Original Article

Optimization for Rapid Detection of *Staphylococcus aureus* using Real-time LAMP

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Abstract
Introduction: Bacterial food poisoning is considered a global concern in terms of economical and human health. *Staphylococcus aureus* produces low molecular weight extracellular toxins. These enterotoxins (SEs) are similar in structure and bioactivity. Today, there are several methods to detect SE genes. Nevertheless, culture-based and immunological methods are simple and cheaper than molecular techniques, they are time-consuming, with low sensitivity and specificity. In this field, loop-mediated isothermal amplification (LAMP) is a fast and simple method for gene amplification. The aim of this work was to optimize the LAMP reaction using the Taguchi method.
Materials and Methods: For this, in order to improve and accelerate the LAMP diagnostic process, essential factors for the identification of enterotoxin, including $MgSO_4$ concentration, time, and temperature reaction were optimized separately and using Taguchi experimental design.
Results: The results showed that 57 µg/ml of *S. aureus* genome as template is suitable for the replication, and in optimization of LAMP assay in separate condition the best replication rate was observed at 6 mM $MgSO_4$, 45 min, and 65 °C. Whereas using Taguchi methods, the optimum condition was at 8 mM $MgSO_4$, 60 min and 65 °C.
Conclusions: The one-step-visual developed LAMP assay with the optimum conditions could be of interest for screening functions in food analytical laboratories, and a portable detection method could be used to design a suitable identification kit for *S. aureus* without the need for special equipment or trained personnel.
Keywords: Food Poisoning, *Staphylococcus aureus*, Enterotoxin, Detection, Optimization
Citation: Sheikhi F, Zeinoddini M, Jalili S. Optimization for Rapid Detection of *Staphylococcus aureus* Using Real-time LAMP. J Appl Biotechnol Rep. 2023;10(2):984-991. doi:10.30491/JABR.2023.368333.1575

Introduction

Bacterial contamination is known to induce food poisoning, a problem that would be crucial for human well-being. There are a variety of causes for food poisoning, including unsafe food, insufficient cooking, inadequate storage temperature, contaminated equipment, and poor personal hygiene. In some countries, concerns about food contamination with chemical and microbial agents have led to inhibit food imports.[1,2] *Escherichia coli*, *Shigella dysenteriae*, *Bacillus cereus*, *Listeria monocytogenes*, *Vibrio cholera*, and *Staphylococcus aureus* are the most important bacteria in terms of food contamination.[3,4] *S. aureus* is a Gram-positive and optional anaerobic that is medically important genus of the *Staphylococcal* family. The bacterium is mesophilic, which grows well at temperatures between 25 °C and 38 °C, however; it is generally able to grow at temperatures between 6 °C to 48 °C.[5] This bacterium causes disease in two models: through proliferation and extensive diffusion in tissues and through the production of multiple cellular nutrients.[6] *S. aureus* produces low molecular weight extracellular toxins (SEs). Although the SEs are similar in

structure and bioactivity, they are different in antigenic properties. These SEs divided into classical and new-fangled species. More than 95% of the SEs of these bacteria are responsible for food poisoning and belong to the A-E group. A and D groups are the most vital SEs that cause staphylococcal food poisoning.[7,8] Since SEs are resistant to heat, their thermal resistance in food is higher than in laboratory culture environments. Also, the bioactivity of toxins will remain after the food preparation heating stages. SEs are in dairy products such as milk, cheese, raw meats, and vegetables that cause probable food poisoning. The occurrence of food poisoning by SEs requires 10^5-10^8 CFU of bacteria.[9-11] Enterotoxin B (SEB) is one of the main responsible agents in food poisoning and is relatively stable and soluble in water. Therefore, to deactivate that, it is necessary to boil it at 100 °C for several minutes.[12] Inhalation of SEB can cause severe pathophysiological changes, including extensive systemic damage, general and severe illness and even septic shock. The notable point is that SEB can cause poisoning within 3 to 12 hours.[12] Today,

several methods have been used for the identification of SEs. For instance, immunological methods including ELISA, agglutination, and radioimmunoassay are sensitive and have a limit of detection up to 1 picogram of SEB.[13] However, they are time-consuming and laborious. Molecular detection methods such as PCR, real-time PCR, and ELISA PCR have been reported for SEB diagnosis.[14-18]

