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Optimization for Rapid Detection of *E. coli* O157:H7 Using Real-time Loop-mediated Isothermal Amplification

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ABSTRACT

Background: *Escherichia coli* O157:H7-related food poisoning is one of the most well-known causes of bloody diarrhea illness around the world. Therefore, devising a rapid, highly sensitive, and convenient detection technique for this species is crucial. In this work, we optimized a colorimetric loop-mediated isothermal amplification (LAMP) for detecting the intimin gene from *E. coli* O157:H7.

Materials and Methods: In this study, *eae* (intimin), one of the virulence factors of *E. coli* O157:H7, was selected as the target gene, and specific primers were designed for the sequence of this gene using the Primer Explorer software, version 5. The LAMP reaction was optimized with three variable factors of $MgSO_4$ concentration, temperature, and incubation time, in a traditional (separate) way and by Taguchi experimental design. Finally, the LAMP products were visualized by 2% agarose gel electrophoresis stained with ethidium bromide or green fluorescence (SYBR green I) and the pink fluorescence (KBC power load), which can be observed using the naked eye.

Results: The LAMP reaction was optimized at 63°C and 8 mM $MgSO_4$ for 40 min. Also, the naked eye can readily visualize the LAMP products within 40 minutes and have a detection limit of 3.2×10^4 CFU/mL according to 0.38 fg from the genome. Designed primers based on the gene sequence proved their specificity by testing 4 species of other common foodborne pathogenic microorganisms.

Conclusion: The rapid, sensitive, one-step-visually developed LAMP assay could be of interest for screening functions in food analytical laboratories without special equipment or trained personnel.

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Introduction

So far, over 250 completely different foodborne diseases due to consuming contaminated foods such as raw meat and unpasteurized dairy products have been reported; the leading cause of these diseases is bacteria [1, 2]. *Escherichia coli* O157:H7, with more than 150 human pathogenic serotypes and an infectious dose of 10^1–10^3 CFU, is one of the causative agents of foodborne diseases and urinary tract infections (UTIs) [3, 4]. Moreover, Shiga toxin-producing in at least 95 serotypes of *E. coli* is associated with hemorrhagic colitis and hemolytic uremic syndrome [5]. On May 2, 2017, the World Health Organization (WHO) estimated that *E. coli*-mediated gastroenteritis is responsible for approximately 1.7 billion diarrheal illnesses, 8500 hospitalizations, and 760000 deaths in children under 5 years old each year [6, 7]. Therefore, sensitive and rapid methods to detect *E. coli* O157 are required to supplement conventional methods based on selective culture media, biochemical identification, and other approaches [8]. However, polymerase chain reaction (PCR) has long been used as an indispensable and popular instrument for clinical applications in the diagnosis kits for infectious agents, environmental monitoring, and food quality control. Still, it has shortcomings, such as requiring a complicated thermal cycle instrument [9, 10].

In the past two decades, loop-mediated isothermal amplification (LAMP) was appraised as a powerful molecular amplification technique for detecting an extensive range of pathogens associated with foodborne diseases. It has high sensitivity, specificity, and easy operation at isothermal temperatures [11-13]. LAMP technology amplifies a limited value of DNA copies in less than 2 hours by using 4 to 6 key sets of specific primers consisting of outer primers (forward outer primer, F3, and backward outer primer, B3) and inner primers (forward inner primer, FIP, and backward inner primer, BIP) which recognize and amplify 6 (or 8) different zone in the target DNA then synthesize large amounts of target sequence [14-16]. In contrast to serological methods, the validated LAMP assay with high specificity and sensitivity could detect the DNA pathogen in the first few days of infection even when the clinical signs have not yet been apparent [17-19]. Compared to other molecular methods such as PCR and real-time PCR, the LAMP assay is low-cost due to reduced consumption of reagents and no need to choose an enrichment culture medium and subsequent recovery of colonies [20-22]. Moreover, without the risk of PCR-inhibiting substances, this method allows for direct nucleic acid amplification in places without extraction steps, laboratory equipment for precise thermal-cycle control or result reading, labeled DNA probes and trained personnel [18, 19]. Another notable advantage of this technique is various visual monitoring methods, such as turbidity monitoring and fluorescent or colorimetric dyes, without using an expensive tool such as a thermocycler [21]. Furthermore, it is superior to the other methods because of its high diagnostic sensitivity with 10 CFU/mL of pathogens and amplified 10^9-10^{10} times of target DNA in the shortest time possible [21, 22]. In the present study, LAMP methodology was established and optimized in isothermal conditions to rapidly detect *E. coli* O157:H7 strains, including the *eae* virulence factor gene responsible for colonizing the pathogen. The intimin receptor (Tir) protein is one of the essential virulence factors coded by most pathogenic strains of E. coli. This protein significantly affects colonizing the host epithelial layer and is required to cause diarrhea [23].

