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Xylenol orange-based loop-mediated DNA isothermal amplification for sensitive naked-eye detection of *Escherichia coli*

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ABSTRACT

Loop-mediated isothermal amplification (LAMP) can amplify DNA specifically and sensitively. Under minimal buffering conditions, it produces hydrogen ions that lower the pH of the solution upon DNA amplification. This characteristic was applied to visually detect amplified DNA of *Escherichia coli* through the use of Xylenol Orange, a pH-dependent dye. Under the optimal conditions, 120 min at 63 °C, the Xylenol orange-dependent colorimetric LAMP revealed a detection limit as low as 1 CFU, namely 100,000 times more sensitive than typical multiplex PCR, and showed no cross-reactions with other foodborne pathogens. The colorimetric assay was successfully exploited to detect *E. coli* contaminations in milk samples, showing high reliability and the same high sensitivity with naked-eye readout. Together with robustness, simplicity, and visual detectability of amplification, this assay can serve as an alternative tool to PCR for detecting *E. coli*, which is suitable for both laboratory and on-field applications.

1. Introduction

Escherichia coli, Gram-negative, facultative anaerobic bacterium, is a foodborne pathogen commonly found in human and warm-blooded animal feces (McFeters et al., 1974). It has been established as the most reliable indicator of human fecal contamination to predict the quality of potable water since 1986 (Maheux et al., 2009). Since then, several polymerase chain reaction (PCR)-based detection protocols have been developed to serve as a substitute to conventional culture-based methods (APHA, 2005) to detect *E. coli* (Juck et al., 1996; Tsen et al., 1998; Frahm and Obst, 2003; Horakova et al., 2008; Sandhya et al., 2008; Pavlovic et al., 2011). However, these protocols require the specialized thermal cycling equipment and technical expertise, which often limit their availability to centralized reference laboratories.

Loop-mediated DNA isothermal amplification (LAMP) is an alternative approach for rapid and specific DNA amplification under single temperature conditions (usually in the 60–65 °C range) (Notomi et al., 2000). Analysis of LAMP products (amplicons) is usually carried out by gel electrophoresis (AGE), lateral flow dipstick (LFD) (Kiatpathomchai et al., 2008; Jaroenram et al., 2009; Puthawibool et al., 2009; Arunrut et al., 2011), or in real-time (Lin et al., 2015; Mashooq et al., 2016; Sakai et al., 2017), which typically require sophisticated equipment and extended workflow time. Interestingly, during LAMP amplification, large amounts of pyrophosphate moieties and hydrogen ions are formed as by-products (Notomi et al., 2000; Mori et al., 2001). In the presence of weakly buffered or non-buffered solutions, such by-products significantly lower the initial alkaline pH of the LAMP solution to a final acidic pH, typically around 6.0–6.5 (Tanner et al., 2015). This significant change in pH value offers the possibility of visually detecting DNA amplification through the use of pH-sensitive dyes (Tanner et al., 2015).

Xylenol orange (XO) is one of the pH-dependent indicators that are low-priced, simple to use, exhibit easy-to-see color changes (yellow < pH 6.7 < violet), and commonly used in laboratory for metal titration (http://www.csun.edu/~hcchm003/321/321120313.pdf). To the best of our knowledge, combining this dye with LAMP has not been used to detect any pathogens. Therefore, the approach was examined here to determine whether it would efficiently and colorimetrically detect *E. coli*.

2. Materials and methods

2.1. Bacterial strain and DNA template preparation

Escherichia coli used in this study was a reference strain ATCC® 10536™ purchased from Microbiologics® (MN, USA). It was re-cultured on LB agar medium at 37 °C for 17 h. A single colony was selected for further amplifying in 2 ml LB broth medium for 16 h. One milliliter of

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the bacterial suspension was ten-fold serially diluted in distilled water and utilized for colony-forming unit (CFU) assay by the standard spread-plate technique. The remaining suspension (1 ml) with known concentration (10^6 CFU/ml) by the spread-plate technique was subjected to DNA extraction using DNAzol® Reagent (ThermoFisher Scientific, CA, USA). Briefly, the *E. coli* suspension was pelleted at 5000*g* for 10 min. The pellet was re-suspended in 1 ml of DNAzol® Reagent. After incubating for 15 h at room temperature, and boiling for 10 min to allow a complete digestion of *E. coli* cells, 500 µl of 100% Isopropanol were added with vigorous mixing. The tube was placed on ice for 10 min before being centrifuged at 12,000*g* for 10 min. The DNA pellet was washed with 70% (v/v) ethanol, air dried and dissolved in 300 µl of DNase-free water, followed by incubation at 65 °C for 10 min to allow the DNA dissolve completely. Quality and concentration of DNA, relative to the CFU value obtained above, were measured by spectrophotometric analysis at 260 and 280 nm. The DNA was serially diluted to prepare stocks containing 5×10^6–5×10^{-4} CFU/µl. Two microliters of the templates were used in the following experiments, unless otherwise stated.