The loop-mediated isothermal amplification (LAMP) was first introduced in 2000 by Notomi. This molecular method also has been reported for identifying bacterial toxin genes. LAMP is a fast and simple method for gene amplification and immediate detection of microbial pathogens.[19,20] This method has some advantages in comparison to PCR. For example, the time for the reaction process is considerably shorter than conventional PCR, as well as; having more sensitivity. Furthermore, as the LAMP method is an isothermal amplification, the required temperature for the reaction could provide using a simple water bath which is more cost-effective than PCR thermal cycler device. Also, one of the unique features of the LAMP method is that, the amplification is observable by the naked eye.[19-21] This technique is one of the most specific molecular methods

because during the amplification four primers should be designed to identify six particular regions in the target gene.[22] In the present work, optimization of the LAMP assay for effective factors in this reaction including time, temperature, and MgSO$_4$ concentration was carried out for the detection of the SEB gene from *S. aureus* separately and using Taguchi experimental design.

Materials and Methods
Primer Design
Specific primers for LAMP assay were designed according to the sequence of the enterotoxin B variant v$_1$ (SEB) gene from *S. aureus* strain KLT6 (Accession No. KX168628.1), using PrimerExplorerV5 (https://primerexplorer.jp) software (Table 1). Also, we used multiple sequence alignment MEGA6 (Molecular Evolutionary Genetics Analysis Version 6.0) software and find the consensus sequences of the *SEB* gene for primer design. Each set of primers contained two external primers (F3 and B3), and two internal primers (FIP and BIP). The BIP and FIP included an AAAA spacer between the B1/B2c sequences, and F1c/F2 sequences (Figure 1).

Table 1. The Sequences of Specific Primers

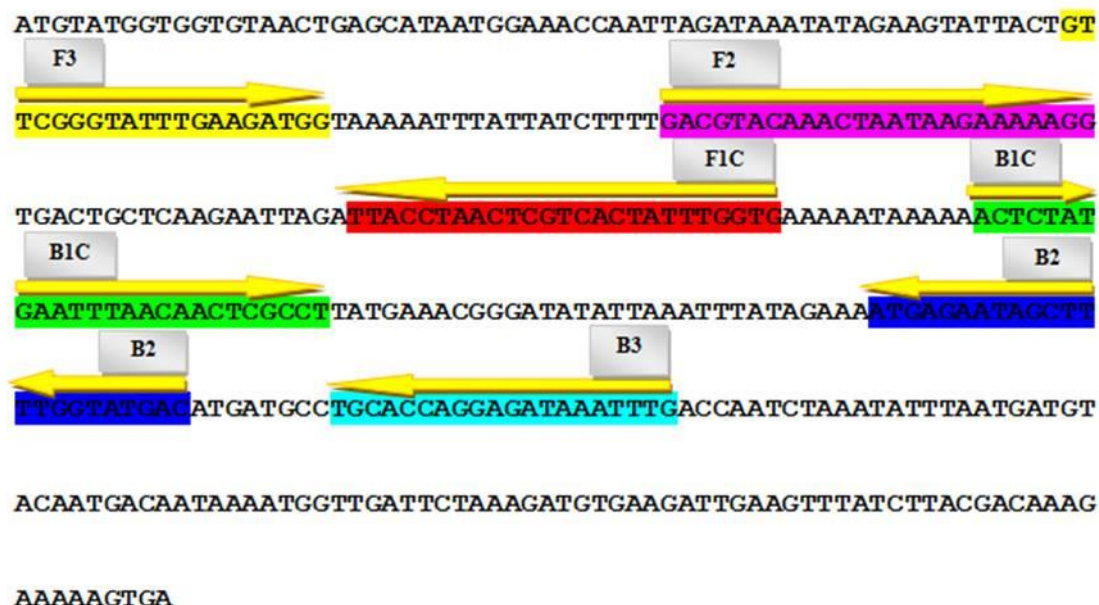| Primer	Sequence
F3	GTTCGGGTATTTGAAGATGG
B3	CAAATTTATCTCCTGGTGCA
FIP	CACCAAATAGTGACGAGTTAGGTAAAAAAGACGTACAAACTAATAAGAAAAAGG
BIP	ACTCTATGAATTTAACAACTCGCCTAAAAGTCATACCAAAAGCTATTCTCAT

