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Point-of-care detection of 16S rRNA of *Staphylococcus aureus* based on multiple biotin-labeled DNA probes

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ABSTRACT

A visual method that combines multiple biotin-labeled DNA probes and lateral-flow nucleic acid biosensor was developed to detect *Staphylococcus aureus*. The 16S rRNA from *Staphyloccocus aureus* (*S. aureus*), coupled with multiple biotin-labeled DNA probes, was functionalized in a signal structure for lateral-flow point-of-care detection. The secondary structure of the 16S rRNA was unwound by two specific capture probes modified by Fam and multiple bridge probes, which extended additional sequences for use as initiators. By utilizing the initiators, each target 16S rRNA with multiple DNA probes could tether a number of biotin molecules, so that a large number of streptavidin-labeled gold nanoparticles could be introduced in the lateral flow assay. The images of the lateral flow detection results obtained using a smartphone were transmitted to a computer via Wi-Fi or Bluetooth connection for quantitative processing by ImageJ. The limit of detection was 10^3 cfu/mL without sample enrichment, and decreased to 0.12 cfu/mL following a 3-h enrichment of samples in growth medium. Notably, this method presented high specificity and applicability for the detection of *S. aureus* in food samples. In short, the developed visual non-specific operation method is very suitable for point-of-care diagnosis of pathogens in resource-limited countries.

1. Introduction

Many pathogenic bacteria found in the environment, food, water, and soil can often spread to humans and cause food-borne diseases. A total of 31 major pathogens have been identified to cause food-borne diseases in millions of people each year through contaminated-food consumption [1]. Among them, *Staphylococcus aureus* is a widely distributed pathogenic bacterium in nature, which is considered as one of the most serious threats to public health [2,3], affecting people all over the world, especially those living in poor areas [4]. Therefore, to control infectious diseases caused by *S. aureus*, there is an urgent need to develop a simple and cost-effective point-of-care test (POCT) for *S. aureus* detection.

Owing to their high sensitivity, ideal specificity, and good reliability, bacterial culture and colony counting method are used as the gold standard for the identification and quantitative analysis of *S. aureus;* however, they are not suitable for POCT because of the requirement of 3–5 days to yield results. In contrast, immunoassays such as conventional ELISA, as a popular and widely used technique for detecting pathogenic bacteria, require shorter detection time of several hours, but present sensitivity of 10^6–10^7 CFU/mL [5]. Specific nucleic acid sequence detection plays an important role in pathogen detection, because the nucleic acid sequence is individualized and associated with pathogenic specificity. The most widely used PCR is a valuable tool for the detection and analysis of pathogenic nucleic acids, PCR could be more sensitive than immunoassays because of its ability to amplify trace amounts of DNA to detectable levels. However, the high cost and confined resources limit the use of PCR technology in developing and underdeveloped countries. Several low cost nucleic acid detection methods based on isothermal amplification (e.g., loop-mediated amplification, nucleic acid sequence based amplification), as an alternative approach, have the ability to provide a platform for POCT in a low resource environment because of their high sensitivity and specificity [6]. These methods are performed under the condition of constant temperature, which simplifies the reaction process and is conducive to POCT.

While target nucleic acid amplification assays are sensitive, they require enzymatic amplification and are therefore easily affected by inhibitors and prone to false-positives. In contrast, signal amplification assays have certain specific advantages; for example, they typically do

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not require target amplification step, thus simplifying the detection workflow, and are more resistant to inhibitors and less prone to false-positives in the absence of enzymes [7]. In the past few decades, various signal amplification methods have been developed for direct detection of specific nucleic acids, such as fluorescence assay, electrochemical analysis, and biosensor. Furthermore, considerable efforts have been made to develop sensitive biosensors for nucleic acid detection based on signal amplification [8]. Direct multiple labeling of target nucleic acid is an effective signal amplification method. In a previous study, 64 DNA strands were directly hybridized with 16S rRNA to form 32 deoxy ribozyme catalytic nuclei to generate fluorescence signals, and achieved detection of about 3×10^4 bacterial cells [9]. Furthermore, to image mRNA expression patterns in fixed biological specimens, fluorescence amplification polymers formed by self-assembly of hybrid chain reaction were added to the target mRNA of interest through complementary DNA probes [10–12].