Materials and Methods

DNA preparation and primer designed

The *E. coli* O157:H7 (ATCC 43894) as a positive control was verified by 16S rRNA sequencing. The *E. coli* O157:H7 strain used in this study was resuscitated on a Luria-Bertani liquid medium in a shaking incubator (Stuart) at 37°C overnight. Then, genomic DNA was extracted using the manufacturer's handbook of Gene Transfer Pioneers Kit (DM04050), and the concentration of DNA was determined using a NanoDrop spectrophotometer instrument (Thermo Scientific). The sequence of the *eae* gene was obtained from the National Center for Biotechnology Information GenBank database. LAMP-specific sets of primers containing the two outer primers (F3 and B3) and the two inner primers (FIP and BIP) were designed according to the *E. coli* O157:H7 consensus genomic sequence (*eae* gene) in GenBank (ATCC 43894) using the PrimerExplorer software, version 5 [12]. They were purchased from Pishgam Biotech Co (Iran, Tehran). The sequences of LAMP primers are summarized in Table 1. Figure 1 demonstrates the schematic position of LAMP primers within the *eae* gene.

LAMP assay

The LAMP mixture was performed in a total of 12.5 µL reaction volume containing 1.9 µL of 1.6 µM each of FIP and BIP (inner primers), 0.5 µL of 0.4 µM each of F3 and B3 (outer primers), 1 µL (38 ng/µL) of DNA template, 2.5 µL of 0.8 µM Betaine (Sigma-Aldrich, MO, USA), 1.4 µL of 1.4 mM of deoxyribonucleotide triphosphates (dNTP) (Biotechrabbit, Hennigsdorf, Germany), 1.25

μL of 10X isothermal amplification buffer (New England Biolabs, Ipswich, MA), 0.5 μL of 8 mM $MgSO_4$ (New England BioLabs, MA, United States), 1.25 μL of Bst polymerase (8 U/μL), and sterile water to make the final volume. Also, no template-free water was used as the negative control; then the reaction mix was ready, gentle centrifugation and 1 μL of SYBR green I or 2.5 μL of KBC power load (Kawsar Biotech Company, Iran) inwardly dotted at the tube cap as the fluorescent indicator. The reaction microtubes were incubated at 65˚C for 90 min. The LAMP amplicons of target DNA results were analyzed using a fluorescent indicator under UV light (after a brief spin tube) or 2% agarose gel electrophoresis [12].

Optimization of LAMP assay based on Taguchi experimental design

The effects of three factors (temperature, reaction periods, and concentrations of $MgSO_4$) on the function of the LAMP products were studied at three levels (Table 2) using Taguchi experimental design, and the obtained data were analyzed with Qualitek-4 software. Then, The LAMP temperature was optimized by incubating the LAMP mixture at 60˚C, 63˚C, and 65˚C. The Mg^{2+} ion concentration was optimized by adding $MgSO_4$ at a final 6, 8, or 10 mM concentrations to the LAMP mixture. Also, the time of the LAMP reaction was optimized by incubating the LAMP mixture for 40, 60, and 90 min. After finishing the LAMP procedure at different conditions, all the LAMP amplicons were initially separated by 2% agarose gel electrophoresis, and the optimal ratios of three parameters were determined. If amplification was successful, we could observe a ladder-like pattern [12].

