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ORIGINAL PAPER

Rapid detection of *Staphylococcus aureus* via a sensitive DNA hybridization assay based on a long-lifetime luminescent europium marker

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Abstract A two-probe tandem nucleic acid hybridization assay for detection of *Staphylococcus aureus* is presented. It is based on a europium(III) complex as a marker that has a long fluorescence lifetime, high quantum yield and can be easily conjugated to an oligonucleotide signaling probe. The amino-modified capture probe was associated with the signaling probe to form a two-probe tandem DNA pattern that is complementary to the target DNA. The method was optimized in terms of hybridization temperature, hybridization time and washing time. This resulted in good specificity and sensitivity when detecting such bacteria in food samples.

Keywords *Staphylococcus aureus* · Capture probe · Signaling probe · Long lifetime biomarker · DNA hybridization · Pathogenic microorganisms monitoring

Introduction

The food poisoning caused by *Staphylococcus aureus* (*S. aureus*) is characterized by the symptoms including nausea, vomiting, abdominal cramps, and diarrhea lasting from 24 to 48 h. And the complete recovery usually needs within 3 days. In spite of being as a mild and low mortality rate accident, it has been considered one of the most important diseases throughout the world [1–4]. Routine detection of *S. aureus* in food sample is usually carried out by traditional methods and the biological characteristics of the pathogen require a long time to get the biochemical identification [5, 6]. Therefore, a rapid and sensitive method of detection *S. aureus* bacteria is demanded in the field of safety food industry. This requirement has lead to develop multiple detection and identification approaches that have considerably contributed to shorten the analysis time [7–13]. They include a wide range of strategies that have been applied at the first examined step, such as the inclusion of colored substrates in selective media, the use of polymerase chain reaction (PCR) technique for the purposes of amplification a single or few copies of target DNA [14–16].

In recent years, the fluorescence analysis methods integrated with DNA-based molecular techniques have shown great potential for the specific detection and easily differentiation of pathogenic microorganisms [17–21]. But most conventional fluorophores has the fluorescence lifetime of about 10 ns or less. The background fluorescence from biological samples, buffer components, and carrier surface also has similar lifetimes with those fluorophores, which can greatly limit assay sensitivity. And the background noise becomes a main problem in most fluorescence analysis methods. To overcome this problem, dyes with considerably longer lifetimes have been used in assays based on time-resolved fluorescence (TRF) whereby the detection and measurement of assay signal is delayed until background fluorescence has dissipated. Among those dyes, lanthanide chelates exhibit a relatively efficient long-lived fluorescence lifetime of about 200–

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1500 μs. And they have been widely used in the research of nucleic acid field [22, 23].

A bifunctional europium complex of $Eu(TTA)_3(5\text{-}NH_2\text{-}phen)$, lanthanide chelate, was synthesized by our group [24]. Compared to other dyes of lanthanide chelate [25, 26], the complex has a long fluorescence lifetime, high fluorescence quantum yield, and is easy to label oligonucleotides for time-resolved fluorescence bioanalysis. It can be directly labeled with oligonucleotides as a long lifetime biomarker and used to the biological detection. In this study, the europium complex was labeled with the designed oligonucleotide of signaling probe and then with the capture probe forming a two-probe tandem DNA pattern were employed to the detection of target nucleic acid (Fig. 1). In the time-resolved fluorometry detection mode, the europium complex could effectively eliminate the background noises and improve the sensitivity of the method. In addition, the two-probe tandem DNA hybridization pattern used to investigate the pathogen microbiology has been approved to be an easy operation, satisfactory sensitivity and specificity approach [24]. In the present work, this analytical method was employed to directly monitor the actual *S. aureus* bacteria instead of the synthetic oligonucleotide, and it was re-optimized and successfully applied to examine *S. aureus* bacteria in the food samples. To the best of author's knowledge, this was the first time that the present method was carried out without any biotin-streptavidin for signal amplification and enrichment measure. And the satisfactory results of sensitivity and specificity were obtained from the study. The authors believe that the present approach would be widely applied in the detection pathogenic microorganism.

