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A New Specific DNA Target Sequence for Identification of *Staphylococcus epidermidis* using Modified Comparative Genomic Analysis

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Abstract

Background: *Staphylococcus epidermidis (S. epidermidis)* is the most frequently isolated pathogen from prostheses infections in the body. Therefore, improving its diagnostic methods, including rapid Nucleic Acid Amplification Tests (NAAT), seems necessary. Since the first step in designing a NAAT is to find a specific sequence and all DNA targets that have been introduced so far are not completely specific, we introduced a new 100% specific DNA target sequence to identify *S. epidermidis* in this study.

Methods: Modified comparative genomic analysis was used to find the best specific target sequence to detect *S. epidermidis*. A PCR method was designed for the evaluation of this target. To determine the detection limit and analytical specificity, pure genomic DNA of 18 bacteria include 12 standard strains (one *S. epidermidis* and 11 non-*S. epidermidis*) and six clinical isolates (five *S. epidermidis* and one non-*S. epidermidis*) were used.

Results: The 400 *bp* sequence of *S. epidermidis* ATCC 14990 was identified as the most specific sequence (Se400), having a 100% sequence similarity to *S. epidermidis* genomes but not with other bacteria. The detection limit of Se400-PCR was 10 *fg*, equal to about 4 copies of *S. epidermidis* genomic *DNA/µl*. All pure DNA templates from *S. epidermidis* generated a detectable amplicon by 264 *bp* length, but the PCR test was negative for the non-*S. epidermidis* group.

Conclusion: The Se400 sequence can be considered as a specific target for detecting *S. epidermidis,* based on our findings.

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Keywords: Comparative genomic analysis, Detection, Pathogen, Polymerase chain reaction, Se400, *Staphylococcus epidermidis*

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Introduction

One of the most isolated members of the coagulase-negative staphylococci (CoNS) group is *Staphylococcus epidermidis (S. epidermidis)*. This bacterium colonizes mucous membranes and the skin, accounting for the majority of the bacterial flora in this environment [1-3]. Genome study of *S. epidermidis* revealed that it is fully equipped with genes supposed to offer resistance from the severe circumstances faced in surrounding environment, allowing it to remain longer in dry conditions in hospitals [4]. *S. epidermidis* is the most commonly implicated pathogen in infections related to any form of an indwelling medical device [5]. This micro-organism has been detected with a relatively high prevalence from the Central Nervous System (CNS) shunts, joint prostheses, and prosthetic valves [6,7]. Also, the mentioned bacterium has been repeatedly isolated from different specimens, such as blood, skin, wound, urinary tract, soft tissue infections, endocarditis, bacteremia, and pneumonia [8,9]. According to scientific documents, CoNS cause half of all cases of Prosthetic Valve Endocarditis (PVE) [10]. More than 20% of people with implanted cardiac devices are infected by *S. epidermidis*, which in turn causes pain and purulence at the infection site and sepsis [6]. The sepsis mortality rate resulting

from *S. epidermidis* in infants could be as high as 4.8 and 9.4% [11]. The mortality rate due to endocarditis caused by CoNS is reported to be about 36% [6], while it is estimated at 30% for septic shock [12].

Traditionally, *S. epidermidis* diagnosis has been performed according to the biochemical tests and morphological characteristics. Such methods are time-consuming (sometimes up to several days) and do not reliably distinguish *S. epidermidis* from other CoNS. Therefore, finding faster and more reliable methods has always been required. The development of Nucleic Acid Amplification Tests (NAATs) such as Polymerase Chain Reaction (PCR) in recent decades, has greatly increased the speed, sensitivity, and specificity of diagnostic tests [13-15]. One of the critical points for designing a NAAT is a completely specific DNA sequence for the desired micro-organism. The specific sequence should be present in all strains of such micro-organism but not found in any other micro-organism or has very low similarity [16]. In recent years, various PCR tests have been designed to detect *S. epidermidis* based on genes such as *serp0107, gseA, Staphostatin A, and sesC* [17-20]. Our bioinformatics evaluation showed that all genes introduced so far as diagnostic targets, are not 100% specific, and have many similarities with other species of *Staphylococcus*. Therefore, finding a specific target sequence that can be applied to design a completely specific PCR is still needed. Modified comparative genomic analysis or modified genome comparison is among the methods for finding a specific target sequence for organisms and was introduced by our team in 2018 [16,21]. So, this study aimed to introduce a novel target gene that is specific for the *S. epidermidis* complex, as well as to design highly specific and sensitive primers for the rapid detection of *S. epidermidis* using modified comparative genomic analysis.