Figure 1. Locations of Primer-binding Sequences Enterotoxin B Variant v$_1$ (SEB) Gene from *S. aureus* Strain KLT6, (Accession No. KX168628.1).

DNA Extraction and Optimization
The 24-h post-culture cell biomass of *S. aureus* in tryptic soybean digest agar medium (Merck company) at 37 °C was

collected by centrifuge at 8000 rpm for 5 min. In order to optimize DNA extraction, different methods including boiling, extraction column, sonication, and DNA extraction

kit (Sina Clone, Iranian company) were used to extract of the total bacterial genome. The extracted genome was analyzed by 1% agarose gel electrophoresis in order to determine the best genomic extraction method.

PCR Assay

The PCR assay was performed in a total reaction volume 5 µl containing 57 ng (1 µl) of DNA template, 1 µM (0.25 µl) from each of primers B3, F3, and FIP, BIP, 2.5 µl from a master mix of Taq DNA polymerase (amplicon PCR kit, Denmark). The PCR assay was carried out according to the program below: Initial denaturation at 94 °C for 4 min, 30 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR procedure was carried out using an automated thermocycler (Techgene, Germany) and the PCR products were analyzed by 1% agarose gel electrophoresis.

LAMP Assay

LAMP assay according to Figure 2, was performed in a total 12.5 µl volume reaction containing 1.6 µM of the inner primers (FIP/BIP), 0.4 µM of the outer primers (F3/B3), 1.4 mM dNTPs, 1.25 µl of 10X isothermal amplification buffer, 6 mM of $MgSO_4$, 0.8 M of betaine, 4 U of Bst 2.0 DNA polymerase (New England Biolabs), and 57 ng of target DNA. The mixture was incubated at 65 °C for 90 min and heated at 80 °C for 10 min to stop the reaction. LAMP products were analyzed using SYBR Green staining and 2%

agarose gel electrophoresis.

Optimized LAMP Assay

For optimization of the LAMP assay, three factors of $MgSO_4$ concentration, temperature, and incubation time were performed separately and using the Taguchi method. The concentration of Mg^{2+} ion was optimized by adding $MgSO^4$ at a final concentration of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 mM to the LAMP mixture and incubating at 65 °C for 90 min. The LAMP temperature was optimized by incubating the LAMP mixture at 59, 61, 63, 65, 67, 69, and 71 °C for 90 min. The time of the LAMP reaction was optimized by incubating the LAMP mixture at 0, 15, 30, 45, 60, and 90 min. On the other hand, the LAMP assay was optimized using the Taguchi design experiment, by 9 combined tests that were designed by Qualitek-4 software. In this experiment, three factors included $MgSO_4$ concentration (4, 6, and 8 mM), incubation temperatures (60, 63, and 65 °C), and incubation time (40, 60, and 90 min) were considered, and for each factor, 3 levels were selected (Table 2). The reaction mixture in 12.5 µl volume contains 1.25 µl of 10X Bst buffer, 0.8 M of betaine, 1.6 µm of the FIP and BIP primers, 0.4 µm of the F3 and B3 primers, 4 units of enzyme Bst polymerase, 1.4 µl dNTPs, 2, 4, and 6 mM $MgSO_4$ and 1 µl (57 ng) of the genome were prepared. According to designed combined assays for 40, 60, and 90 min at 63, 60, and 65 °C, it was heated to 80 °C for 10 min to stop the reaction. Finally, the LAMP product was examined by SYBR Green staining and