In this study, based on 16S rRNA appended with multiple biotin-labeled DNA probes and lateral flow nucleic acid biosensor (LFNAB), a visual POCT method for *S. aureus* detection was developed. It must be noted that 16S rRNA has been widely used in bacterial classification and identification, mainly because of its bacterial specificity, with each bacterium containing 5,000–75,000 ribosomes. In the present study, the 16S rRNA extracted from *S. aureus* was hybridized with two specific capture probes (CPs) and multiple bridge probes (BPs), which were connected with prefabricated biotinylated report probes (RPs), and the signal was output by LFNAB. Each target 16S rRNA yielded multiple biotinylated DNA probes in this process, thus functionalizing the signal structure, and numerous streptavidin (SA)-labeled gold nanoparticles (AuNPs) were introduced in the LFNAB for visual detection. The images of the lateral flow detection results, obtained by using a smartphone, were transmitted to a computer via Wi-Fi or Bluetooth connection for quantitative processing by ImageJ software (Fig. 1).

2. Experimental

2.1. Reagents

Bacterial RNA Extraction Kit was supplied by BioFlux (Japan), lysozyme was purchased from Biosharp (Guangzhou, China), and biotin-11-dUTP was obtained from Thermo Scientific (USA). DNA polymerase, dNTPs, and DNA ladder were purchased from TaKaRa Bio Inc. (Dalian, China). Colloidal AuNPs was bought from Hualan Chemical Co., Ltd. (Shanghai, China) and SA was obtained from Sangon Biotech. (Shanghai, China). Bovine serum albumin (BSA)-biotin and anti-Fam antibody were purchased from Ruiqi Biotech Co., Ltd. (Shanghai, China). All the reagents were of analytical grade. TAE and TE buffers were prepared by using ultrapure water. All DNA probes were synthesized by Sangon Biotech (Shanghai China) and primers were obtained from Invitrogen (Shanghai, China). The oligonucleotide sequences used in this study are listed in Tables S1 and S2.

2.2. Bacterial strains and culture conditions

S. aureus (CP020354), *Salmonella typhimurium* (Z49264), *Escherichia coli* (Z83204), *Campylobacter jejuni* (L14630), *Shigella flexneri* (CP024473), *Listeria monocytogenes* (KJ765692), *Enterobacter sakazakii* (AB435579), and *Proteus mirabilis* (AB626123) were all provided by Institute of Military Veterinary (China). All the bacteria were cultivated in LB medium (5 g/L NaCl, 10 g/L peptone, and 5 g/L yeast extract; pH 7.0) in a rotary shaker at 37 °C and 170 rpm for 18 h. The bacterial counts were expressed in CFU/mL.

2.3. Total RNA extraction

The pathogenic bacterial cells were collected by centrifugation at 7,500 rpm and 4 °C for 5 min. The total RNA was extracted with TRIzol method. In brief, the pelleted cells were resuspended in EP tube containing 1 mL of TRIzol and incubated for 5 min. Then, about 1/2 volume of chloroform was added to the suspension, vigorously shaken for 15 s, incubated for 3 min, and centrifuged at 12,000 × *g* for 15 min. The