Materials and Methods

Staphylococcus epidermidis-specific target mining

According to the described method in our previous studies [16,21], the genomic sequence of *S. epidermidis* ATCC 14990 was compared with the available genomic sequences on the nucleotide collection database [22,23], and the most specific sequence was selected (Figure 1). The steps are described below:

1. Genomic sequences of *S. epidermidis* on nucleotide collection database were determined. Then, one case which was preferably the NCBI reference sequence, was regarded as the reference.
2. The sequence of *S. epidermidis* ATCC 14990 (NZ_CP035288.1) was selected as the reference, and the sequence was obtained and cut to 5000 *bp* independent fragments, producing about 493 fragments.
3. Separately, each fragment was compared with other available sequences of nucleotide collection database by Basic Local Alignment Search Tool (BLAST). BLAST discovers similar regions between DNA sequences. The nucleotide sequences is compared with available sequences on database and the statistical significance is calculated by the program [22].
4. After each analysis, results were screened, and the best fragments were selected. Evaluation of results was performed by two criteria:

a. Presence of all *S. epidermidis* NCBI reference sequences in search results, having both identity and query cover of 100%.

b. No other microorganism except *S. epidermidis* would appear with the query cover >90%. The selection of these two criteria was based on our experiences and evaluation of the first 200 fragments.

5. Selected fragments of the previous step were compared separately with non- *S. epidermidis* sequences of the nucleotide collection database, and conserved parts of each fragment were determined.
6. Finally, the longer specific part was selected, and we named it Se400.

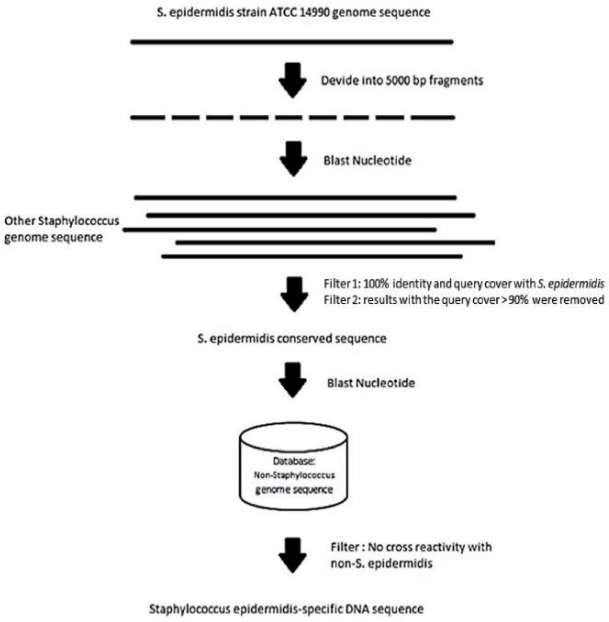

Figure 1. Method for mining *S. epidermidis*-specific nucleotide sequences.

Primer design and PCR

To evaluate the specificity of Se400, an end-point PCR was designed with Oligo7 software [24]. The primers were then tested for secondary structure and anticipated melting temperature using Oligo Analyzer 3.1 (https://eu.idtdna.com/calc/analyzer) and were manufactured by DENAzist Asia Company. 264 *bp* amplicon and Primer sequences are provided in table 1. PCR reaction was prepared in 25 μl containing 2.5 μl of 10×PCR buffer (100 *mM* Tris-HCl [pH=8.3], 500 *mM* KCl), 1 μl of each 10 μM forward and reverse primers, 1 μl of DNA sample, 0.5 μl of 200 μM (each) of the four dNTPs, 1.5 μl of 25 *mM* $MgCl_2$, 0.625 *U* of *Taq DNA polymerase*, and PCR grade water. 10 *ng* of