Table 2. Factors and their Levels Employed in the Taguchi Experimental Design for LAMP Assay

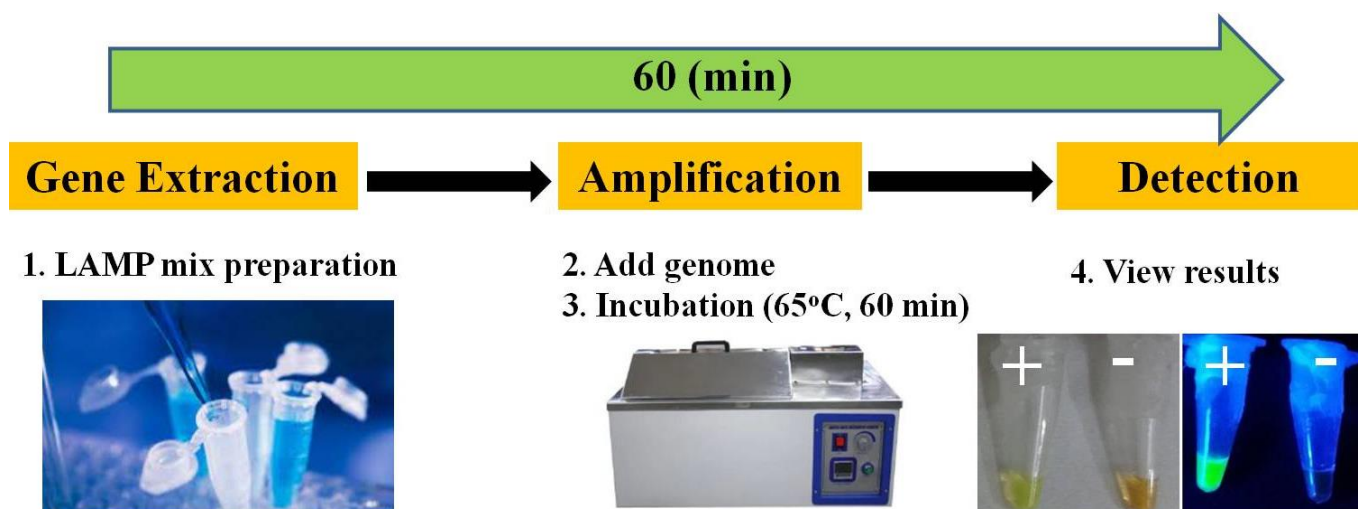| Elements	Exp1	Exp2	Exp3	Exp4	Exp5	Exp6	Exp7	Exp8	Exp9
$MgSO_4$ (mM)	4	6	8	4	6	8	4	6	8
Time (min)	40	60	90	60	90	40	90	40	60
Temperature (°C)	60	60	60	63	63	63	65	65	65