2.2. LAMP primer design and initial LAMP condition

LAMP primers were designed from the outer membrane protein-encoding sequence (*yaiO* gene) of *E. coli* genome (GenBank accession no. NC_000913.3). The details of the primers are shown in Table 1. The primers were synthesized by Integrated DNA Technologies, IA, USA. To determine the optimal temperature for LAMP, reactions were performed on T100™ Thermal Cycler (BIO-RAD, CA, UAS) at 60, 63 and 65 °C for 1 h, followed by heat inactivation at 90 °C for 2 min. The products were analyzed by 2% agarose gel electrophoresis (AGE). The LAMP reaction mixture contained 2 µM each of inner primers (FIP and BIP) and loop primers (LF and LB), 0.2 µM each of outer primers (F3 and B3), 1.4 mM of dNTP mix (New England Biolabs, MA, USA), 0.6 M Betaine (Sigma-Aldrich, MO, USA), 4 mM MgSO4 (NEW England Biolabs, MA, USA), 8 U *Bst* 2.0 DNA polymerase (New England Biolabs, MA, USA), 1× supplied buffer, and the specified amount of template DNA in a final volume of 25 µl. DNA-free LAMP reactions were included as negative controls.

2.3. Optimization of colorimetric LAMP assay

To combine Xylenol Orange (XO) (Sigma-Aldrich, MO, USA) with LAMP into a colorimetric assay, LAMP requires minimal buffering conditions. Therefore, the commercial strong buffer presenting in a standard LAMP reaction (see Section 2.2) need to be changed to minimal buffer solution. In this regard, 10× low-buffer solutions with different pH values (8.0, 8.5 and 9.0) were prepared. The buffer solutions were made up from 100 mM $(NH_4)_2SO_4$, 500 mM KCl, 20 mM $MgSO_4$, and 1% v/v Tween-20, and were adjusted to the desired value pH by KOH. They were then used in determining the optimal pH for colorimetric LAMP assays. To do so, reactions containing 1× low buffer-solution having the initial pH of 8.0, 8.5 and 9.0 were performed at the optimal temperature for 2 h (to allow maximum amplification) using 10^4 CFU-derived DNA (2 µl of 5×10^3 CFU/µl stock) as template. The results were analyzed by AGE. The products were then dipped with Litmus papers in order to evaluate the drop in pH caused by LAMP amplification. The resulting pH values were compared to the pH key.

To optimize XO concentration, LAMP reactions containing 10^7 (positive control), 1, 0.1 CFU/reaction, and distilled water (negative control) were prepared. After adding XO to achieve final concentrations of 0.12, 0.06, 0.012 mM, the reactions were incubated at the optimal temperature for 60 min. Dye-free reactions were also included as reaction negative controls. The mixtures were examined visually for any color change after the reactions completed, and the results were compared to those by AGE.

2.4. Comparative molecular sensitivity of LAMP-colorimetric assay, LAMP-AGE, and multiplex PCR-AGE

Various amounts of *E. coli*-DNA (10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10, 1, 0.1, 0.01 CFU) were used as templates for colorimetric LAMP assays and LAMP-AGE. Both protocols were carried out at the optimal temperature for 45, 60, 75, 90, 120 and 180 min. The same DNA templates were also amplified by multiplex PCR following the protocol described previously (Molina et al., 2015), and the results were compared to those from LAMP assays.

2.5. Specificity of colorimetric LAMP assay

The specificity of *E. coli*-LAMP colorimetric assay was examined under optimal conditions using 100 ng of DNA extracted from pure culture of *E. coli*, *Vibrio parahaemolyticus*, *Bacillus cereus*, *Salmonella enterica*, *Campylobacter jejuni*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Clostridium perfringens* as templates. Reactions with 10^7 CFU-derived DNA and distilled water were included as positive and negative control, respectively. The results were confirmed by AGE.