Experimental

Bacterial strains, culture conditions and medium

The *S. aureus* (No. GIM1.221) and *Staphylococcus epidermidis* (*S. epidermidis*) strains (No. GIM1.143) were purchased from Guangdong Microbial Culture Collection Center (Guangzhou, China). The *Escherichia coli* strain (No. CCTCC 200068) was from China Center For Type Culture Collection (Wuhan, China). And they were routinely grown on nutrient broth or agar at 37°C overnight. Nutrient broth medium contained 10.0 g peptone, 5.0 g nutrient broth, 5.0 g NaCl, 15.0 g agar (if necessary) per litre (pH 7.0–7.2), and the medium was sterilized by a LDZX-30FBS vertical heating pressure steam sterilizer (Shanghai Shenan Medical Instrument, http://www.shenan.com.cn) before being used. Solution pH was measured by a pHS-3B pH meter (Shanghai Precision & Scientific Instrument Co. Ltd., http://www.spsic.com).

Capture probe, signaling probe and target DNA sequence

Sequences of the oligonucleotide were designed by Primer Premier 5.0 software. The specificity of those sequences was checked by the program 'Probe Match' provided by the Ribosomal Database Project II and the NCBI Blast 2 alignement tool in GenBank database. Sequences of the capture and signaling probe were complemented with the specified target DNA sequences. Melting temperature of the two probes was designed in the approximate value to insure the uniform condition of renaturation in hybridization. Ten bases of 'T' were added on the 3′end of capture probe and 5′end of signaling probe, which could avoid the sterically

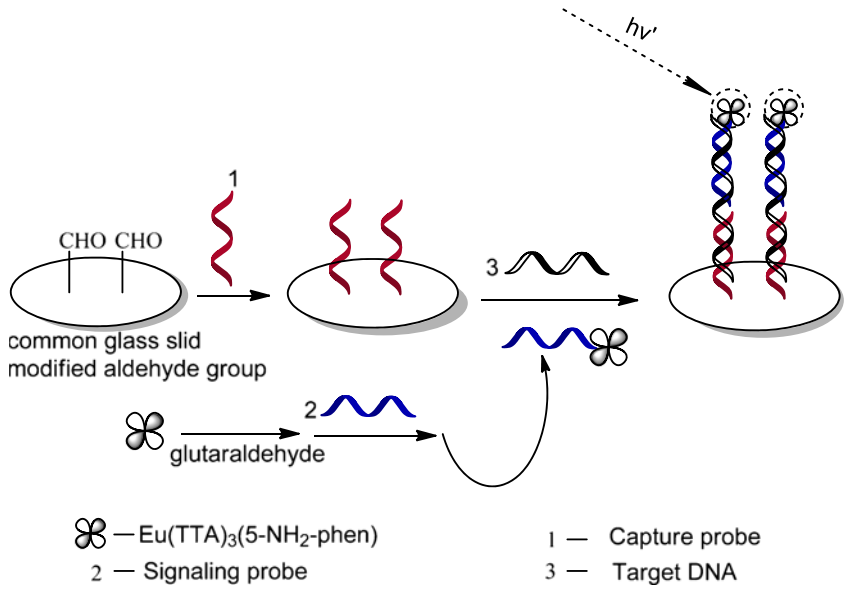

Fig. 1 Schematic illustration of sensitivity detection *S. aureus bacteria* based on a long lifetime biomark hybridization method

hindered from the immobilization substrate. For the purpose of coupling, an amine group was introduced to the end of two probes, respectively. The selected sequences of capture probe and signaling probe were 5′-CAC TTT TTC TTA AAT GTT GTTC $(T)_{10}$-3′-NH_2 and 3′-TTC GCT TTT TTC TCT TTTA $(T)_{10}$-NH_2-5′, respectively. All the oligonucleotide sequences were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. (Shanghai, China, http://www.sangon.com).

Capture probe conjugation onto glass slide surface

Common glass slides were modified with aldehyde groups according to the literature [27]. The clean glass slides were immersed in 25% ammonium hydroxide for 2 h and rinsed with double distilled water. Then they were soaked in the solution of 2% 3-aminopropyltrimethoxysilane at pH 4.5 adjusted by glacial acetic acid. And they were ultrasonically washed with double distilled water 30 min later. Finally, they were immersed in 2.5% glutaraldehyde for 3 h and washed with PBS buffer and double-distilled water in turn and dried at room temperature.