Specificity and sensitivity of the LAMP assay

To verify the specificity of LAMP primers for *E. coli* O157:H7 detection, we performed reactions in a total volume of 12.5 μL with 1 μL of different genomic DNA of bacterial strains, including *Salmonella typhi* (PTCC No.1609), *Pseudomonas aeruginosa* (PTCC No.1557), and *Vibrio cholera* (PTCC No. 1611). Afterward, the microtubes were incubated at 65˚C for 90 min, and amplified products were analyzed under UV light. Furthermore, the sensitivity of the LAMP reaction was evaluated by testing different concentrations of *E. coli* O157:H7 genome serial dilution at levels ranging from 3.2×10^{10} to 3.2×10^{1} CFU/mL. Eventually, after incubation of all dilutions in the optimal condition of the LAMP assay, the products were analyzed under UV light [12].

Design of positive control constructs (TA-*eae*)

One of the intelligent strategies in molecular kit design is establishing positive control structures from conserved regions of the pathogen gene sequences. The design of such structures in the absence or lack of access to the standard genome of pathogens helps a lot in diagnostic methods. Thus, in the first step, the PCR was performed in a total volume of 25 μL with specific F3 and B3 primers containing 1 μL DNA templates (the concentration of pathogen was 38 ng/μL), 12.5 μL Taq Master Mix (Thermo Fisher Scientific DreamTaq), 1.25 μL from each primer and nuclease-free water. The PCR amplification procedure was as follows: an initial denaturation at 94˚C for 4 min, followed by 30 cycles of 1 min at 94˚C, 1 min at 58˚C, 1 min at 72˚C, and a final extension of 10 min at 72˚C. The expected size of the PCR product was around 206 bp. The PCR products were analyzed by agarose 1.5% gel electrophoresis. Afterward, PCR products were cleaned using the gene DNA recovery GTP Kit (Gene Transfer Pioneers, IRAN) according to the manufacturer's instructions. The fragment was cloned into the TA vector, with the ligation reaction carried out for 16 h in a total volume of 10 μL purified PCR product, 1 μL (20 ng) TA vector, 1 μL ligation buffer (2x), and 2 μL (5 u) T4-DNA ligase (Promega Co.). The TA-*eae* construct was then transformed into chemically competent *E. coli* DH5a cells.

Results

Optimization of the LAMP reaction

The LAMP reaction was performed according to the methods section, then LAMP product detection was performed for fluorescent interpretation. A positive amplification with SYBR green I showed brilliant green fluorescence. In contrast, in reaction tubes with the addition of KBC power load, positive amplification showed bright pink, and no glowing fluorescence was observed in the negative control (Figure 2). Also, in the present studies to enhance the efficiency of the LAMP method for detecting *E. coli* O157:H7, the influence of various parameters on the performance of this technique, such as $MgSO_4$, incubation time, and the reaction temperature, were optimized simultaneously by Taguchi methodology. Taguchi experimental design helps investigate the interactions between various factors and reduces the materials and time required to design the experiments to determine the best parameters [24]. Based on the results shown in Figure 3, LAMP assays successfully amplified the *eae* gene of *E. coli* O157:H7 DNA under isothermal conditions (60˚C, 63˚C, 65˚C). Under UV light, LAMP

Table 1. Oligonucleotide primers used for LAMP assay

Target Gene	Primer	Type	Sequence (5′–3′)	Length (bp)
eae	F3	Forward outer	ATGGAACGGCAGAGGTTA	18
	B3	Backward outer	AATGAAGACGTTATAGCCCA	20
	FIP (F1c+F2)	Forward inner	CCTGACCAAATGCCAGCATTTTT-GAGTGGTAATAACTTTGACGG	44
	BIP (B1c+B2)	Backward inner	TCGGAGCGCGTTACATTGACT-ACATGTTTGCAGGAAGGAA	39