2.6. Colorimetric detection of milk contaminations

The colorimetric LAMP was exploited for the detection of *E. coli* contaminations in milk using 83 milk samples: 51 were spiked with various concentrations of *E. coli* and 32 were non-adulterated. To prepare the contaminated milk samples, the *E. coli* suspension, cultured as previously indicated (Section 2.1), was serially diluted in water to prepare the bacterial stocks of 10^7, 10^6 and 10^5 CFU/ml. Ten microliters of each stock were further diluted in 990 µl uncontaminated milk to the final concentration of 10^5, 10^4 and 10^3 CFU/ml, respectively. The CFU number was checked by the plate count assay (reference method). The DNA extraction for the contaminated milk samples was performed according to the previously described protocol (Section 2.1). One microliter of each resulting extraction product (100, 10, 1 CFU/µl) was tested with the optimized colorimetric LAMP, followed by AGE. The non-adulterated samples were submitted to the same procedure. The results were used to evaluate the diagnostic performance of the colorimetric LAMP protocol following equations given in Table 2.

Table 1
Primer sequences for the *E. coli*-LAMP/colorimetric assay.

Name of primers	Position on *yaiO* gene	Sequence (5′-3′)
yaiO2.F3	169–188	CCAGTCATAGGTGTAAGCAC
yaiO2.B3	447–428	CGTCAGGATATTAACCTGGC
yaiO2.FIP	286–267/TTTT/208–227	CAGCGATGCAGGTGGTAGTT/TTTT/TAGTTGCGTATAACCAGTGC
yaiO2.BIP	298–319/TTTT/379–360	GGTATAGCGGTAGCTGGTGATC/TTTT/TTACGATGATGTCGAAGTCG
yaiO2.LF	252–235	TCCGTGCGTCTGAATGAC
yaiO2.LB	324–344	GGCCAGTATAGAGTGATACGC

Table 2
Diagnostic performance of the colorimetric LAMP protocol.

Colorimetric LAMP	*E. coli* status by reference assay (spread-plate technique)	
	Positive	Negative
Positive	51 (TP)[a]	0 (FP)
Negative	0 (FN)	32 (TN)
%	100 (sensitivity)	100 (specificity)
%	100 (accuracy of colorimetric LAMP result)	

Sensitivity = [TP/(TP + FN)] * 100, specificity = [TN/(TN + FP)] * 100.
Accuracy = [(TP + TN)/(TP + TN + FN + FP)]*100.
[a] TP, true positive; FP, false positive; FN, false negative; TN, true negative.

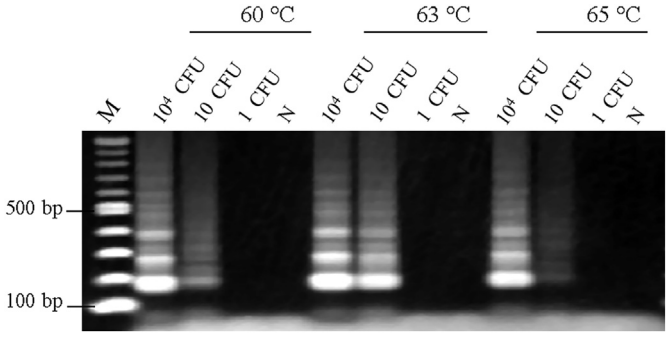

Fig. 1. Optimization of LAMP reaction temperature using various amounts of CFU-derived DNA of *E. coli* as template. The reactions were incubated at the given temperatures for 1 h. Lanes M and N: molecular marker and negative control (DNase-free water), respectively.

3. Results

3.1. Determination of initial LAMP conditions

LAMP reactions at 60, 63 and 65 °C with the templates of 10^4, 10, and 1 CFU/reaction showed that 63 °C yielded the highest amounts of DNA products (Fig. 1). Therefore, 63 °C was selected as the optimal temperature.

3.2. Optimization of colorimetric LAMP assay

LAMP reactions carried out using low-buffer solutions with different initial pH values (8.0, 8.5, 9.0) identified the pH 8.5 as optimal, as it yielded the highest amounts of DNA products when 10^4 CFU-derived DNA template/reaction was used (Fig. 2A). When LAMP products from this optimal pH were dipped with Litmus paper, a significant drop in pH from the initial value of approximately pH 8.5 (negative control, N) to a final value of pH 6.0–6.5 (10^4 CFU/reaction) was detected (Fig. 2B). This suggests that detecting DNA amplification through the change in pH value is possible.