The capture probe diluted in TE buffer with the volume o f 40 μL was activated and pipetted onto the aldehyde glass slides surface. After incubated at room temperature for 5 h, the glass slides were washed with 0.2% sodium dodecyl sulfate and double distilled water twice to remove unbound DNA. Subsequently, the glass slides were dried under a filtered air-stream. To block the surplus aldehyde groups on the surface, the glass slides were immersed in a glycin solution for 1 h. The final glass slides coupling with capture probe were dried after washed with 0.2% sodium dodecyl sulfate and double distilled water twice, respectively.

Signaling probe conjugation with europium complex

The europium complex of $Eu(TTA)_3$(5-NH_2-phen) using 2-thenoyltrifluoroacetonate (TTA) and 5-amino-1,10-phenanthrolin (5-NH_2-phen) as ligand reagents was synthesized by our group. The complex has the lifetime of 0.688 ms and a very high fluorescence quantum yield of 0.62 [24]. With the characteristics of large Stokes shift and sharp emission profile, the europium complex can greatly improve the signal-to-noise ratio by decreasing the background in time-resolved fluorescence detection mode.

Firstly, the europium complex was dispersed in a 2.5% glutaraldehyde solution and stirred for 3 h at room temperature. Then, the mixture was centrifuged (by a Centrifuge 5415R, Eppendorf, http://www.eppendorf.com) and washed with double distilled water twice, and finally suspended in PBS buffer. A certain concentration of signaling probe was added to the complex suspended solution with continuous stirring at room temperature for 5 h and then centrifuged and washed with PBS buffer to remove the unbound oligonucleotides. Finally, the mixture was treated with glycin solution in order to inactivate the unreacted aldehyde groups.

Genomic DNA preparation

Total genomic DNA was extracted by a Generay™ Genomic DNA Extraction Kits following the instructions of the manufacturer (http://www.generay.com). An overnight culture of the bacterial cells in fresh nutrient broth was grown to 0.6–1.0 OD_{600}. The purity of the collected DNA was verified by a spectrophotometer (DU®640Bechman, US, http://www.beckmancoulter.com) using the A_{260}/A_{280} ratio.

Hybridization assay

The primitive nucleic acid was heat-denatured at 95°C for 5 min and immediately immersed in mixture of ice and water for 5 min to obtain the single strand of target DNA. A hybridization box was prepared filled with cotton soaked in 6×SSC solution to guarantee a uniform moistening of the glass slides surface. Target single stranded DNA 10 μL, hybridization buffers (6×SSC) 10 μL and dye modified signaling probe 10 μL were pipetted onto the glass slides and mixed thoroughly. Then the box was incubated at designed temperatures in a thermostat. After hybridization reaction is completed, the glass slides were rinsed by washing buffers. All the assays were performed in triplicates. Unless otherwise stated, blank controls were used of sterile double distilled water instead of samples.

Washing strategy

A stringent washing strategy was performed to disrupt undesired hybrids after hybridization. The preliminary washing step (high salt concentrations and low temperatures) could remove nonspecifically bound probe but the low homology hybrids would not be disrupted in this procedure. The final washing step (low salt concentration and high temperatures) could remove undesired hybrids of low homology. Protocol of stringent washing is shown in Table 1. Stock solutions of 20×SSC and 10% SDS were used as washing buffers.

Time-resolved fluorescence detection and data acquisition

In this research, time-resolved fluorescent intensity was monitored by a detection system which consisted of a Perkin-Elmer LS-55 spectrofluorimeter, a personal computer, a two-arm of the bifurcated optical fibers and a home-made poly (tetrafluoroethylene) detection cell. The optical fibers were fixed in the detection cell of the spectrofluorimeter to

Table 1 Strategy of stringent washing

Step	Volume	Final concentration	Temperature
Preliminary washing	10 mL 20×SSC 600 μL 10% SDS	1×SSC, 0.03%SDS	RT
	189.4 mL double-distilled water		
Final washing	2 mL 20×SSC 198 mL double-distilled water	0.2×SSC	50°C
	500 μL 20×SSC 199.5 mL double-distilled water	0.05×SSC	50°C

carry the excitation and emission light. The detection cell acted as an immobilized platform for the glass slide and optical fibers. It was divided into two sections from its cylinder center. One was dug a penetrative hole for locating the optical fibers, and the other was made into a chamber (deep 5.0 mm, side length 15.0 mm) for holding the glass slide with samples. The two sections were fixed by the mounting screw nut before detection.