Table 1. The Se400 sequence, the designed primers for PCR, and amplicon

Specific DNA target sequence (Se400)
>NZ_CP035288.1:242200-242600 *Staphylococcus epidermidis* strain ATCC 14990 chromosome, complete genome GAGAGCCTACACCAAAAATTGTAATAAACACGAGGACAGTGATTTCTAACATCTTGCCTCGTTTTGAAGACCAAAAATCC ATCATTTATTCATCTCACATCTTTAAATTTTTGTTGATAAAAAAAGCAACTTAATCATTATAACGCTAATGGTAAGGTGGG TAAATAAACTGATATGATGATATTAAAAATGAAAAAGAAAATCTTAATATATTTTATTTTTAAAAAGATAATGTAATTATT TATACTGTTAAAGATATGAAAATGGCATGGTATTTCATTAAAAATATAATAACTAAACTTTGGAATCAATGACCAATAACC ATAAATACGTTATCCTTACATAGAAGGTTATTAATGAATTCGAATGGAGGATAATACGTTATGATGGATTTTTTACAT
Forward primer
5′-TTGTAATAAACACGAGGACA
Reverse primer
5′-TAATGAAATACCATGCCAT
264 *bp* amplicon
5′TTGTAATAAACACGAGGACAGTGATTTCTAACATCTTGCCTCGTTTTGAAGACCAAAAATCCATCATTTATTCATCTCACA TCTTTAAATTTTTGTTGATAAAAAAAGCAACTTAATCATTATAACGCTAATGGTAAGGTGGGTAAATAAACTGATATGATG ATATTAAAAATGAAAAAGAAAATCTTAATATATTTTATTTTTAAAAAGATAATGTAATTATTTATACTGTTAAAGATATGA AAATGGCATGGTATTTCATTA

S. epidermidis ATCC 14990 pure DNA was used as the positive control, and water as the negative control.

For DNA amplification, 5 *min* of initial denaturation at 95°*C* was followed by 30 cycles of (i) 45 *s* of denaturation at 95°*C*, (ii) 45 *s* of annealing at 49°*C* for, (iii) 60 *s* of extension at 72°*C* for, and (iv) final 5 *min* of extension at 72°*C*. Finally, 3 μl of PCR product was visualized using 1.5% agarose gel electrophoresis and DNA green viewer. The presence of a 264 *bp* amplicon specifies the positive result.

Bacterial isolates

In this study, pure genomic DNA of 18 bacteria, including 12 standard strains (one *S. epidermidis* and 11 non-*S. epidermidis*) and six clinical isolates (5 *S. epidermidis* and one non-*S. epidermidis*) were used. All standard strains were acquired from the microbial bank in the Antimicrobial Resistance Research Center of Mashhad University of Medical Sciences, and all clinical isolates were acquired from Imam Reza Hospital of Mashhad (Table 2).

Analytical sensitivity (limit of detection)

The pure DNA concentration of *S. epidermidis* ATCC 14990 was calculated by a spectrophotometer (Thermo Scientific). A serial dilution of pure DNA was then prepared in water (10 *ng*, 1 *ng*, 100 *pg*, 10 *pg*, 1 *pg*, 100 *fg*, 10 *fg*, 1 *fg*) and the volume of 1 μl was applied as the template. The process was repeated three times to ensure the results.

Analytical specificity

The primer specificity was investigated by Blastn software to determine cross-reactivity with other human or bacterial genomes. To determine the analytical specificity for Se400-PCR, pure DNA of six *S. epidermidis* and 12 non-*S. epidermidis* were used (Table 2). 10 *ng* of pure DNA was applied in each reaction. Finally, sequencing was performed on all positive PCR products.

