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Thiolated AuNP probes and multiplex PCR for molecular detection of *Staphylococcus epidermidis*

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ABSTRACT

The emergence of nanotechnology in biology helps to apply the gold nanoparticle probes for fast and accurate identification of pathogens compared to the time-consuming and non-precise phenotypic methods. In this study, two molecular methods have been established for the accurate identification of *staphylococcus epidermidis* from other coagulase-negative staphylococci. Multiplex PCR was performed using designed primers for *Gmk2* and *pta* housekeeping genes, and *SESB* specific gene of *S. epidermidis*. Colorimetric detection by gold nanoparticle probes was carried out using two 20-base thiolated probes designed based on the sequence of *pta* housekeeping gene of *S. epidermidis*. The specificity of multiplex PCR and colorimetric assays were determined using genomic DNA of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* as negative controls and no alteration was detected. To investigate the sensitivity of the primers and gold nanoparticle probes, different concentrations of the extracted DNA from *S. epidermidis* were used. Based on the results, the minimum required quantity of target DNA for multiplex PCR amplification was 1 ng/μL and for color and absorption alteration of solution in colorimetric assay was 20 ng/μL. Our results revealed that both methods were sufficiently specific and sensitive to detect *S. epidermidis*.

1. Introduction

The *Staphylococcus* genus comprises nonmotile and invasive gram-positive cocci. These bacteria are associated with various pus-forming diseases in humans and other animals [1,2]. Ubiquitous colonization on different parts of the human skin and mucous membranes of the host is the key source of endogenous infections caused by coagulase-negative staphylococci (CoNS). However, they are transmitted mainly by medical and/or nursing procedures [3]. Surface proteins of *Staphylococcus epidermidis* (SES) are a group of important pathogenic factors to facilitate bacterial binding to host cells, the first basic step in colonization or infection. The most important SES protein encoding gene in the genome of *S. epidermidis* are SesB, SesC, SesD, SesE, SesF, SesG, SesH, SesI. However, accurate function and possible role of these proteins as virulence factor is still unknown. SES proteins are considered as candidate to develop new therapies against staphylococcal infections [4,5]. *Gmk2* housekeeping gene incodes guanylate monokinase enzyme that is necessary enzyme in the synthesis of guanosine [6] and *pta* housekeeping gene incodes Phosphate acetyltransferase that involved in acetate metabolism [7].

Today, infections caused by *S. epidermidis* are listed as one of the top five microbial nosocomial infections [8]. Therefore, rapid and accurate detection of this organism is believed to be very important [9,10]. There are many different molecular and biochemical methods for CoNS detection. Phenotypic methods for the identification of staphylococci have poor discriminatory power, because of variable expression of characters [9] and overlapping phenotypes [11,12]. Molecular DNA-based methods with high sensitivity and specificity may provide an alternative means of accurately identifying *Staphylococcus* species [2,9]. Polymerase chain reaction (PCR) assay-based methods are rapid and accurate [10] with few drawbacks. One disadvantage of the assay is its high sensitivity and a lot of precautions must be taken in order to avoid contamination [13]. Real-Time PCR, nucleicacidsequence-basedamplification (NASBA), loop-ediatedisothermalamplification (LAMP), oligonucleotide DNA microarray, enzyme-linkedimmunosorbentassay(ELISA) are other molecular technics have been used for identification of the bacteria

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Today, applications of nanotechnology in detection systems make use of nano-particles for pathogen detection by the combination of nanotechnology with biology and medicine to develop ultrasensitive detection [16]. Gold nanoparticles (GNPs) have been used as appropriate nanoprobes with potential uses in analytical and/or biological sciences because of their unique optical, physical, and chemical properties [16–18]. Gold nanoparticles have exceptionally high absorption coefficients, allowing greater sensitivity in optical detection techniques than routine colors [19]. The color alterations related with nanoparticles aggregation were initially used by Mirkin et al. who presented that ssDNA-stabilized nanoparticles could be used to colorimetrically detect the complementary oligonucleotide [16]. The use of thiol-linked ssDNA-modified gold nanoparticles for the colorimetric detection of specific DNA sequences may represent an inexpensive and easy method to perform alternative to fluorescence or radioactivity-based assays [19,20].

For the past few years, many GNP-based methods have been developed for the detection of several targets, including metal ions [21], nucleic acids [22–28], proteins [29,30], cells [31–34]. In the present study, we report two molecular methods for the specific detection of *S. epidermidis* DNA, a multiplex PCR method for *Gmk2* and *pta* housekeeping genes and *SESB* specific gene of *S. epidermidis* and a colorimetric method using oligonucleotide-Au modified.

2. Materials and methods

2.1. Bacterial strains and growth conditions

All of the bacterial strains (*S. epidermidis* ATCC 12228 obtained from Pasteur Institute of Iran, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Acinetobacter baumannii* ATCC 19606 obtained from Bouali Hospital in Hamadan) were grown on LB (Luria Burtani) agar (Merk, Germany) medium and incubated in incubator (Memmert, Germany) at 37 °C for 18–24 h.