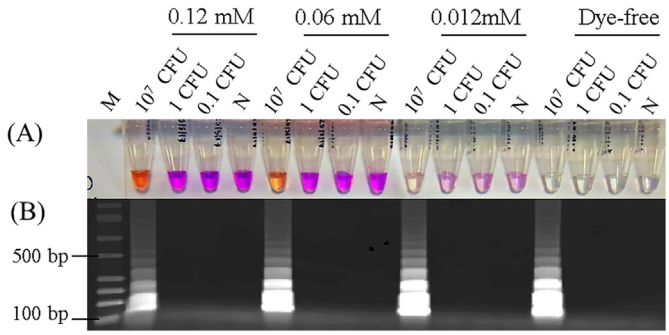

Fig. 3. Effect of Xylenol Orange (XO) on LAMP reactions performed at 63 °C for 60 min using various amounts of *E. coli*-DNA as template. (A) Colorimetric results for LAMP products at different concentrations of XO (B) AGE results corresponding to the LAMP result shown in (A). Lanes M and N: molecular marker and negative control (DNase-free water), respectively.

As for XO optimization, LAMP reactions containing different amounts of XO (0.12, 0.06, 0.012 mM) were performed, and the result showed that the final concentration of 0.12 and 0.06 mM resulted in clear color differences between positive (orange for 0.12 mM, and yellow-orange for 0.06 mM) and negative test results (purple) (Fig. 3A). However, 0.06 mM gave slightly higher amplification yield (Fig. 3B); thus, it was chosen as optimal.

3.3. Comparative sensitivity of LAMP-colorimetric assay, LAMP-AGE, and multiplex PCR-AGE

From colorimetric LAMP reactions incubated for various times (45–180 min) using different amounts of DNA template (Fig. 4A), the color change from purple (negative result) to reddish/orange (positive result) started to be detectable at 100 CFU/reaction after 45 min, while those for 10 and 1 CFU become visible after 60 and 90 min, respectively. All reactions with templates lower than 1 CFU remained purple even after 180-min incubation. The results were in accordance with those by AGE (Fig. 4B). However, to allow maximum DNA amplification and maximum purple-to-yellow color development, 120 min was selected as the standard assay time. The naked-eye detection limit (DL) of our assay was thus 1 CFU/reaction. Using the same set of DNA template, the DL of multiplex PCR was 10^5 CFU/reaction, namely

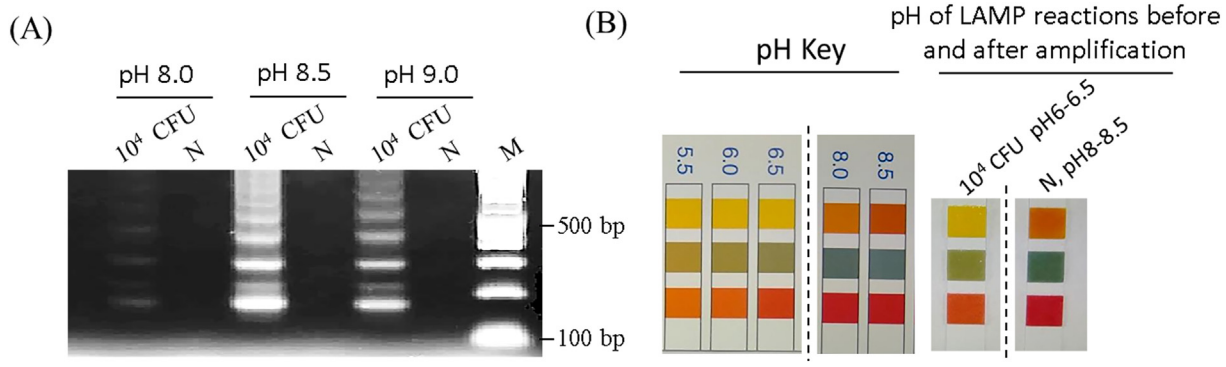

Fig. 2. Effect of initial pH values on LAMP. To allow maximum amplification, the reactions were carried out at 63 °C for 2 h. (A) AGE results of LAMP reactions at different initial pH values with the template of 10^4 CFU-derived DNA/reaction. Lane M and N: molecular marker and negative control (DNase-free water), respectively. (B) pH values corresponding to the AGE results showed in (A). After AGE analysis, the leftover products from the initial pH 8.5 reactions were dipped with Litmus papers, and their pH was read. Comparison with a pH legend gives an estimated final pH of 6.0–6.5, representing an approximately 2 pH unit drop due to amplification. pH strips and key were recorded by photographing, with the dashed line on them indicating a cut/paste junction.