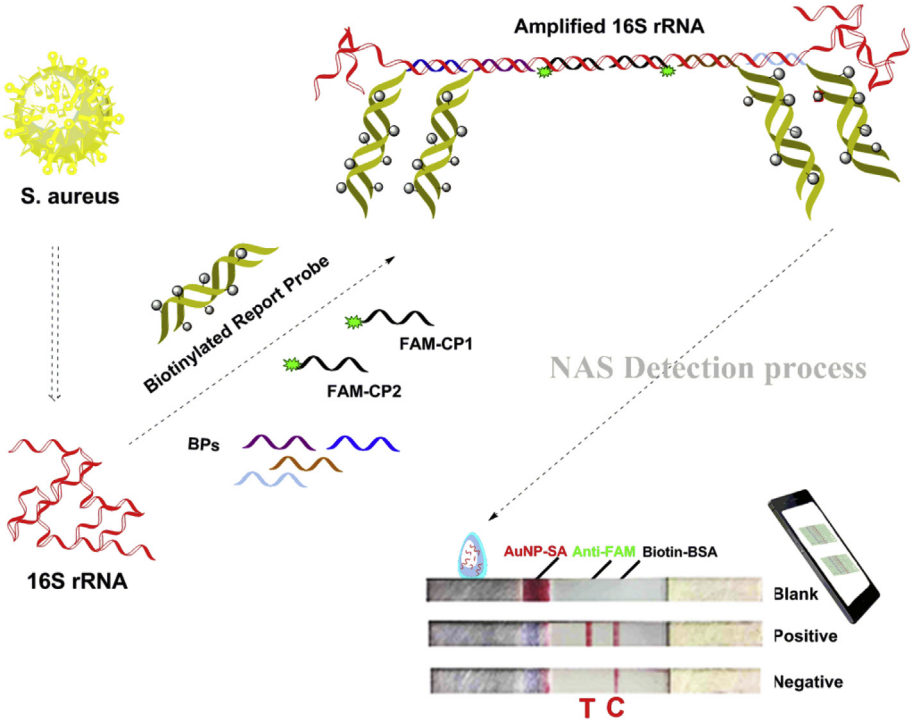

Fig. 1. Schematic representation of POCT of 16S rRNA of *S. aureus* based on multiple biotin-labeled DNA probes, lateral flow, and smartphone.

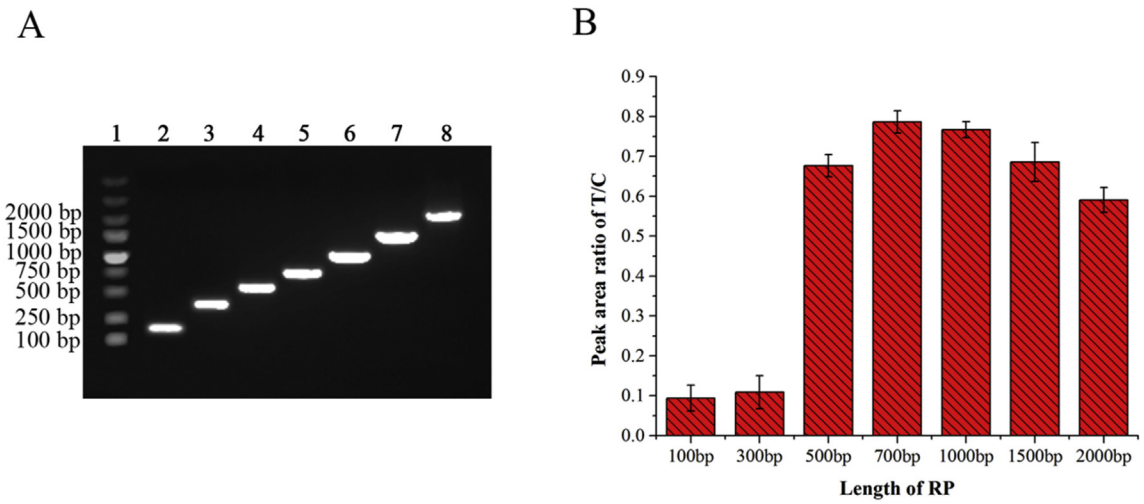

Fig. 2. Effect of different-length RPs on *S. aureus* detection. (A) Preparation of RPs with different lengths (101, 333, 526, 732, 1,035, 1,515, and, 2187 bp). (B) Corresponding results of 16S rRNA ligated with different-length RPs. Vertical bars indicate standard deviation (n = 3).