Table 2. Factors and their levels utilized in Taguchi's orthogonal array design for LAMP assay

Factor	Level 1	Level 2	Level 3
$MgSO_4$ (mM)	6	8	10
Temperature (˚C)	60	63	65
Reaction time (min)	40	60	90

amplicon products appeared in a positive pink color in the closed tube with KBC power load. In contrast, the remaining negative purple color represented the failure of amplification. In addition, results demonstrated that the optimum temperature by LAMP assay was 63˚C (level 2) for 40 min (level 1). The $MgSO_4$ concentration at 8 mM gave the optimal amplification (level 2), whereas, at a lower concentration of $MgSO_4$ (level 1), the LAMP assay did not react. This condition might be due to the effect of Mg^{++} as a cofactor in the activity of the Bst DNA polymerase by enabling the incorporation of dNTPs during polymerization.

Determination of specificity and sensitivity method

The specificity of the LAMP assay for intimin protein detection was assessed employing the DNA genomics of untargeted bacteria as negative controls, including *S. typhi*, *P. aeruginosa*, and *V. cholera*. After spinning reaction tubes with SYBR green I for visual inspection of LAMP products under UV light, bright green fluorescence as a positive sign was only observed in *E. coli* O157:H7 DNA, whereas no amplification was observed in LAMP reactions with other bacteria DNA (Figure 3C, Left). These results indicated that cross-reactivity with any of the bacteria was unlikely. The specificity test was repeated with KBC Power Load dye to affirm the LAMP assay with SYBR green I outcomes. The data indicate that the repeated specificity test with KBC power load dye had a similar result, and LAMP products

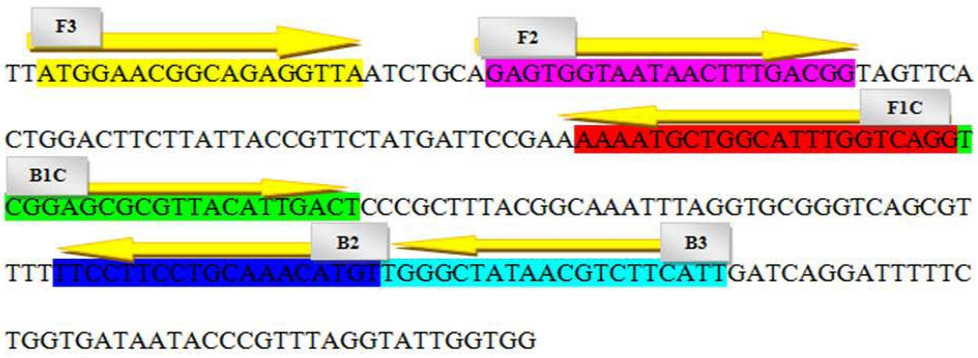

Figure 1. Location and the primers sequences of *eae* gene used to LAMP
The nucleotide sequences of the primer sites are underlined.

