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Lateral Flow Assay for *Staphylococcus aureus* Detection in Cosmetics

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Abstract

In this study, a rapid and simple assay for the detection of *Staphylococcus aureus*, the important bacteria found in cosmetic contamination, was successfully developed. The assay was relied based on the use of an asymmetric PCR for the specific amplification of target DNA from *S. aureus* combined with a lateral flow-based assay for the rapid and visual detection of the amplified target DNA. A *nuc* gene of *S. aureus* was selected as a target gene and used to design the DNA primers and probe by targeting a 279-bp DNA fragment. The specificity tests showed that only sample containing *S. aureus*-specific DNA amplicons yielded a positive signal, indicating a high specificity of the assay for detecting *S. aureus* without cross-reactivity to other tested bacteria. The sensitivity of the assay was found as low as 1 pg of total bacterial DNA or equivalent to 3.4×10^2 CFU per tested reaction, implying the detection limit of the assay towards *S. aureus* detection. The feasibility of the assay for detecting *S. aureus* in cosmetic samples was determined, and the results were analyzed against a gold standard assay for the microbiological detection of *S. aureus*. Both methods showed comparable results, implying a high potential of the assay to be used for detecting *S. aureus* contamination in cosmetic samples.

Keywords: Asymmetric PCR, Lateral Flow Assay, *Staphylococcus aureus*, Cosmetics

Introduction

Staphylococcus aureus is one of the important pathogens associated with human and animal infections. This bacterium also can cause food spoilage and poisoning worldwide, and it is also found to contaminate in cosmetic products as well. The analysis of re-call cosmetic products in the European Union from years: 2005-2018 evidenced the contamination of *S. aureus* in eye balm, massage cream, and other personal care products (Lundov & Zachariae 2008; Neza & Centini, 2016; Stewart et al., 2016; Michalek et al., 2019). A high level of *Staphylococcus* spp. contamination in the in-used skin products (powder and cream) and eye products (mascara and eyeliner) in Iran has been indicated (Dadashi & Dehghanzadeh 2016). In addition, several cosmetic products (shared and used of the expired products) has also been reported to contaminate with *Staphylococcus* spp. in Poland (Skoron et al., 2017). Thus, the absence of this bacteria in cosmetic samples is necessary to limit its spread and hazard to the consumer's health.

To identify *S. aureus*, a standard method based on the culturing methods, directly observing the characteristics of colonies, and biochemical tests are required. However, these techniques are laborious and time-consuming, and may give non-specific results, yielding the false-positive or false-negative results. Thus, to cope with these limitations, a rapid and simple method is important to establish, but its specificity, sensitivity, and cost-effective are also required.

To date, numerous rapid assays have been carried out to detect *S. aureus*. Polymerase chain reaction (PCR) is one of such that examples. It was firstly described in 1991 to recognize *S. aureus* strains, related with methicillin-resistant phenotype (Tokue et al., 1991), and since then several PCR-based techniques were also developed, dealing with many target genes and sample types (Brakstad et al., 1992, Martineau et al., 1998, Jimenez et al., 2000, Liu et al., 2007, Samadi et al., 2007, Hussain et al., 2008, Fooladi et al., 2010). However, the application of a rapid assay for the detection of *S. aureus* associated with its contamination in cosmetic samples has been limited. So far, a report on the development of PCR-based assays for the detection of *S. aureus* and other pathogens in cosmetics as

well as in the pharmaceutical samples including their raw materials used has been literized (Jimenez et al., 1999, Jimenez et al., 2000, Samadi et al., 2007).

In this report, by taking the advantage of our knowledge on a PCR-based assay, a rapid method relying based on an asymmetric PCR reaction for the specific amplification of target DNA from *S. aureus* in conjunction with a lateral flow-based assay for the rapid and visual detection of *S. aureus* DNA were described. The specificity and sensitivity of the assay were studied. The feasibility of the assay for detecting *S. aureus* in cosmetic samples were also determined. The results were also compared with those analyzed by a gold standard method for the microbiological detection and confirmation of *S. aureus* parallelly.

Research Methodology

1. Bacterial Strains and DNA Isolation

S. aureus (ATCC 25923) was used as a reference strain, and it was kindly provided by Assoc. Prof. Dr. Wijit Wonglumsom (Department of Clinical Microbiology and Applied Technology, Faculty of Medical Technology, Mahidol University). The bacterium was grown on a Tryptic soy agar at 37 °C for 18 h. For non-*S. aureus* bacteria, all of them were cultured under their specific media and growth conditions for about 24-48 h. After cultivation, the bacterial cells were collected and used for the DNA isolation using a commercial kit. The DNA concentration was checked based on the OD_{260}/OD_{280} ratios, and then kept at -20 °C until used.