aqueous solution was transferred to a new EP tube and slowly mixed with equal amount of isopropanol. After incubation at room temperature for 10 min, the solution was centrifuged at 12,000 × *g* for 10 min to remove the supernatant. Subsequently, the pellet was gently washed with 1 mL of pre-chilled 75% ethanol and centrifuged at 7,500 × *g* for 5 min. The obtained supernatant was discarded and the pellet was allowed to stand for 15 min at room temperature to evaporate the remaining ethanol. Finally, the total RNA was dissolved in preheated DEPC water at 60 °C and stored at −40 °C.

2.4. Design of probes

Probes played important roles in this study, and were designed as follows. The target 16S rRNA of specific sequences of *S. aureus* was determined through alignment with the responding sequences of different bacteria by using DNAMAN software. Subsequently, two specific segments were screened and modified with fluorescence at the 5′ and 3′ ends, respectively. Then, several short probes were designed according to both the sides of the sequences to change the fold structure of the 16S rRNA, and an additional sequence was supplemented to the end of the short probes to connect with biotin-labeled RPs that were synthesized based on primers and can be used for a long time.

2.5. Preparation of LFNAB

LFNAB was manufactured and assembled based on the detailed description in relevant literature with some modifications [13]. Four components, including absorbent pad, conjunction pad, nitrocellulose (NC) filter membrane, and residue absorbent pad, were assembled. A total of 18 μg/mL SA was mixed with 20 nm AuNPs (0.01%, m/v) to obtain the SA − AuNPs complex. Then, the complex was impregnated on the absorbent pad of NC membrane, and anti-Fam monoclonal antibodies with 4 and 1.2 mg/mL BSA-biotin were respectively sprayed on the NC membrane at a concentration of 1 μL/cm on the test line (T line) and control line (C line). The LFNAB was cut into pieces (0.5-cm width and 5-cm length) and the end sealed products were stored at room temperature until further use.

2.6. Synthesis of RPs

Biotin-modified double-stranded DNA was employed along with BPs to obtain biotinylated amplified 16S rRNA, and the target product was combined with SA in the absorbent pad to form a readable signal on the LFNAB. Then, 1 mmol dUTP-biotin was first mixed with dNTP and subjected to PCR, in which part of dUTP-biotin compete with the complementary sites of dATP and replace dTTP bases to form high copies of biotin-labeled dsDNAs. The reaction was performed in a 50-μL system, comprising 5 μL of buffer, 4 μL of dNTP including dUTP-biotin, 1 μL of respective primers, 0.5 μL of DNA polymerase, and distilled water to a final volume of 50 μL.

2.7. General procedure

Equal volumes of Fam-CP1, Fam-CP2, and multiple BPs at concentrations of 0.5 μM were mixed with total RNA (400 ng/μL), and 40 μL of this mixture were denatured at 95 °C for 5 min. Afterwards, the re-prepared biotinylated RPs were added to the CP/16S rRNA/BP complex at 65 °C for 3 min to promote nucleic acids hybridization reaction, and respectively inoculated for more than 30 min at room temperature to form sandwiched structures of Fam-CPs/16S rRNA/bio-RPs. The target 16S rRNA was mixed with a certain amount of running buffer (25% saline sodium citrate buffer containing 4% BSA) and then dropped on the sample pad of LFNAB. The LFNAB detection result for the 16S rRNA was determined after 10 min. The appearance of visible red lines at both the C and T lines was regarded as positive detection of amplified 16S rRNA. A negative test result was indicated by the presence of a red line solely at the C line. For quantitative analysis, photos of the LFNAB detection results were taken using a smartphone and the signal strength of the T/C lines (peak area ratio of T/C, the intensity of the test band divided by the intensity of the control band) was quantitatively processed in ImageJ software [14].