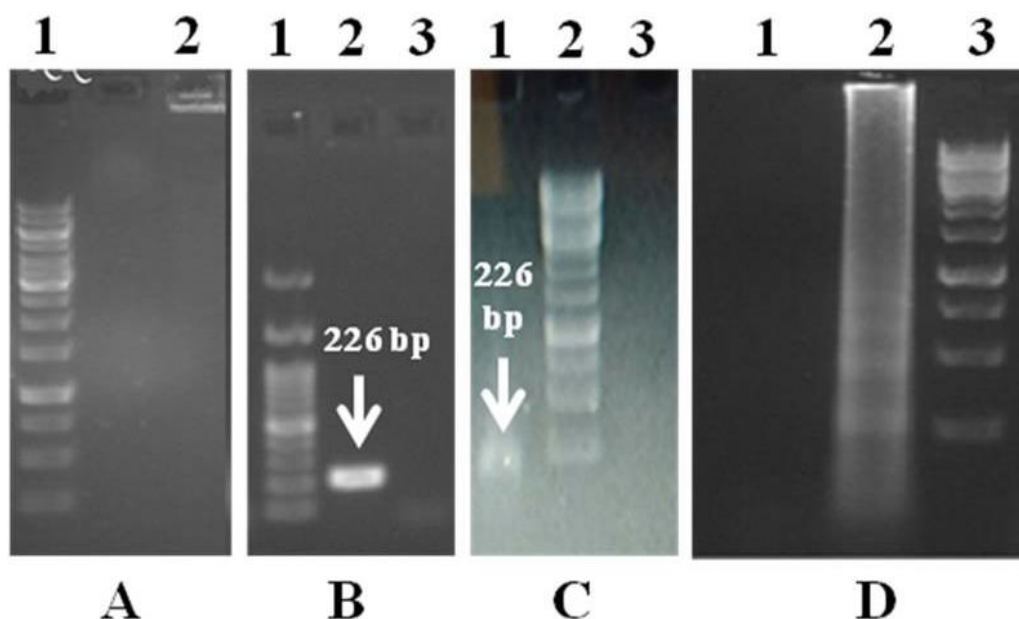**Figure 2.** Schematic Representation from LAMP Steps.

Figure 3. (A) 1% Agarose Gel Electrophoresis with Ethidium Bromide for Observation of Extracted Genomic DNA. Lane 1: extraction using sonication; Lane 2: DNA ladder (1 Kb). **(B)** PCR product analysis using F3/B3 primers. Lane 1: DNA ladder (100 bp); Lane 2: positive control; Lane 3: negative control. **(C)** PCR product analysis using BIP/FIP. Lane 1: positive control; Lane 2: DNA ladder (1 Kb); Lane 3: negative control. **(D)** Analysis of the LAMP reaction product by 2% agarose gel electrophoresis. Lane 1: negative control without genome; Lane 2: positive control; Lane 3: DNA ladder (1 Kb).

2% agarose gel electrophoresis.

Specificity of the LAMP Assay

The specificity test was carried out in the optimized condition of amplification to validate the selectivity of the designed primers. For this, F3/B3 and FIP/BIP primers and extracted genomes of *Yersinia enterocolitica*, *Bacillus cereus*, and *S. aureus* bacteria used in the LAMP assay. Each tube contains a total volume of 12.5 μl of the reaction mixture with the conditions of 6 mM MgSO$_4$ at 65 °C and 90 min incubation and finally, stained by SYBR-Green and visualized under ultraviolet light.

Results

DNA Extraction

The bacterial genomic DNA was extracted by several protocols according to material and methods section and results were analyzed by 1% agarose gel electrophoresis. The data showed that the genome extracted by sonication has a better quality than other methods (Figure 3-A).

Detection of SEB Using PCR and LAMP Assay

The PCR reaction was performed with B3/F3 and FIP/BIP primers, and the PCR product was analyzed using 1% agarose gel electrophoresis. The presence of 226 bp bands related to the SEB fragment confirms the successful amplification of this sequence and the correctness of the primers designed (Figure 3-B, Figure 3-C). Also, the results of the initial LAMP with designed primers, in the conditions of 6 mM MgSO$_4$ at 65 °C and 90 min incubation time were performed on a 2% agarose gel, indicating proliferation as a ladder-like pattern with these conditions (Figure 3-D).