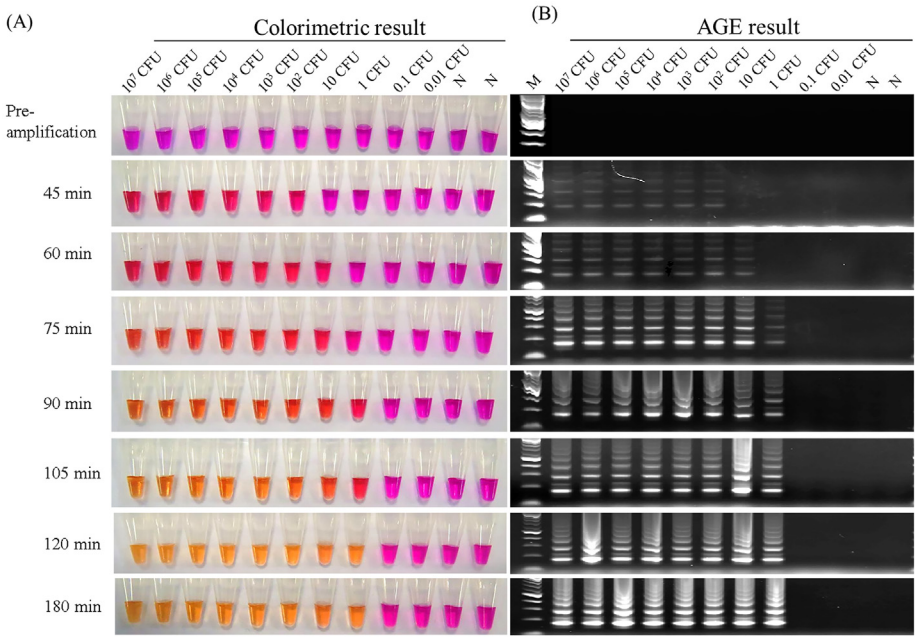

Fig. 4. Sensitivity of the colorimetric LAMP assay and LAMP-AGE. (A) Colorimetric results read by naked-eye at different time points after amplification. The LAMP reactions were performed at pH 8.5 using 2 µl of 10-fold serially diluted CFU-derived DNA extracts (5×10^{6}–5×10^{-3} CFU/µl) to make the final concentration of 10^{7}–10^{-2} CFU/reaction. (B) LAMP-AGE results using the same templates as in (A). Lanes M and N: molecular marker and template-free LAMP reaction (negative control), respectively.

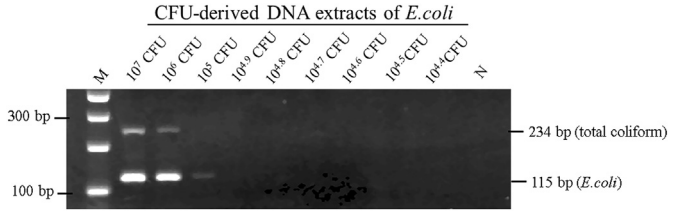

Fig. 5. Sensitivity of multiplex PCR carried out using various amounts of *E. coli*-DNA as template. Lanes M and N: molecular marker and negative control (DNase-free water), respectively. The expected size of *E. coli* was 115 bp.

100,000 times less sensitive than our LAMP assays (Fig. 5).

3.4. Specificity of colorimetric LAMP assay

Colorimetric LAMP assay with 100 ng of DNA of various foodborne pathogens gave a yellow orange positive result only for *E. coli*. With all other pathogens, reactions remained purple (Fig. 6A). These were supported by AGE (Fig. 6B), indicating that the colorimetric LAMP assay was highly specific for *E. coli* detection.

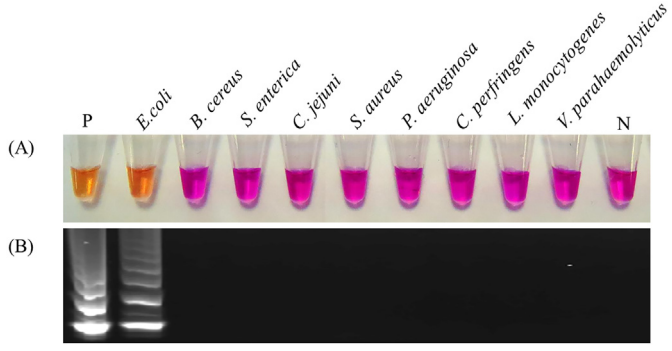

Fig. 6. Specificity of colorimetric LAMP assay. (A) Colorimetric results for LAMP products obtained from amplifying 100 ng of DNA extracted from various pathogens. (B) AGE results for the same LAMP amplicons as in (A). Lanes P and N: 10^{7} CFU/reaction of *E. coli* (positive control), and distilled water (negative control), respectively.