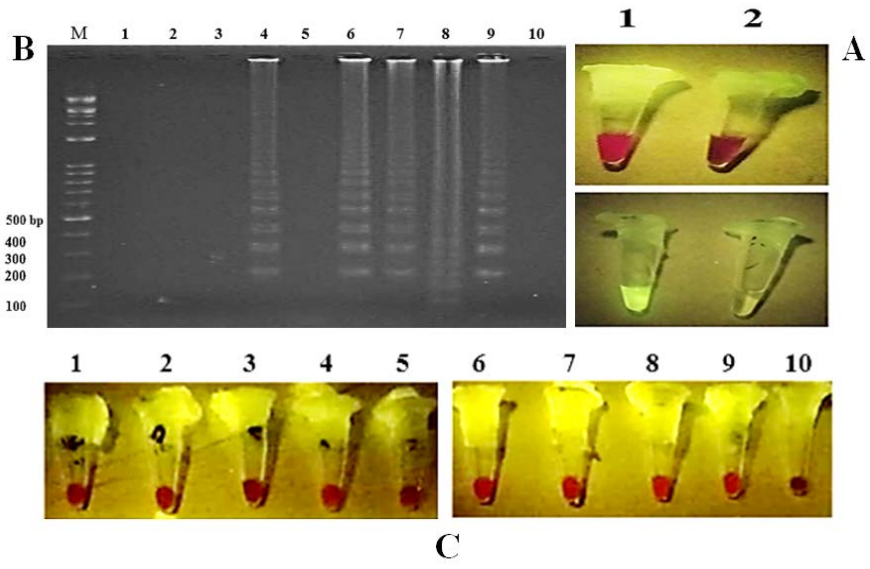

Figure 2. A) Fluorescent LAMP reaction using the *E. coli* O157:H7 DNA template (ATCC 43894)

Top) KBC power load: The color changes from violet (positive reaction, lanes 1) to pink (negative reaction, lanes 2); Lower) SYBR green: Bright fluorescence indicates a positive reaction.

B) Optimization of the LAMP reaction for detection of *E. coli* O157:H7

Amplified products were analyzed by gel electrophoresis. Lane M: 100bp DNA ladder (fermentas); Lanes 1, 2, and 3, $MgSO_4$ 6 mM; Lanes 4, 5, and 6, $MgSO_4$ 8 mM; Lanes 7, 8, and 9, $MgSO_4$ 10 mM.

Effect of temperature on the LAMP reaction: Lanes 1, 2, and 6, amplification at 60°C; Lanes 3, 4, and 9, amplification at 63°C; Lanes 5, 7, and 8, amplification at 65°C.

Effect of incubation time on the LAMP reaction: Lanes 1, 2, and 4, amplification for 40 min; Lanes 3, 5, and 6, amplification for 60 min; Lanes 7, 8, and 9, amplification for 90 min; Lane 10: Negative control showing no ladder-like pattern.

C) LAMP results indicated by KBC power load to the visualized under UV light.

were only produced when the primer sets reacted with their respective *E. coli* O157 genome and the color of KBC power load dye in the positive tube changed from violet to bright pink, under UV light (Figure 3B, Left) compared with visible light (Figure 3A, Left). Thus, the primer set was designed to detect the *E. coli* O157:H7 serotype.

On the other hand, four different *E. coli* O157:H7 DNA concentrations were prepared in the LAMP mixture for sensitivity assessment. For this reason, 2.5 µL of the KBC power load was applied directly to the tube cap. Without opening the tubes, the analysis of the results showed that color change was observed even at a concentration of 3.2×10^4 (0.38 fg) CFU/mL (Figure 3, Right). No positive detection and color difference did not occur in other *E. coli* O157:H7 concentrations.

Also, we designed a positive construct (TA-*eae*, GenBank: MW584345.1) for the next investigation to use the diagnostic kit for *E. coli* O157:H7. The presence of the *eae* fragment in the recombinant plasmids was verified by colony PCR using the F3 and B3 set primer (Figure 4A). Then the TA-*eae* plasmid is used as a template in the LAMP reaction, and the color of KBC Power Load dye in the positive tube changed from violet to bright pink under UV light (Figure 4C).

Discussion

The number of fatalities caused by foodborne infections due to resistant pathogens like *E. coli* O157:H7 increases every year. Also, *E. coli* O157:H7 is responsible for cases of hemolytic-uremic syndrome, kidney failure, and bloody diarrhea [2, 25]. The latest CDC report indicates that *E. coli* O157 causes an estimated number of 63100 illnesses, 2100 hospitalizations, and 20 deaths every year. The prevalence rates of this bacterium as a zoonotic waterborne pathogen are more than 31% in Africa, 7.35% in Northern America, 6.85% in Oceania, 5.15% in Europe, and 4.69% in Asia [26, 27]. Some isothermal amplification techniques with specific features, low-cost, and accurate operations, like LAMP techniques, are suitable alternatives to conventional detection methods for infective agent screening [21, 28]. The LAMP method requires less detection time (<1 h) than PCR or real-time PCR [29]. Baraily et al. used the