3. Results and discussion

3.1. Optimization of the length of biotinylated DNA RPs

To enhance their identification capacity, the length of the biotinylated DNA RPs was optimized in this study. Biotinylated DNA RPs with different molecular weights (101, 333, 526, 732, 1,035, 1,515, and 2,187 bp) were synthesized by PCR (Fig. 2A). As shown in Fig. 2B, the signal value increased with the increasing segment length of the biotinylated DNA RP, reaching the maximum at almost 700 bp and then decreasing slowly with the increasing segment length. Hence, 700 bp was chosen as the optimal fragment length of biotinylated DNA RPs. It must be noted that short segment probes (100 or 300 bp) may not carry sufficient biotin, resulting in low signal value. In contrast, with the increasing length of the biotinylated DNA RPs, the signal intensity gradually weakened, indicating that excess biotin produced

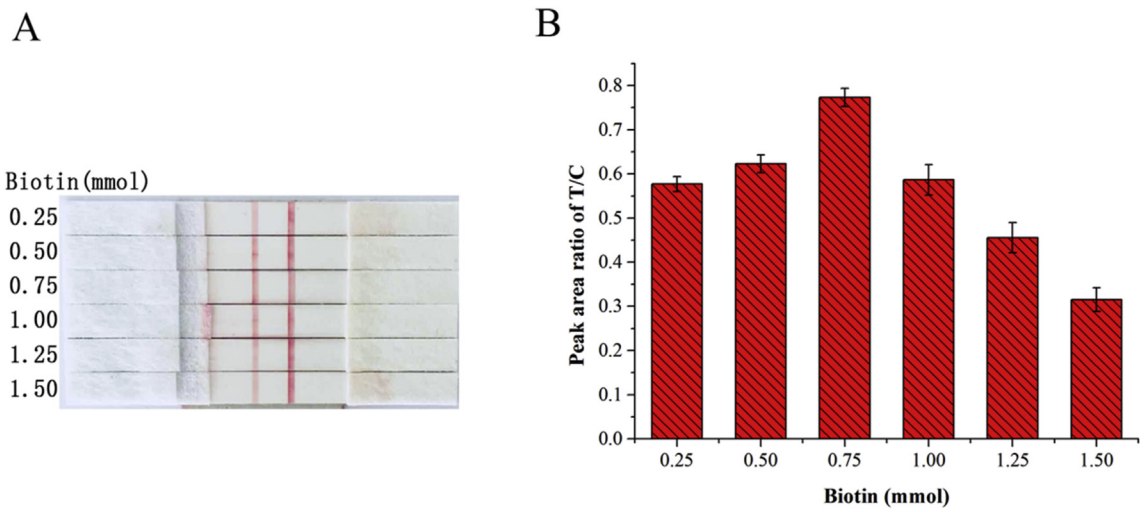

Fig. 3. Optimal concentration of biotin in RP. (A) Effect of a 700-bp RP modified with different amounts of biotin on *S. aureus* detection. (B) Detailed statistics of the LFNAB output results from histograms. Vertical bars indicate standard deviation (n = 3).

intramolecular competition, thus affecting the function of the probe. Therefore, 700 bp was considered as the optimal length of RP for the detection of *S. aureus*.

3.2. Optimization of biotin concentration

The concentration of biotin in the biotinylated DNA RPs determines the signal value of the assay. In the process of preparing biotinylated DNA RP fragments by PCR, the concentration of dUTP-11-biotin defines the amount of biotin in the DNA fragment. As shown in Fig. 3A, the signal value increased with the increasing concentration of dUTP-11-biotin, and the highest signal value was obtained with 0.75 mM dUTP-11-biotin. However, a further increase in the concentration of dUTP-11-biotin decreased the signal value. This result may be owing to the insertion of excess biotin in the fragment, which may affect the binding of AuNPs to each other.