3.5. Colorimetric and sensitive detection of E. coli in milk samples

We prepared 83 milk samples to be tested with the colorimetric LAMP. Interestingly, all the 51 contaminated specimens with different contamination levels (100, 10 and 1 CFU/reaction) displayed a yellow color (positive result), while all the 32 negative controls had a purple color (negative result, Fig. 7A). These colorimetric results, further confirmed by AGE (Fig. 7B), underlined that the 1 CFU/reaction sensitivity was preserved in milk matrices, and that the reaction was 100% reliable. Table 2 summarizes the diagnostic performance of the colorimetric LAMP assay. As clearly seen from 83 samples tested, false positive (FP) and false negative (FN) results were not detected by both spread-plate technique (reference method) and our assay. Assuming the reference method was correct, the statistical sensitivity and specificity (i.e. the ability of the method to identify correctly the actual *E. coli*-positive and negative samples tested, respectively) and the accuracy of the colorimetric LAMP to produce test result was 100%.

4. Discussion

The *E. coli* detection platform described herein employs LAMP for sensitive and specific amplification of a target nucleic acid fragment, and XO-dependent colorimetric reaction for visualizing the presence or absence of the amplicon by naked-eye. The assay targets the consensus sequence of orphan gene *yaiO*, which is conserved across diverse lineages of *E. coli* and is not shared by other foodborne pathogens,

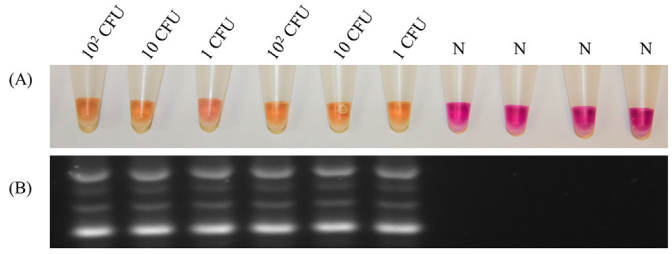

Fig. 7. Colorimetric detection of *E. coli* contaminations in milk samples. (A) Representative colorimetric results for LAMP reaction on 6 contaminated milk samples: 10^{2}, 10 and 1 CFU/reaction, and 4 non-contaminated (N). (B) Corresponding AGE results for the LAMP products in (A).

based on BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/).

The initial LAMP temperature was optimized to 63 °C, as this temperature gave the highest amplification efficiency among 60, 63, and 65 °C. For visual detection, we first investigated the amplification efficiency of LAMP in minimal buffering conditions and observed a range of pH tolerance (pH 8.0–9.0), with the most efficient LAMP reactions occurring at pH 8.5 (Fig. 2A). The LAMP products from this optimal pH showed a significant drop in pH, from the initial value of approximately pH 8.5 to a final value of pH 6–6.5 (Fig. 2B). Such ≥2 pH unit decrease enables, in principle, the use of pH-sensitive dyes to monitor amplification success in LAMP reactions as suggested by Tanner et al. (2015).

We next evaluated the effect of XO concentrations on LAMP reactions. Performing LAMP using low-buffer, pH 8.5, with a control DNA template (10^7 CFU/reaction) and 0.012–0.12 mM XO, had effects on color changes; in particular, 0.12 and 0.06 mM resulted in significant color differences between positive (orange for 0.12 mM, and yellow for 0.06 mM) and negative test results (purple) (Fig. 3A). However, 0.12 mM gave slightly lower amplification yield (Fig. 3B). This may indicate the loss of reaction efficiency when XO at 0.12 mM or higher is presented. Thus, 0.06 mM was selected as optimal. Having demonstrated that XO is compatible with LAMP reactions, we next examined the detection sensitivity at different time points. A detection limit (DL) when using XO is shown in Fig. 4A where a range of *E. coli*-DNA amounts (1–10^7 CFU/reaction) was detected. At 90–120 min, all template concentrations indicated amplification (orange/yellow), except those below 1 CFU/reaction remaining negative (purple) (Fig. 4A). Further increasing the incubation time did not improve the sensitivity, the color only shifted from reddish (at 90–105 min) to yellow (at 120 min), and remained stable till the end of experiment (180 min), indicating that the colorimetric reactions reached its end point. At 120 min (standard amplification time), the colorimetric LAMP assay showed a reproducible DL of 1 CFU, which was 100,000 times more sensitive than the multiplex PCR (Fig. 5). The protocol also possessed high specificity against non-related DNA templates (Fig. 6). The high degree of specificity demonstrated by this assay was attributed to the use of 6 accurately designed primers recognizing eight independent sequences to capture target DNA specifically.