Optimization of the Reaction Using Taguchi Method

Firstly, LAMP assay was optimized by three factors, separately: MgSO$_4$ concentrations, temperature, and the incubation time of the reaction. Optimization was performed using MgSO$_4$ in different concentrations ranging from 2 to 12 mM and the best amplification was achieved by 6 mM MgSO$_4$ (Figure 4-A). In order to determination of the optimum incubation time for the LAMP reaction, LAMP amplification was performed in 45, 60, 75, and 90 min. The results showed that LAMP amplification was performed well for 45 min (Figure 4-B). Also, the effect of temperature on the LAMP reaction was determined at 59, 61, 63, and 65 °C. The data showed that 65 °C was better than other temperatures for amplification (Figure 4-C). The reaction optimization results were observed using agarose gel and SYBR Green staining.

In the second step, we used a combination of these factors in the Taguchi design experiment. The result of agarose gel analysis for 9 designed experiments with MgSO$_4$ concentration, temperature, and incubation time at three different levels was estimated and indicated that the optimal amount of these factors was in the condition of 8 mM MgSO$_4$, 60 min incubation time and 65 °C incubation temperatures that had the best amplification. But in the first experiment, with the following conditions 4 mM MgSO$_4$, 40 min heat-up time, and 60 °C amplification did not observe. The results of proliferation were also determined with SYBR green staining (Figure 5).
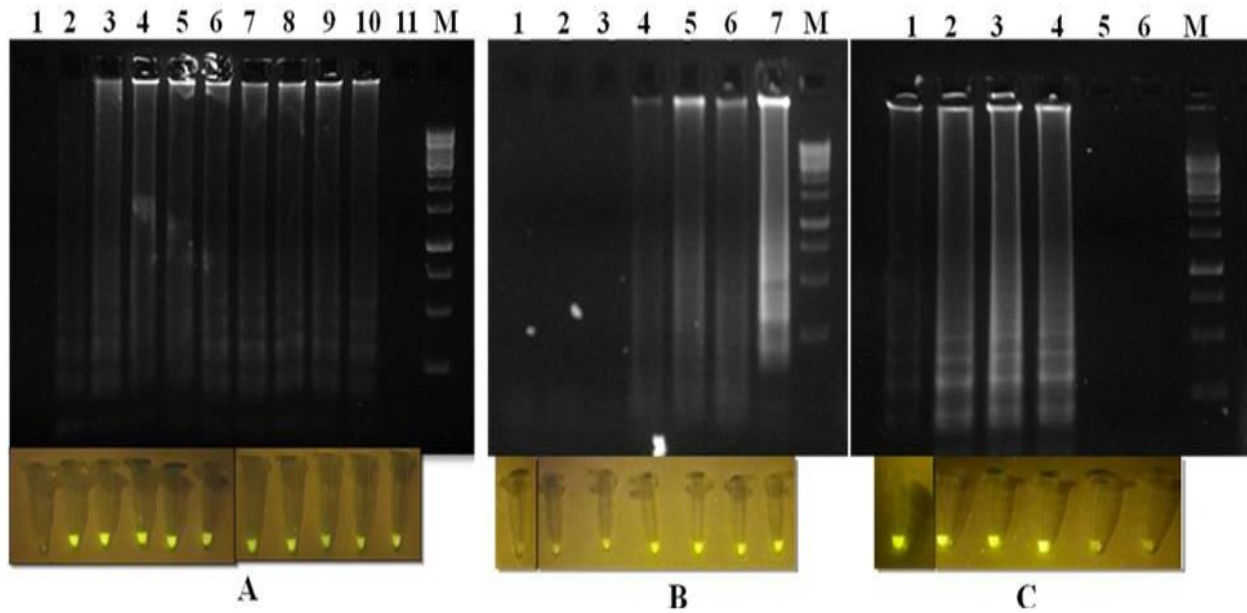
Figure 4. (A) Optimization of MgSO₄ in the LAMP Reaction. Lane 1 to 11 contain concentrations of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 mM MgSO₄, respectively. **(B)** Optimization of duration time in LAMP reaction. Lane 1 to 7 the arrangement incubation times consists of 0, 15, 30, 45, 60, 75, 90 min; Lane M: 1 kb DNA marker. **(C)** Optimization of temperature in LAMP reaction. Lane 1 to 6 the arrangement incubation temperatures consist of 59, 61, 63, 65, 67, 69 °C; Lane M: 1 kb DNA marker.
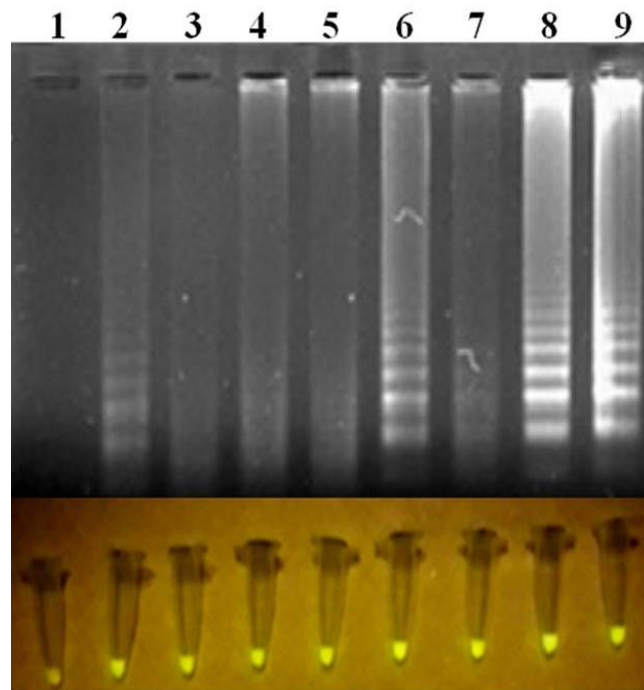

