-ups at

the Reproductive Health and Wellness Center in Angeles City, Pampanga using PCR with primers TvK3 and TvK7. The developed LAMP assay had a 10-fold lower limit of detection (0.036 ng/µl *T. vaginalis* DNA) compared to PCR using primers TvK3 and TvK7 (0.36 ng/µl *T. vaginalis* DNA). Previous studies have proven that LAMP has a 10- to 1,000-fold lower limit of detection compared to conventional PCR. [6–9,28–30] A LAMP assay designed to detect *Cyrptosporidium parvum* was even shown to have a 100,000-fold lower limit of detection compared to PCR [11].

In a separate study, Reyes et al. [31] detected *T. vaginalis* DNA in spiked genital swab samples and spiked urine samples using a LAMP assay with different sets of primers. The detection limit of LAMP was 10^0 trichomonads/ml or 1 trichomonad, either by gel electrophoresis, turbidity, or addition of SYBR Safe. In comparison, the detection limits for PCR were 10^3 trichomonads/ml or 1000 trichomonads and 10^1 trichomonads/ml or 10 trichomonads for spiked genital swab samples and spiked urine samples, respectively. Thus, it was stated that higher sensitivity of LAMP for detection of trichomonads was established due to the tolerance of *Bst* DNA polymerase to NAAT inhibitors.

It is possible that the LAMP assay may be able to detect DNA from very small numbers of *T. vaginalis* as well as dead cells from previous infections. This high sensitivity also makes LAMP more susceptible to contamination [15]. However, LAMP was replicated for a subset of vaginal swab extracts with extra care given to have the least amount of contamination possible (e.g. wiping the surface of the laminar flowhood and pipettors with DNAse away®, quickly opening and closing tubes) and the results obtained were consistently similar. Moreover, the developed LAMP assay was highly specific to *T. vaginalis*. It did not amplify the DNAs of two other common microorganisms that infect human vaginas (*S. aureus* and *C. albicans*) as well as other two trichomonads known to infect humans (*T. tenax* and *P. hominis*). In particular, Crucitti et al. [32] have reported detection of *P. hominis* – which commonly infects the digestive tract of humans – in self-obtained vaginal swabs. LAMP and PCR had similar analytical sensitivity when used on Chelex DNA extracts of 1:10 dilutions of *T. vaginalis* cells (100 cells/ml). However, the presence of mucus in the vaginal swabs may have trapped more *T. vaginalis* cells or DNA compared to serially diluted *T. vaginalis* cells in PBS even though the same DNA extraction method was used in clinical samples. Thus, it is highly likely that *T. vaginalis* prevalence in sex workers in this study is higher than previously expected. Clinical sensitivity and specificity were not computed because of the difference in analytical sensitivities of LAMP and PCR. NAATs (e.g., PCR, DNA probes) should have equal sensitivities when comparing results since differences can lead to biases in computing for clinical sensitivity and specificity [33]. Positive results of tests with higher sensitivity cannot be properly confirmed by tests with lower sensitivity leading to false positives [34]. The LAMP assay did not detect presence of *T. vaginalis* in 2 PCR-positive samples. These conflicting results could be due to different effects of inhibitors present in the vaginal swabs to LAMP and PCR. More than 1% contamination of biological substances such as blood and urine or more than 30% of saline solution or PBS can inhibit LAMP as well as PCR reactions [35]. Raw milk contaminants can even inhibit LAMP but not PCR [36].

Conclusions

In conclusion, the developed LAMP assay in this study is a rapid, specific, and highly sensitive method for detecting *T. vaginalis* in vaginal swab samples. Studies on effects of various inhibitors on the LAMP assay as well as comparison with NAAT with equal sensitivity (e.g. real-time PCR) can further elucidate its efficiency in diagnosis in clinical samples.

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