Table 2. The list of bacteria used in this research

Groups	Bacterial names
Staphylococcus epidermidis	
	S. epidermidis ATCC 14990
	S. epidermidis (clinical isolate 1)
	S. epidermidis (clinical isolate 2)
	S. epidermidis (clinical isolate 3)
	S. epidermidis (clinical isolate 4)
	S. epidermidis (clinical isolate 5)
Non-Staphylococcus	
	Staphylococcus aureus ATCC 25923
Epidermidis bacteria	
	Staphylococcus haemolyticus ATCC 29970
	Staphylococcus saprophyticus ATCC 15305
	Staphylococcus schleiferi ATCC 43808
	Streptococcus pyogenes ATCC 12344
	Streptococcus agalactiae ATCC 12386
	Enterococcus faecalis ATCC 29212
	Streptococcus oralis (clinical isolate)
	Listeria monocytogenes ATCC 35152
	Lactobacillus casei ATCC 393
	Pseudomonas aeruginosa ATCC 27853
	Escherichia coli ATCC 25922

Results

Target mining

The 400 *bp* sequence containing nucleotides 242, 200 to 242,600 of *S. epidermidis* ATCC 14990 (NZ_CP035288.1) was recognized as the most specific sequence, having a 100% sequence similarity to *S. epidermidis* genomes but not with other bacteria. Se400 is a non-coding sequence located between *EQW00_01195* and *EQW00_01200* genes (Figure 2). The blastn search showed that Se400 could detect all strains of *S. epidermidis* among the registered complete genomes in the nucleotide collection database, and no similarity was observed with other micro-organisms.

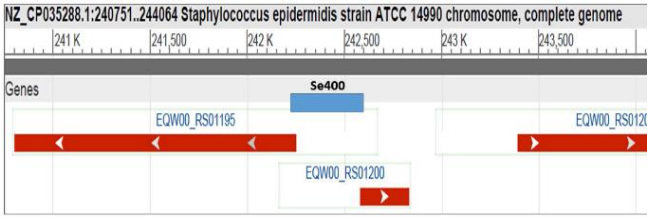

Figure 2. Schematic location of Se400 between *EQW00_01195* and *EQW00_01200* genes.

Analytical sensitivity (limit of detection)

The detection limit is defined as the lowest analyte concentration that can be reliably detected. Consequently, the Se400-PCR detection limit was 10 *fg*, equal to about 4 copies of *S. epidermidis* genomic *DNA/µl* (Figure 3).

The analytical specificity

PCR amplification using Se400-specific primers was performed with 18 bacteria using pure genomic DNA as the template to consider the analytical specificity of the Se400-PCR. As presented in figure 4, all genomic DNA templates from *S. epidermidis* generated a detectable amplicon by 264 *bp* length, but the PCR test was negative for the non-*S. epidermidis* group; subsequently, the Se400 primer was specific for detecting *S. epidermidis*. Finally, analysis of sequencing results for PCR products from 6 positive samples showed that the produced amplicon is related to the Se400 sequence of *S. epidermidis*.

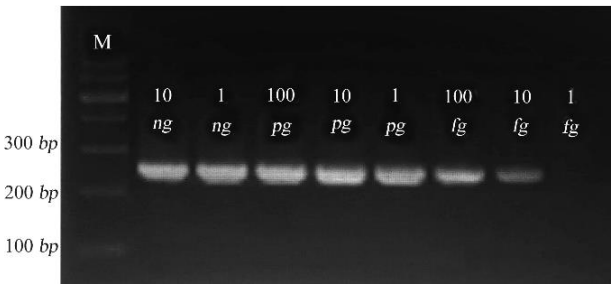

Figure 3. Gel electrophoresis of Se400-PCR product on 1.5% agarose gel. 264 *bp* amplicons are shown at various concentrations of *S. epidermidis* pure DNA as template. Amplicons using 10 *ng*, 1 *ng*, 100 *pg*, 10 *pg*, 1 *pg*, 100 *fg*, 10 *fg*, and 1 *fg* are shown in lanes.

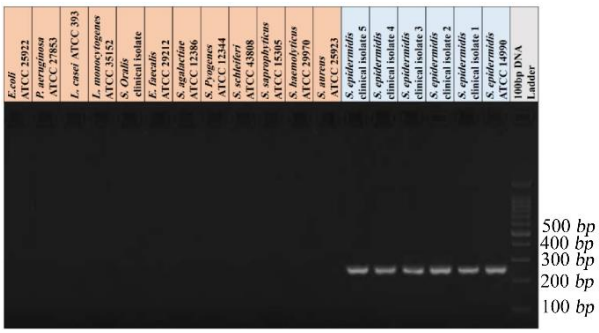

Figure 4. Gel electrophoresis of Se400-PCR products. The amplicon size is 264 *bp*. M: 100-*bp* molecular DNA ladder (Pars Tous, Iran).