2. Oligonucleotide Primers and DNA Probe

Oligonucleotide primers and DNA probe were specifically designed to target a *nuc* gene of *S. aureus*, as previously reported (Brakstad et al., 1992), and their specificity was searched against the GenBank database using the BLAST program (Altschul et al., 1990). Nucleotide sequences of the designed primers and DNA probe will be detailed if requested. The primers were targeted a 279 bp of *nuc* gene from *S. aureus*, as counted based on the *nuc* gene nucleotide sequences. To further facilitate the DNA detection with a lateral flow-

based assay, the 5'-ends of reverse primer and DNA probe were modified with biotin and fluorescein isothiocyanate, respectively. All oligonucleotides were custom synthesized.

3. Asymmetric PCR reaction

Asymmetric PCR was used to amplify a target DNA from *S. aureus*. A mixture (25 µL) consisted of 5 µL of 5X PCR master mix II (GMbiolab Co. Ltd., Taiwan), 2.5 µL of primers mixed in 1:10 ratios, 2.5 µL of DNA template, and nuclease-free water. Control reaction contained sterile water instead of a DNA template, while the isolated DNA from *S. epidermidis* was used as a negative control. All reactions were conducted in a PCR thermal cycler (model: T100™, Bio-Rad Laboratories, USA) with the following programs: initial denaturation at 94 °C for 3 min, target DNA amplification: 94 °C for 15 s, 55 °C for 15 s, 72 °C for 15 s (10 cycles), 94 °C for 15 s, 56 °C for 15 s, 72 °C for 15 s (10 cycles), and 94 °C for 15 s, 58 °C for 15 s, 72 °C for 15 s (20 cycles), and a final extension at 72 °C for 1 min. After amplification, 5-µL of the reaction mixtures were taken and analyzed by gel electrophoresis containing 1.8% agarose to correct the amplicon's size.

4. Lateral flow-based Assay

A lateral flow-based assay was used to detect a specific amplicon from *S. aureus*, as previously reported (Poonlapdecha et al., 2018). A mixture (25 µL) contained 5 µL of the amplified DNA, 1.0 µM of DNA probe, 2.5 µL of hybridization buffer, and a sterile water. After heating at 95 °C for 3 min, the reaction mixtures were cooled down to 60 °C for 10 min to allow the hybridization reaction, and then they mixed with a 100 µL of DNA running buffer, followed by applying to the DNA test strip device. After 5-10 min, the results were obtained, which can be detected by visualization with the naked eye. All images were photographed by a smartphone camera. The presence of two-red lines at Test (T) and Control (C) positions denoted a positive result, while the presence of only one-red line at C position was a negative result.

5. Specificity and Sensitivity tests

A specificity of the assay was tested using the DNA template isolated from various bacteria, as denoted by the DNA cocktail 1-5 in this report, as well as several isolates of *S. aureus* (*S. aureus* WT 1-7) and *Staphylococcus* spp. (*S. epidermidis* and *S. lugdunensisi*).

In each DNA cocktail, the final DNA concentration of each bacterium was about 1 ng per reaction tested, and the information for all target bacteria used will be available if requested. A sensitivity of the assay was carried out using the purified DNA from *S. aureus* with various concentrations, ranging from 4.5 ng to 45 fg or equivalent to 10 ng to 0.1 pg per test reaction.

6. Detection of *S. aureus* in spiked cosmetic samples

A facial gel containing 0.05% of sodium hyaluronate was prepared, and then used as a cosmetic sample to spike with *S. aureus* (n = 5). The concentration of bacterial cells was adjusted by a McFarland No. 0.5, which was initially estimated to be 10^3 CFU/mL. One g of the sample was taken, neutralized with Eugonic LT100 medium (Himedia, India) containing Tween 80 (pH 7.0) in 1:10 ratios, and then incubated at 32.5 ± 2.5 °C for about 20 h. Non-spiked samples (n = 5) were also analyzed. After cultivation, the bacterial cells (0.1-mL cultures) were collected, and then treated with the ZEROprep™ DNA reagent (Geneaid, Taiwan). The bacterial lysates were used for asymmetric PCR amplification, followed by colorimetric detection by a lateral flow assay, as described above. A gold standard method for the microbiological detection and confirmation of *S. aureus* was also performed in a parallel experiment, and the results were compared.