Finally, we validated our colorimetric LAMP on real samples. We selected milk as a matrix because it is one of the major staples consumed worldwide, and contamination of *E. coli* in milk and dairy products is a major concern for health-related hazards in human being (Kumar and Mondal, 2015). By detecting spiked *E. coli* in milk at different levels of contamination, the colorimetric results displayed that the complex matrix of the starting material did not interfere with the reaction performances and sensitivity (1 CFU/reaction). Therefore, there was not necessary to introduce additional sample processing/purification steps in the DNA extraction procedure. Moreover, the absence of false positive and false negative results on 83 replicates indicates that the statistical sensitivity, the specificity and the reliability of the reaction are outstanding and suitable for on-field applications (Table 2).

In summary, the colorimetric LAMP assay described herein required ~120 min to complete, about half that of multiplex PCR-AGE (Molina et al., 2015). The method abrogates the need for gold-nanoparticle-functionalized probes and post-amplification steps required for colorimetric analysis previously combined with LAMP for the detection of other pathogens (Jaroenram et al., 2012; Seetang-Nun et al., 2013). Pre-enrichment of *E. coli* in appropriate media as required in conventional culture-based methods is not necessary in our colorimetric LAMP, bringing the diagnosis of *E. coli* one step closer to the ideal point-of-use and point-of-care testing. High sensitivity and specificity, and the relatively short detection time are key advantages of this protocol. In addition, it serves as a model platform that could be adapted easily to detect other pathogens or genetic markers of interest using basic laboratory equipment. However, to make it fully practical, simple and robust DNA extraction methods should be combined. Since LAMP has been reported to be less sensitive to inhibition by sample components than PCR, and that the DNA extraction step can be omitted (Kaneko et al., 2007; Teng et al., 2007; Jaroenram et al., 2009), future studies should focus on investigating the feasibility of using minimally processed samples with our colorimetric LAMP assay.

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References

APHA/AWWA/WEF, 2005. Standard Methods for the Examination of Water and Wastewater, 21st ed. American Public Health Association/American Water Works Association/Water Environment Federation, Washington, DC.

Arunrut, N., Prombun, P., Saksmerprome, V., Flegel, T.W., Kiatpathomchai, W., 2011. Rapid and sensitive detection of infectious hypodermal and hematopoietic necrosis virus by loop-mediated isothermal amplification combined with a lateral flow dipstick. J. Virol. Methods 171, 21–25.

Frahm, E., Obst, U., 2003. Application of the fluorogenic probe technique (TaqMan PCR) to the detection of *Enterococcus spp*. and *Escherichia coli* in water samples. J. Microbiol. Meth. 52, 123–131.

Horakova, K., Mlejnkova, H., Mlejnek, P., 2008. Specific detection of *Escherichia coli* isolated from water samples using polymerase chain reaction targeting four genes: cytochrome bd complex, lactose permease, beta-D-glucuronidase, and beta-D-galactosidase. J. Appl. Microbiol. 105 (4), 970–976.

Jaroenram, W., Kiatpathomchai, W., Flegel, T.W., 2009. Rapid and sensitive detection of white spot syndrome virus by loop-mediated isothermal amplification combined with a lateral flow dipstick. Mol. Cell. Probes 23, 65–70.

Jaroenram, W., Arunrut, N., Kiatpathomchai, W., 2012. Rapid and sensitive detection of shrimp yellow head virus using loop-mediated isothermal amplification and a colorogenic nanogold hybridization probe. J. Virol. Methods 186, 36–42.

Juck, D., Ingram, J., Prevost, M., Coallier, J., Greer, C., 1996. Nested PCR protocol for the rapid detection of *Escherichia coli* in potable water. Can. J. Microbiol. 42, 862–866.