Figure 5. Optimization of the LAMP Reaction Using the Taguchi Method. Lane 1: 4 mM MgSO₄, 60 °C, 40 min; Lane 2: 6 mM MgSO₄, 60 °C, 60 min; Lane 3: 8 mM MgSO₄, 60 °C, 90 min; Lane 4: 4 mM MgSO₄, 63 °C, 60 min; Lane 5: 6 mM MgSO₄, 63 °C, 90 min; Lane 6: 8 mM MgSO₄, 63 °C, 40 min; Lane 7: 4 mM MgSO₄, 65 °C, 90 min; Lane 8: 6 mM MgSO₄, 65 °C, 40 min; Lane 9: 8 mM MgSO₄, 65 °C, 60 min.

Specificity of the LAMP Assay

The specificity of the LAMP assay for the *SEB* gene detection was assessed employing the DNA genomic of untargeted bacteria as negative controls, including *Yersinia enterocolitica* and *Bacillus cereus* bacteria. After spinning reaction tubes with SYBR green for visual inspection of LAMP products under UV light, bright green fluorescence as positive was only observed in *S. aureus* DNA, whereas no amplification was observed in LAMP reactions with other bacteria DNA (Figure 6). These results indicated that cross-
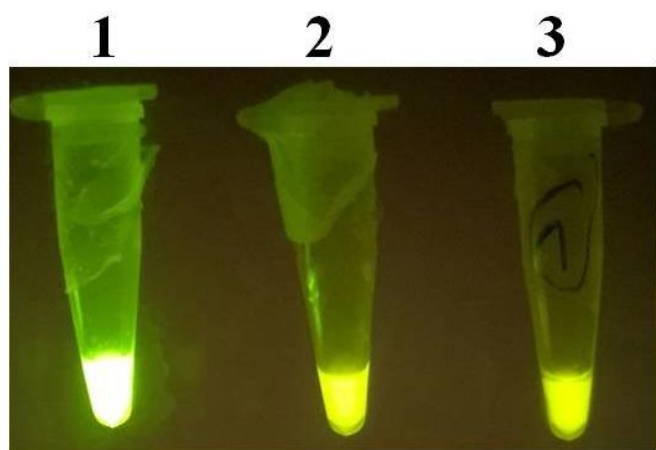
Figure 6. Specificity of the LAMP Assay and Visualization under UV Light with SYBR Green. Lane1: *S. aureus*; Lane 2: *B. cereus*; Lane 3: *Y. enterocolitica.*

reactivity with any of the bacteria was unlikely.