Results

A *nuc* gene of *S. aureus* was selected as a target gene to amplify the DNA fragment of about 279-bp in length, as determined by the nucleotide sequences analysis. Based on the alignment of various *nuc* gene sequences, a new pair of gene-specific primers and DNA probe were re-designed from the previous reported (Brakstad et al., 1992) to avoid nucleotide-mismatch amplification. The specificity of the designed primers and DNA probe was checked against the GenBank database using the BLAST program (Altschul et al., 1990), and they were also confirmed by asymmetric PCR tests to be specific for only *S. aureus* without cross-amplification with other tested bacteria such as *S. epidermidis* (data not shown). As expected, the presence of DNA band with corrected size was seen on the agarose gel in the sample containing *S. aureus* DNA only (data not shown). No DNA band was observed in a negative

control reaction, where a sterile water was used instead of the DNA template (data not shown). Moreover, it should be noted that the modification of 5'-end of reverse primer and DNA probe with biotin and fluorescein isothiocyanate, respectively that were facilitated the detection of the amplified target DNA by a later flow assay had no effects on the amplification reaction, which is in accordance with the previous report (Poonlapdecha et al., 2018).

To visual and rapid detection of the amplified DNA targeting a *nuc* gene of *S. aureus*, a lateral flow-based assay was applied, and a commercial DNA test strip was used as a reporting device in this report. As the results shown in Figure 1, in the presence of a target DNA template from *S. aureus*, a positive signal was generated on the DNA test device, and the result can be visually observed by the naked eye within 5-10 min. By contrast, a negative result was obtained when a non-target DNA from *S. epidermidis* was used as a template. A negative control reaction containing a sterile water also gave a negative result. These results indicated the succeed application of a lateral flow assay to detect *S. aureus* DNA, and it was specific for *S. aureus* without cross-detection with other tested bacteria.

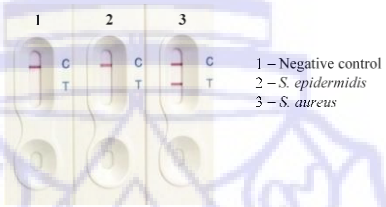

Figure 1 Specific detection of a *nuc* gene from *S. aureus*. As a DNA template tested in the asymmetric PCR reaction, the purified DNAs from *S. aureus* (3) and *S. epidermidis* (2) were used as target and non-target samples, respectively. A sterile water (1) was included as a negative control reaction.

The specificity tests carried out with various DNA templates from several isolates of *S. aureus* (*S. aureus* WT 1-7) as well as other non-target bacteria (such as *S. epidermidis* and *S. lugdunensis*, and DNA cocktail 1-5) are resulted in Figure 2. It was observed that only samples containing *S. aureus* DNA yielded positive results, while other tested DNA samples from non-target bacteria showed negative results. These results could demonstrate a high

specificity of the assay for *S. aureus* detection only without cross-reaction to other bacterial tests. With the purified genomic DNA from *S. aureus* as a DNA template used, the results from the sensitivity tests showed that the lowest amount of DNA to be detected was about 1 pg (Figure 3) or equivalent to 3.4 x 10^2 CFU per tested reaction, suggesting the limit detection of the assay towards *S. aureus* DNA detection. In this report, a genome size of *S. aureus* containing 2.69 Mb of DNA sequences was used to calculate the bacterial numbers in term of a colony forming unit (CFU) (Park et al., 2020).

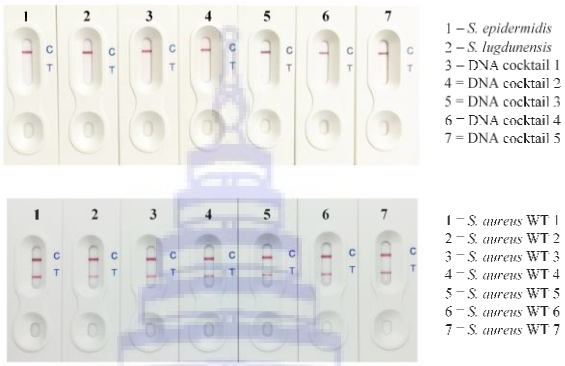

Figure 2 A panel of bacterial DNA templates were used for the specificity test. The purified DNAs from *S. epidermidis* and S. *lugdunensis*, and DNA cocktail 1-5 were included as non-target bacteria. The purified DNAs from several isolates of *S. aureus* (WT 1-7) were used as a target bacterium.