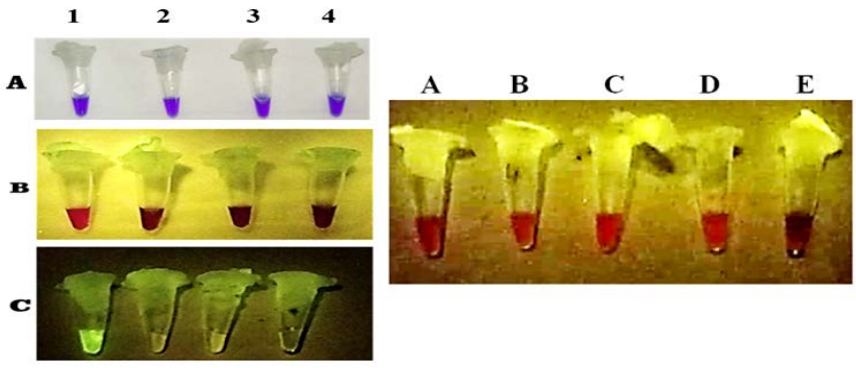

Figure 3. Left, specificity of the LAMP assay

A) Visualization on a visible light, B) Under UV light with KBC power load, C) SYBR green

1: *E. coli* O157:H7; 2: *V. cholera*; 3: *S. typhi*; 4: *P. aeruginosa*.

Right-sensitivity of LAMP assay for different concentrations of total *E. coli* O157:H7 DNA prepared and used as template. Lane A: 3.2×10^7 CFU/mL, Lane B: 3.2×10^6 CFU/mL, Lane C: 3.2×10^5 CFU/mL, Lane D: 3.2×10^4 (0.38 fg) CFU/mL, Lane E: 3.2×10^3 CFU/mL.

LAMP assay by targeting *stx1* and *stx2* genes for inexpensive and rapid detection of Shiga toxin-producing strains of *E. coli* in animal-origin food. This report showed that the LAMP assay is 10 times higher sensitivity than conventional PCR and could be detected at 1.11×10^2 CFU/mL [5]. In 2017, Wang, for the detection of the 7 serogroups (O26, O45, O103, O111, O121, O145, and O157) from *E. coli*, developed a LAMP assay by targeting wzx and wzy genes that could be detected 0.1-1 pg per reaction (25-250 CFU/reaction) of *E. coli* [30]. Also, Xue-Han et al. developed a specific and sensitive LAMP method to detect all known intimin variants harbored by pathogenic *E. coli* strains. In this study, the LAMP method detected all 27 tested *eae* variants with highly specific reactions and a detection limit of about 10 copies per reaction which was 100 times more sensitive than conventional PCR [31]. In 2020, Fei et al. used a LAMP assay and coupled it to a new bioluminescent pyrophosphate assay to detect *E. coli* O157:H7. In this novel assay, sensitivity and limit of detection were determined by about 10 copies/uL (5 CFU/mL), which is higher (100 times) than traditional LAMP methods [32]. In comparison, a study by Zhao et al. utilized LAMP to rapidly identify *E. coli* (ETEC) by targeting the F4 fimbriae in 60 min [8]. The specificity of the LAMP assay was assessed by concentrated DNA of four bacterial species strains, including *E. coli* O157: H7. A positive result was observed from *E. coli* O157: H7, while other strains showed negative results, confirming the 100% specificity of the assay. Also, in 2010, Zhao et al. used the LAMP technique to detect *E. coli* O157 and Shiga toxins with a 10 pg DNA/tube sensitivity for *rfbE* [33]. Zhao et al. reported the limit of detection for propidium monoazide (PMA)-LAMP was as low as 1.6×10^2 CFU per reaction tube in detecting the *rfbE* gene of the *E. coli* O157 genome [34]. Accordingly, the present