3.3. Optimization of BPs quantity

The BPs complementary region of 16S rRNA was selected on both the sides of the CP complementary region of 16S rRNA. Hybridization of 16S rRNA with BPs can unwind complex secondary structures of 16S rRNA, and the resulting single chain region can be easily combined with CPs. As the amount of BPs bound by 16S rRNA determines the number of RPs indirectly carried by 16S rRNA and that hybridized by 16S rRNA affects the sensitivity of the developed method, the quantity of BPs was optimized. As shown in Fig. S1A, with the increasing number of probes, the signal value continued to increase. An increase in the BPs from 1 to 4 caused a significant enhancement of signal intensity, and a further increase in the number of BPs from 5 to 7 produced a smooth increase in the signal intensity; however, when the number of BPs reached 8, the signal intensity decreased. This result suggested that the optimum quantity of BPs for the developed method was 7.

3.4. Optimization of CPs and BPs concentrations

The CPs explicitly recognize specific region of 16S rRNA from *S. aureus*. To improve the sensitivity of the developed method, two short chain specific CPs were designed. As shown in Figs. S2A and 5B, the optimal concentrations of CP1 and CP2 were 0.01 and 1.0 μM, respectively. The mixtures of seven BPs were continuously diluted for optimal concentration screening. As shown in Fig. S2C, the signal value increased with the increasing concentration of the probe mixture, reaching the maximum at a probe concentration of 0.3 μM, but decreasing with further increase in the concentration of the probe mixture. This result may be owing to excess probe inhibition, causing a decline in signal values. Subsequently, optimization of incubation time for 16S rRNA and probes was performed. As shown in Fig. S2D, the background signal intensity gradually enhanced owing to the extension of incubation time at room temperature. At an incubation time of 150 min, the background signal was prominent, and the signal intensity remained stable with a further increase in incubation time. Therefore, 150 min was considered to be the optimal incubation time.

3.5. Sensitivity analysis

To verify the sensitivity of the detection strategy, *S. aureus* was serially diluted (log-dilution in each step) at concentrations ranging from 10^{-2}–10^{8} CFU/mL. As shown in Fig. S3

A and 6B, the limit of detection (LOD) was 10^{3} CFU/mL. There was a good linear relationship between dilutions 10^{3} and 10^{7} CFU/mL, and the linear equation was $Y = 0.1153X + 0.0023$; $R^2 = 0.9875$. According to the US Food and Drug Administration, the concentration of *S. aureus* in food must not exceed 10^{4} CFU/mL [15]. The sensitivity of the method developed in the present study met the safety standard requirement of *S. aureus* in food. In a previous study, a straightforward, fast, and inexpensive POCT of pathogenic bacteria with LOD of about 3×10^{4} bacterial cells had been designed based on 16S rRNA hybridization with 64 DNA strands and fluorescence signal output [9]. In recent years, lateral flow nucleic acid biosensors (LFNABs) combined with different signal amplification approaches have been developed for analytical detection because of their cost-effectiveness, short assay time and easy-to-use attributes compared to intricate instrumentation [16,17]. In addition, Liu et al. [18] developed an ultrasensitive and simple lateral flow immunoassay for *Salmonella* sp. detection with LOD as low as 10^{4} CFU/mL. In the present study, multiple biotin-labeled DNA probes were used to improve the detection sensitivity by an order of magnitude. To further improve the sensitivity of the assay, sample with concentration gradient below 10^{3} CFU/mL was enriched for a minimum of 3 h. As shown in Figs. S3C and 6D, after 3 h of enrichment of the sample, the LOD reached 0.12 CFU/mL, with a good linear relationship between 10^{-1} and 10^{3} CFU/mL.