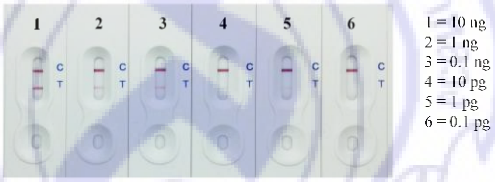

Figure 3 A series of the purified genomic DNA from *S. aureus* (10 ng to 0.1 pg) were used for the sensitivity test.

To test the feasibility of the assay to detect *S. aureus* in contaminated cosmetic samples, the artificial samples were prepared, and then spiked with *S. aureus* cells. The results showed all spiked samples produced the positive results, while the non-spiked samples were found to be negative. These results implied the succeed application of the assay to detect *S. aureus* in real cosmetic samples such as facial gel containing 0.05% of sodium hyaluronate, as reported herein. These results were also similar with those analyzed by a gold standard method for the microbiological detection and confirmation of *S. aureus* (data not shown). Moreover, it is interesting to note that the presence of cosmetic ingredients did not show any effect on either bacterial growth or detection methods, as described in this report.

Discussion and Suggestion

A rapid and visual assay to detect *S. aureus* was successfully obtained by using the asymmetric PCR reaction for the specific amplification of target DNA of interest from *S. aureus*, followed by using a lateral flow-based assay to detect the target SNA amplicons, in which the results can be visually observed by the naked eye within 5-10 min without the need of any special instrument. In this report, a *nuc* gene of *S. aureus* encoding for a thermostable nuclease was chosen as a target gene to design the specific primers and DNA probe, since it had previously shown that this gene is very highly specific for *S. aureus*, and no cross-recognition with other *Staphylococcus* species as well as other tested bacteria was achieved, as suggested by several previous reports (Liebl et al., 1987, Brakstad et al., 1992, Chesneau et al., 1993, Cremonesi et al., 2005).

However, by using BLAST analysis against the GenBank database, it was noticed that the lower sequence identities, ranging from 67-87% (data not shown) was observed between a *nuc* gene of *S. aureus* and other *Staphylococcus* species such as *S. argenteus*, *S. capitis*, *S. caprae*, *S. taiwanensis*, *S. saccharolyticus*, and *S. hominis*. These species were of human isolates (Tong et al., 2015, Zhang et al., 2017), and they were also not included in this report. Analyses of the designed primers revealed that the forward primer had 100% identity to *S. argenteus*, but not other *Staphylococcus* spp., as mentioned above. In addition, only the reverse primer and DNA probe sequences showed no sequence identity

to those isolated from the human samples. Thus, based on these results, it can be concluded that these primer sets as well as the DNA probe designed in this report are specific for *S. aureus* DNA amplification and detection.

A high specificity of the assay towards *S. aureus* detection without any cross-reaction with other bacterial tests could not only support those finding results, but also confirmed that a *nuc* gene including its amplified DNA product are solely unique to *S. aureus*, as previously reported (Liebl et al., 1987, Brakstad et al., 1992, Chesneau et al., 1993, Cremonesi et al., 2005). In the point of sensitivity test, the result obtained in this report (1 pg) was satisfied, and it was also found in a similar range (0.6 pg) with the previous report (Brakstad et al., 1992). However, after the completion of PCR reaction, an agarose gel electrophoresis with ethidium bromide staining was used. Gel analysis is thought to be time-consuming for visual the PCR amplicons. For example, it needs about 30 min for gel preparation and about 30 min for gel running with a specific instrument including the buffer used (Brakstad et al., 1992, Chesneau et al., 1993). Moreover, some laboratories still use the ethidium bromide for the visual of DNA band in agarose gel to reduce cost, even many other safe dyes such as fluorescent dyes for gel-staining are available nowadays. It is well-known that this chemical is hazardous and identified as a potent mutagen in human (Saeidnia & Abdollahi, 2013).

In this report, a combination of asymmetric PCR reaction followed by a lateral flow assay were successfully applied to detect *S. aureus* in cosmetic samples, and there were comparable to those analyzed by a conventional method for a microbiological detection and confirmation of *S. aureus*, which is laborious and time-consuming, as it required about 3-4 days to complete the results. Thus, making it as a possible method to be alternatively used as a novel and rapid method for the detection of *S. aureus* in cosmetic samples as well as in other type of samples. In addition, this assay can be used as a novel platform to detect other bacterial contamination in cosmetic samples such as *P. aeruginosa, C. albicans,* and *Clostridium* spp. including other pathogens if required.

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