Discussion

LAMP assay is one of the DNA replication methods that is very simple and from the beginning to the end of the reaction performs at a single temperature and in one step (tube). This technique has been considered for toxins detection and despite its simplicity has a very high selectivity. Since this method does not need expensive devices such as thermocycler, electrophoresis, and gel documentation it is considered as a cost-effective technique. The positive point of the LAMP assay is that the products could be visualized using various staining methods and are observable by the naked eye.[23-25] The LAMP method is proposed for micro-biological clinical laboratories without a thermocycler since does not require any special laboratory equipment. In the present work, we used Taguchi experimental design for the specific detection of *S. aureus* using the LAMP technique. By optimization of various factors in the LAMP reaction, an isothermal and specific mobile diagnostic kit for the detection of numerous pathogens can be designed.[26,27] In 2007, Goto and co-workers used the LAMP technique to study the *SEA*, *SEB*, *SEC*, and *SED* genes in the condition of 60 °C, 60-min incubation time and 8 mM MgSO₄ concentration.[28] In 2011, Ken-Ichi Hanaki used the LAMP technique in the condition of 63 °C, 60-min incubation time, and 8 mM MgSO₄.[29] In 2012, Sowmya et al., used the PCR and LAMP techniques to assess the presence of SEA-SED enterotoxins in food samples such as milk and rice. The results showed that the sensitivity of the PCR method was about 10^4 cfu/ml but the sensitivity of the LAMP method was 10^2 cfu/ml and was performed at 65 °C with 60-min incubation time; and 8 mM MgSO₄ concentration.[30] In another report in 2013, Zhao and co-workers used the LAMP method for 118 clinical samples and 314 food samples. He detected the *FemA* gene in *S. aureus* at 65 °C,

and 45-min incubation time, and the limit of detection was about 100 fg DNA/tube and 10^4 cfu/ml.[31] In 2015, Wang et al., used real-time LAMP assay with the new method of primer design to increase the sensitivity and selectivity in order to 16S rRNA and 23S rRNA detection. Incubation temperature was optimized from 53, 55, 57, 59, and 61 °C and also the amplification was performed in 60 min with 6 mM MgSO₄ concentration, finally the sensitivity was reported at about 10 fg DNA.[32] Yin in 2016, used Multiplex LAMP/LFA to detect *SEA-SEB* genes in food samples. They developed this assay at 60 °C, 30 min incubation time with 6 mM MgSO₄. The sensitivity of this assay was determined at about 102 cfu/ml. Also, they optimized the incubation time at 15, 30, 45, and 60 min, finally 30 min incubation time showed the best amplification.[33] In 2016, Sheet et al. studied the *Nuc* gene of *S. aureus* in food samples with the LAMP technique in the condition of 65 °C and 30 min incubation time. The results showed a sensitivity of about 0.052 pg/μl.[34] In 2020, Zeinoddini et al., used the Taguchi method in order to optimize conditions for the detection of the zonula occludens toxin (zot) gene from *Vibrio cholerae* by LAMP assay. In this study, the optimum condition was 6 mM MgSO₄, an incubation time of 60 min, and a temperature of 65 °C.[35]

Conclusion

In the present work, for the first time, we used the Taguchi design experiment for the identification of the SEB gene by LAMP assay. Our results indicated that the sensitivity of optimized LAMP using the Taguchi experimental design was higher than the PCR assay. Our data has indicated that the best amplification rate was at 65 °C, 60 min incubation time, and 8 mM MgSO₄ condition. Accordingly, this condition could be used for the development of a portable diagnostic kit in order to *S. aureus* identification.

Authors' Contributions
Author contributions are similar for all of them.

Conflict of Interest Disclosures
The authors declare that they have no conflicts of interest.

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