3.6. Selectivity analysis

To demonstrate the selectivity of the assay strategy for *S. aureus* detection, several common pathogens, such as *C. freundii*, *E. coli*, *L. monocytogenes*, *S. typhimurium*, *S. flexneri*, *E. sakazakii,* and *P. mirabilis,*

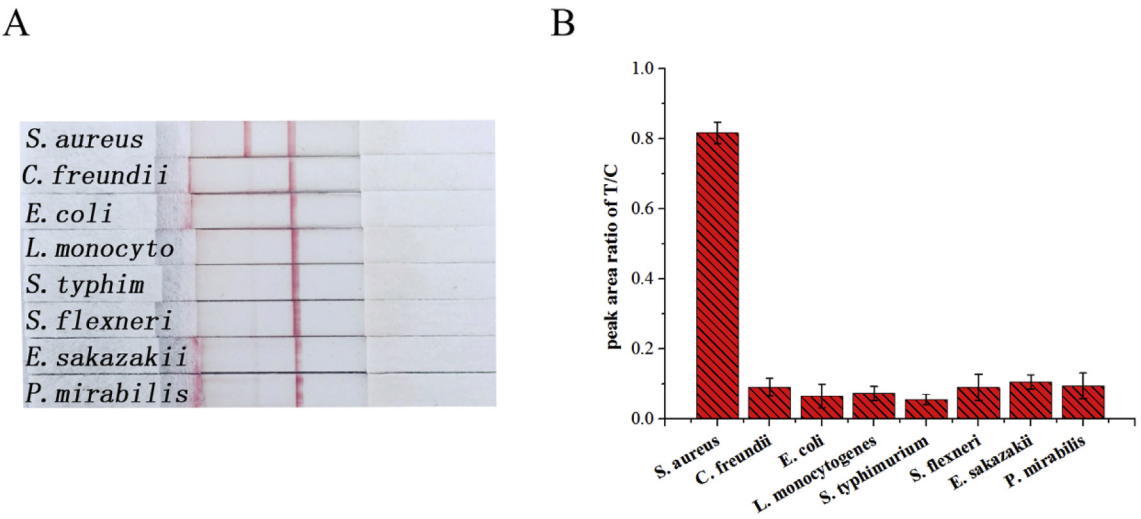

Fig. 4. LFNAB signals and the ratio of T/C peak areas for 10^7 CFU/mL *S. aureus*, *C. freundii*, *E. coli*, *L. monocytogenes*, *S. typhimurium*, *S. flexneri*, *E. sakazakii,* and *P. mirabilis*, respectively. Vertical bars indicate standard deviation (n = 3).

were investigated. The signal values were nearly negligible when 10^7 CFU/mL *C. freundii*, *E. coli*, *L. monocytogenes*, *S. typhimurium*, *S. flexneri*, *E. sakazakii* and *P. mirabilis* were used (Fig. 4), demonstrating the remarkable selectivity of the developed biosensor.

3.7. Detection of S. aureus in milk samples

To verify the ability of the developed assay system to detect target pathogens in food samples, *S. aureus*-spiked milk samples were analyzed. It must be noted that recovery experiments are usually applied to actual samples to verify and evaluate the accuracy of assays. Accordingly, different concentrations of *S. aureus* (10^4, 10^5, and 10^6 CFU/mL) were added to the milk samples, and the recovery rate of bacteria in milk samples was determined based on the calibration curve. The results revealed that the developed assay could successfully detect *S. aureus* in milk with good recovery rate from 98.2% to 108.4% (Table S3). Thus, with a good recovery rate and precision, the assay designed in the present study could have significant potential application as POCT.

4. Conclusion

In this study, based on 16S rRNA conjugation with the prepared multiple biotin-labeled DNA probes and LFNAB, a simple and low cost POCT of *S. aureus* was developed. The results showed that this method was suitable for the detection of pathogens in real samples. The visual test images captured using a smartphone were transferred to a computer based on Bluetooth or Wi-Fi for further quantitatively processing by ImageJ software. The LOD of this method was 10^3 CFU/mL, and the sensitivity was as low as 0.12 CFU/mL after enrichment for 3 h. Thus, this simple, low cost method can be a suitable POCT for *S. aureus* detection, especially in developing and underdeveloped countries.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mcp.2019.101427.

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