Kaneko, H., Kawana, T., Fukushima, E., Suzutani, T., 2007. Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. J. Biochem. Biophys. Methods 70, 499–501.

Kiatpathomchai, W., Jaroenram, W., Arunrut, N., Jitrapakdee, S., Flegel, T.W., 2008. Shrimp Taura syndrome virus detection by reverse transcription loop-mediated isothermal amplification combined with a lateral flow dipstick. J. Virol. Methods 153, 214–217.

Kumar, S., Mondal, K.K., 2015. Visual detection of *Escherichia coli* contamination in milk and fruit juice using loop-mediated isothermal amplification. J. Food Sci. Technol. 52 (11), 7417–7424.

Lin, M., Liu, W., Wang, P., Tan, J., Zhou, Y., Wu, P., Zhang, T., Yuan, J., Chen, Y., 2015. Rapid detection of ermB gene in Clostridium difficile by loop-mediated isothermal amplification. J. Med. Microbiol. 64, 854–861.

Maheux, A., Picard, F., Boissinot, M., Bissonnette, L., Paradis, S., Bergeron, M., 2009. Analytical comparison of nine PCR primer sets designed to detect the presence of *Escherichia coli/Shigella* in water samples. Water Res. 43 (12), 3019–3028.

Mashooq, M., Kumar, D., Niranjan, A.K., Agarwal, R.K., Rathore, R., 2016. Development and evaluation of probe based real time loop mediated isothermal amplification for Salmonella: a new tool for DNA quantification. J. Microbiol. Meth. 126, 24–29.

McFeters, G.A., Bissonnette, G.K., Jezeski, J.J., Thomson, C.A., Stuart, D., 1974. Comparative survival of indicator bacteria and enteric pathogens in well water. J. Appl. Microbiol. 27, 823–829.

Molina, Felipe, López-Acedo, Elena, Tabla, Rafael, Roa, Isidro, Gómez, Antonia, Rebollo, J., 2015. Improved detection of *Escherichia coli* and coliform bacteria by multiplex PCR. BMC Biotechnol. 15, 48.

Mori, Y., Nagamine, K., Tomita, N., Notomi, T., 2001. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem. Biophys. Res. Commun. 289, 150–154.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28, E63.

Pavlovic, M., Luze, A., Konrad, R., Berger, A., Sing, A., Busch, U., Huber, I., 2011. Development of a duplex real-time PCR for differentiation between *E. coli* and *Shigella* spp. J. Appl. Microbiol. 110 (5), 1245–1251.

Puthawibool, T., Senapin, S., Kiatpathomchai, W., Flegel, T.W., 2009. Detection of shrimp infectious myonecrosis virus by reverse transcription loop-mediated isothermal amplification combined with a lateral flow dipstick. J. Virol. Methods 156, 27–31.

Sakai, J., Maeda, T., Tarumoto, N., Misawa, K., Tamura, S., Imai, K., Yamaguchi, T., Iwata, S., Murakami, T., Maesaki, S., 2017. A novel detection procedure for mutations in the 23S rRNA gene of Mycoplasma pneumoniae with peptide nucleic acidmediated loop-mediated isothermal amplification assay. J. Virol. Methods 141, 90–96.

Sandhya, S., Chen, W., Mulchandani, A., 2008. Molecular beacons: a real-time polymerase chain reaction assay for detecting Escherichia coli from fresh produce and water. Anal. Chim. Acta 614, 208–212.

Seetang-Nun, Y., Jaroenram, W., Sriurairatana, S., Suebsing, R., Kiatpathomchai, W., 2013. Visual detection of white spot syndrome virus using DNA functionalized gold nanoparticles as probes combined with loop-mediated isothermal amplification. Mol.

Cell. Probes 27, 71–79.
Tanner, N.A., Zhang, Y., Evans, T.C., 2015. Visual detection of isothermal nucleic acid amplification using pH-sensitive dyes. BioTechniques 58 (2), 59–68.
Teng, P.H., Chen, C.L., Sung, P.F., Lee, F.C., Ou, B.R., Lee, P.Y., 2007. Specific detection of reverse transcription-loop-mediated isothermal amplification amplicons for Taura syndrome virus by colorimetric dot-blot hybridization. J. Virol. Methods 146, 317–326.
Tsen, H.Y., Lin, C.K., Chi, W.R., 1998. Development and use of 16S rRNA gene targeted PCR primers for the identification of *Escherichia coli* cells in water. J. Appl. Microbiol. 85, 554–560.