All the time-resolved fluorescence signals were measured at slits (excitation 10 nm, emission 10 nm), the wavelengths (excitation 368 nm, emission 610 nm) and the delay time at 100 μs. A pulsed Xe lamp was light source and when it excited, measurement of the emission commenced after 100 μs, which allowed all short-lived background fluorescence and light scattering to dissipate. And fluorescence signal from the complex of $Eu(TTA)_3(5\text{-}NH_2\text{-phen})$ is then counted over a fixed time interval before the sample is re-excited. The long-lived fluorescence signal can therefore be measured with very high sensitivity.

Samples detection

Orange juice

The orange juice was purchased from a supermarket and made from a qualified drink produced factory. *S. aureus* cells were artificially inoculated into the sterile orange juice (pH=3.8) , and then 1 mL of the mixture was pipetted into a microtube and centrifuged at 12,000 rpm for 10 min [28]. The bacteria-containing pellet was suspended in 1 mL of phosphate buffer saline (PBS) and centrifuged again at 12,000 rpm for 10 min. The pellet was finally resuspended in 1 mL PBS and used for genomic DNA extraction.

Milk

The milk was purchased from a supermarket and made from a qualified dairy produced factory. *S. aureus* cells were artificially inoculated in 1 mL of pasteurized milk (fat ≥4%, protein ≥3.5%, pH=6.8). The bacterial cells were then isolated from the milk and used for genomic DNA extraction. The method was used to dispose of lipids and proteins from the milk ingredients to isolated *S. aureus* [29]. Firstly, proteinase K and 50 μL of 0.1% Triton X-100 were added to 100 μL of milk samples and they were incubated at 37°C for 1 h to eliminate protein and lipid components. After incubation, 900 μL of 150 mM NaCl was added and then the mixture was centrifuged at 12,000 rpm for 10 min. The pellet containing bacteria cells was collected after the centrifugation. The pellet was suspended in 1 mL of 150 mM NaCl and centrifuged again at 12,000 rpm and was finally resuspended in 150 mM NaCl.

Results and discussion

Influence of the hybridization temperature and time

Temperatures of optimum renaturation, stringent renaturation and non-stringent renaturation were investigated and they were 38°C, 53°C, and 28°C, respectively. As the former expectation, the signals were relatively weak at 38°C and 28°C while it had a satisfactory result at 53°C. As the reported hybridization temperature [30, 31], a stringent temperature would be fit for the renaturation of DNA single strands. And this might result from that high molecular weight of the specimen DNA was hard to outspread at low temperature and thus the reaction of base complementary pairing was incomplete. On the basis of the result, all of the experiments were carried out at 53°C.

The hybridization time was the important element which determined the completeness of hybridization reaction. As shown in Fig. 2, the fluorescence intensity visibly ascended from 3 h to 9 h and got to the summit at 9 h. Theoretically, the effective hybridization time is about 3 h, but there are some interferential elements in a given hybrid system and it needs much time to reach the suitable binding. In this assay, it needed about 9 h to reach the best hybridization efficiency.

Appropriate washing time

When the hybridization procedure is completed, it is necessary to remove the unbinding probe and the undesired hybrids of low homology. A stringent washing strategy was employed to disrupt undesired hybrids in this study. As

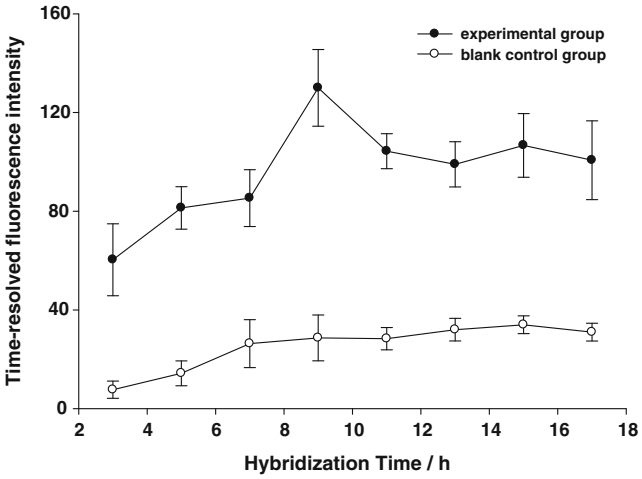

Fig. 2 Optimization of the hybridization time. The blank control group was sterile double distilled water instead of nucleic acid in hybridization detection. And values derived from three independent detections, error bars mean standard deviations

shown in Fig. 3, clear distinctions of fluorescence intensity were appeared at the different checking washing times. According to the principle of signal to noise ratio, the appropriate washing time was 4.5 min and lengthening the washing time did not obtain a better result.