2.2. Primer design

Conserved regions of the genes *Gmk2* (Genbank accession number AY163288.1), *pta* (Genbank accession number AY256976.1), and *SESB* (Genbank accession number EF424054.1) were used to design specific primers for *S. epidermidis* using MP primer (biocompute.bmi.ac.cn/MPprimer). The BLAST program was used for multiple alignments. The quality of designed primers was tested by OligoAnalyzer tool of Integrated DNA Technologies available at (http://www.IDTDNA.com/calc/analyzer). The primers were synthesized by MACROGEN Company, South Korea (Table 1).

2.3. DNA extraction and multiplex PCR amplification

DNA was extracted using boiling method [35]. First, *S. epidermidis* was cultured in liquid LB medium and incubated in shaker incubator (Noorsanat, Iran) with 150 rpm at 37 °C for 24 h. Then, 1000 μL of the bacterial suspension was centrifuged (Eppendorf, Germany) (14000 rpm) for 2min, and the supernatant was discarded. 1000 μL of PBS (Phosphate buffered saline) 1X was added to the pellet and vortexed. Centrifugation was carried out at 14000 rpm for 1min, the supernatant was discarded and 100 μL of deionized water was added to the tube followed by being placed in Termomixer (Eppendorf, Germany) at 98 °C for 10min. The tube was centrifuged at 14000 rpm for 5min and the supernatant (containing DNA) was transferred to a new tube and stored at −20 °C.

Multiplex PCR amplification was carried out with 8 μL of Taq Master Mix RED (Ampliqon, Denmark), 10 pmol of forward and reverse primers and 100 ng of DNA extracted from bacterial strains in a total volume of 15 μL in thermal cycler (Eppendorf, Germany). An initial denaturation was carried out at 94 °C for 4min (the first cycle) followed by 30 cycles at 94 °C for 40s (subsequent cycles of denaturation), annealing at 56 °C for 30s and elongation at 72 °C for 30s. The final elongation was run at 72 °C for 5min. *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Acinetobacter baumannii* ATCC 19606 were used as negative controls. Moreover, the sensitivity of multiplex PCR was investigated using different dilutions of the *S. epidermidis* extracted DNA (1 ng-100 ng) in total volume of 15 μL PCR reaction. After multiplex PCR, all amplified products were analyzed by electrophoresis (Padideh Nojen Pars, Iran) on 2.5% agarose (Sigma-Aldrich, USA(gel. The gel was stained with ethidium bromide (Sigma-Aldrich, USA (for 20min and finally the products were visualized using UV doc (UviTec, Iran).

The sensitivity of multiplex PCR assay was evaluated using 100, 25, 5 and 1 ng/μL concentrations of DNA extracted from *S. epidermidis* ATCC 12228. To determine the specificity of multiplex PCR assay, extracted DNA of *staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Acinetobacter baumannii* ATCC 19606 and *Pseudomonas aeruginosa* ATCC 27853 were used as negative controls.

Table 1
The sequences of primers used for detection of *S. epidermidis*.

Target genes	Sequence (5′- 3′)
Gmk2	Forward- 5′- CAAAGGAAGAATTTGAGGCG- 3′
	Reverse- 5′- GTTCCTCTACCAACAAGACG- 3′
SESB	Forward- 5′- AAATAGTGGTGGCAATCCG- 3′
	Reverse-5′- GGTAAAGTGTAATGAAACCAG- 3′
Pta	Forward- 5′- TCTCTTGCTTCTAAACACGC- 3′
	Reverse- 5′- ACTGCAATTTCAGCAAGTCC- 3′

2.4. Design and synthesis of oligonucleotide probes

PRIMER3 software was employed to design 5′ thiolated probe pta1 (SH- 5′- GTGGTGCTGCACATTCTACT) and 3′ thiolated probe pta2 (GGAGATACAGTCAGACCAGC- 3′- SH) across internal regions of *pta* gene of *S. epidermidis*. The modified oligonucleotides were synthesized by BIONEER Company, South Korea. The dried thiolated oligonucleotides (based on OD equal to 5) were solved in 50 μL of distilled water. 10 μL of 1.0 N dithiothreitol (DTT))Sigma-Aldrich, USA (was added, vortexed and then incubated at room temperature for 15min. Excess DTT and unwanted thiol fragments were emitted by extracting three times with 50 μL of ethyl acetate)Sigma-Aldrich, USA). After the mixture was vortexed, the upper layer was discarded.

2.5. Synthesis of gold nanoparticles

Approximately 20 nm diameter gold nanoparticles were prepared by citrate reduction of tetrachloroauric acid ($HAuCl_4$) [36]. A 90 mL aqueous solution containing 10 mg of $HAuCl_4$)Sigma-Aldrich, USA) was heated at 70 °C while being stirred for 2min. Then, 75 mg of trisodium citrate ($Na_3C_6H_5O_7$))Sigma-Aldrich, USA) was added, and the solution color slowly turned from yellow to red. The size and monodispersity of gold nanoparticles solution were characterized by TEM (Transmission electron microscopy) (Zeiss EM900). Electronic absorption spectra of the gold nanoparticles were recorded using an UV-vis spectrophotometer (UV2100, Germany) at wavelength 450–750 nm.