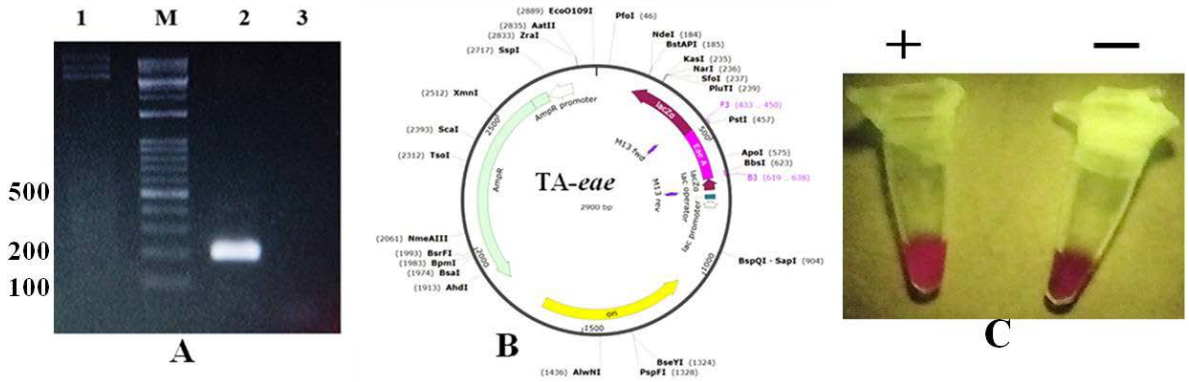

Figure 4. A) Visualization of 1.5% agarose gel electrophoresis for PCR products

Lane 1: TA-*eae* plasmid; Lane 2: PCR products for *eae* gene, 206 bp; Lane 3: Negative control.

B) Schematic representation of positive constructs (TA-*eae*), designed by Snap Gene Software

C) Visualization under UV light with KBC power load

analysis focuses on establishing a reliable and high-sensitivity laboratory protocol by LAMP assay with four different primers to detect *E. coli* O157:H7. LAMP-specific primers sets were designed to target highly preserved regions of the *eae* gene. In addition, KBC power load was used in this LAMP assay as a fluorescent dye for the visual confirmation of a positive reaction which is safer than ethidium bromide and cheaper than SYBR green. Due to the strong inhibitory effects of fluorescent dyes on the LAMP reaction, the dyes were dotted inside the tube cap [35]. Also, the optimization of the LAMP reaction was investigated using the Taguchi statistical method (Table 2) [23]. The Taguchi method evaluates the factors considered and allows the simultaneous study of different effective factors and their interactions [12, 36]. Thus, the experiments were designed on the L9 orthogonal array, and firstly we developed this methodology for rapid detection of intimin using a LAMP assay to screen *E. coli* O157: H7. The optimal temperature and response LAMP detection time of *eae* gene were 63˚C and 40 minutes, respectively. Compared with the previous studies, we could identify *E. coli* O157:H7 in a shorter time (40 min) using the LAMP technique. Because the testing time is critically important for diagnosing infection, this colorimetric LAMP is a perfect choice. The sensitivity of the detecting test in our study was analyzed through a different diluted DNA solution of *E. coli* O157:H7, and the detection limit was established as 3.2×10^4 (0.38 fg). The comparative sensitivity of LAMP and PCR results in several studies demonstrated that LAMP was much more sensitive than PCR. All results showed that the primers we used had high sensitivity and specificity.

Conclusion

The LAMP method effectively amplified *E. coli* specific *eae* gene sequences at 63˚C in 40 min, while the other bacterial control strains tested were negative. The investigation aimed to assess the inclusivity of a developed LAMP technique for simple and quick detection of *E. coli* O157:H7 strains isolated special proved to be a sensitive and inexpensive detection method, which could be of interest for screening functions in food analytical laboratories, without the need for an advanced DNA extraction step and special equipment or trained personnel.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles were considered in this article.

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Authors contribution's

Conceptualization and study design: Mehdi Zeinoddini; Conducting experiments, analysis and interpretation of the data: Alaleh Valiallahi and Mehdi Zeinoddini; Drafting of the manuscript: All authors; Review & editing: Mehdi Zeinoddini; Final approval: All authors.

Conflict of interest

The authors declared no conflict of interest.

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