The optimal washing time was less than that of the reported literature [30]. One possible explanation for this observation was that the present hybridized reaction was taken place on the common glass slide. And it was quite easy to remove the redundant probe or undesired hybrids on glass slides comparing with cellulose acetate membrane or other carriers. Furthermore, the common glass slides would be considered feasible and economical carrier in the high technique detection field, which could make the present method possible to widely generalize and apply.

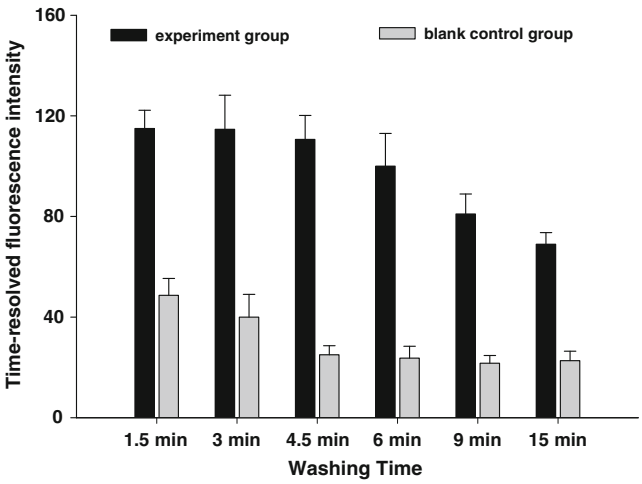

Fig. 3 Optimization of the washing time. Each testing time was the total time of the washing steps at preliminary and final stages. The blank control was sterile double distilled water instead of nucleic acid. And values derived from three independent detections, error bars mean standard deviations

Specificity of the method

The experiments of specificity were carried out by investigated DNA from four kinds of bacteria at the optimum conditions. The *S. epidermidis* strain was chosen, which was known to be closely related to *S. aureus*. The other strains, such as *Paenibacillus polymyxa* and *Escherichia coli* strain, were designed to serve as a random interfered strain in this experiment.

To showing advantages of the long lifetime biomarker, the fluorescence intensity of all samples was monitored with or without time-resolved fluorescence detection. According to the time-resolved fluorescence intensity (Fig. 4), it indicated that the value of positive control signal intensity was the highest and the *S. aureus's* was the second. And the other signals were almost as low as the blank controls. It was apparent that the ratio of signal to noise was desirable and the specificity was satisfactory. Obviously, all the fluorescence intensity without time delay was lower than those with. And the distinction of fluorescence intensity between positive and negative controls was unapparent. It could not meet the need of signal to noise ratio in the detection method.

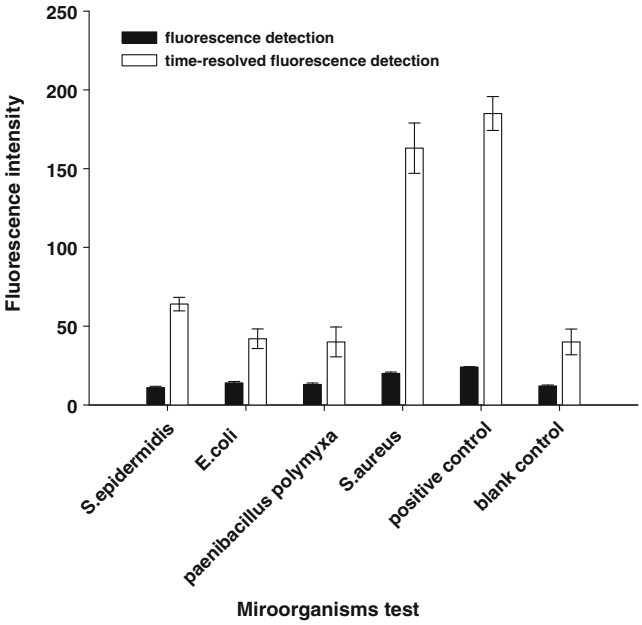

Fig. 4 Investigation of the specificity by this method. Positive control was the synthesized oligonucleotide of target DNA and the blank control was sterile double distilled water. The testing condition at time-resolved fluorometry mode was delay time 0.1 ms, gate time 1.0 ms, λ_{ex}=368 nm and λ_{em}=611 nm. The normal fluorescence intensity was detected at λ_{ex}=368 nm and λ_{em}=611 nm. And values derived from three independent detections, error bars mean standard deviations

Table 2 Results of detection *S. aureus* in food samples

	Time-resolved fluorescence	
	Intensity value	Standard deviation
Orange juice	103.0	10.2
Milk	88.6	12.4
PBS	140.8	6.14
Blank control	42.3	5.65

Sensitivity of the method

The detection limit of this assay was examined genomic DNA isolated from an overnight culture of *S. aureus*. Concentration of the nucleic acid was determined on 10-fold serial dilutions which were prepared by sterile PBS buffer. One milliliter of every dilution was plated on nutrient broth agar plates to obtain the accurate estimation of the cultivable *S. aureus* concentration. The results indicated that linear relationships were found between time-resolved fluorescence intensity and log (CFU mL^{-1} of *S. aureus*) from 1×10^2 to 1×10^6 CFU mL^{-1} ($y=-59+54.8\,x$, $R^2=0.981$). The threshold for the positive detection is set as background (blank) signal plus three times the standard deviation of the blank [32]. According to this criterion, the detection limit of this method is determined as 1.03×10^3 colony forming unit (CFU) per milliliter.

Cancelled the procedure of PCR amplification, the specificity and sensitivity of the method were guaranteed by specificity of the designed oligonucleotide sequences and the excellent time-resolved fluorescence intensity of the europium ternary complex. From the compared results between time-resolved and normal fluorescence intensity in the specificity experiments, it convinced that the long lifetime biomarker, formed by the europium ternary complex labeled with probe, could provide the possibility of specificity and sensitivity to the assay.

Detection of *S. aureus* in samples

For real food samples detection, the cells of *S. aureus* was artificially inoculated in orange juice, and milk samples to reach the concentrations of 1×10^6 CFU mL^{-1} cells. These samples were analyzed by the present assay with time-resolved fluorescence method. As shown in Table 2, in the mode of time-resolved fluorescence detection intensity values of food samples were significantly larger than the blank control's. However, they were less than those in the parallel investigation of PBS solution. The authors suggested that the less sensitivity of detecting *S. aureus* in the food samples compared with that in the PBS solution may be related to a few bacterial cells being lost when the *S. aureus* cells were isolated from the food samples and it can be also related to inhibitors coming from the food. The results indicate that the established assay in the present study is applicable for the detection of *S. aureus* in real food samples.

Comparison to previous methods

In order to show the specific features of this method, the authors compared it with some previous methods about detection of foodborne pathogens. Table 3 lists the respective information of the comparative items. The limit of detection (LOD) is associated with the sensitivity of a method and analysis time and cost levels are also significant features for laboratory productivity. As shown in Table 3, this work has a good LOD index and its experiment cost is considerably economical. Analysis time of this method may be improved by addition of some hybrid promoters or other measures and it would be taken into account in our further studies.

Conclusions

On the basis of results, the authors consider that the two-probe tandem DNA hybridization based on the long

Table 3 Figures of merits of comparable methods for determination of foodborne pathogens

Method used	LOD (CFU mL^{-1})	Application	Analysis time (h)	Cost levels	Ref.
Flow injection piezoelectric detection	1.1×10^7	Foods and waters	5	2	[33]
Gold based immunosensors	1×10^5	N.I.	N.I.	3	[34]
Nanogold linked CdTe nanocrystals	50	Spiked milk samples	N.I.	4	[21]
Au nanoparticles and piezoelectric detection	1.2×10^2	Food samples	N.I.	3	[28]
Long lifetime biomarker and two-probe tandem DNA hybrid	1.03×10^3	Food samples	9	2	this work

N.I. No information, Cost level scale from 1 (cost assigned to a conventional test) to 4 (estimated four times the cost of the conventional test)

lifetime biomarker for detection the food-borne bacterial pathogens of *S. aureus* is a valuable and sensitive method. It could be implemented as an alternative to improve the approach in the routine pathogenic microbiological analysis.

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