Discussion

Before the discovery of molecular methods, phenotypic and biochemical tests were the only powerful methods for the detection and differentiation of various bacteria, including *S. epidermidis*. With the development of molecular methods, especially PCR in recent decades, the problems of traditional methods such as being slow and lacking reliability were solved [13,14]. One of the challenges for designing a PCR test to detect *S. epidermidis* is the lack of a completely specific DNA target. According to the literature, most NAATs in the 1990s used genus-specific targets due to the unavailability of species-specific DNA targets. The amplified sequences based on such genes could only differentiate *S. epidermidis* from other close species when they were analyzed by confirmatory methods. The most famous method for analyzing these fragments was sequencing and then comparing the results with available sequences on GenBank. *16S rDNA*, *sodA*, *hsp60*, and *tuf* were among the most used genes in such methods [25-28]. Although the addition of the sequencing step reduced the speed and increased the costs, some cases were still seen in which the sequenced fragment was quite similar in several species [25,29]. For example, although the *16S rDNA* gene has been suggested as a target gene, it cannot be used as a distinct target to detect *S. epidermidis* in clinical specimens due to the significant similarity to *Staphylococcus aureus (S. aureus)* [30-32].

With an increased detection of *S. epidermidis* infections in the last two decades, the need to find species-specific targets for the detection of this bacterium has been increased significantly. Efforts eventually led to the introduction of *serp0107*, *gseA*, *ecpB*, and *SesC* genes as species-specific targets [17-20]. However, our bioinformatics evaluation before starting this project showed that none of these diagnostic targets was 100% specific (Table 3).

In 2004, the *gseA* gene (GenBank acc. No. AB096695), responsible for the production of glutamic acid-specific 27-*kDa* serine protease (GluSE), was introduced by Ikeda *et al* as a species-specific target of *S. epidermidis*. This protease is involved in degrading human fibronectin, collagen, the complement protein C5, and slime formation. Thus, the protease may be associated with the pathogenesis of *S. epidermidis* [17,33]. Despite an appropriate length of this gene (1214 *bp*), a comparison of its sequence with other available sequences at nucleotide collection database showed that this gene has similarity with some parts of the *Staphylococcus saccharolyticus (S. saccharolyticus)* genome with >68% query cover and >75% identity. This bacterium is a normal flora of the skin, and the pathogenesis is not apparent yet. Furthermore, very similar sequences to the *gseA* gene are present in other bacteria, reducing the specificity of this gene for the detection *S. epidermidis*. These bacteria include *Staphylococcus capitis (S. capitis)*, *Staphylococcus caprae (S. caprae)*, *S.*

Table 3. Comparison of Se400 sequence specificity with other introduced genes for detecting *S. epidermidis* in species level

Target	Length	Similarity to non-*S. epidermidis* genomes with >35% query cover and >60% identity
Se400	400 *bp*	No similarity to any non-*S. epidermidis* microbes
gseA (2004)	1214 *bp*	• 3 strains of *S. saccharolyticus* with >68% query cover and >75% identity • 5 strains of *S. caprae* with >58% query cover and >69% identity • 11 strains of *S. warneri* with 55% query cover and 67% identity • >1300 strain of *S. aureus* with 50% query cover and 67-69% identity • 6 strains of *S. pasteur* with 46-51% query cover and 67-69% identity
serp0107 (2006)	882 *bp*	• 3 strains of *S. saccharolyticus* with 98% query cover and 69% identity • 7 strains of *S. capitis* with 95% query cover and 68% identity • 5 strains of *S. caprae* with 66% query cover and 70% identity • 3 strains of *S. haemolyticus* with 42% query cover and >68% identity • Other microorganisms: *S. warneri S. hominis, S. argenteus*
sesC (2016)	2031 *bp*	• 3 strains of *S. saccharolyticus* with 83-100% query cover and >69% identity • 7 strains of *S. capitis* with 42% query cover and >67% identity • 5 strains of *S. caprae* with 52% query cover and 66% identity
ecpB (2019)	318 *bp*	• 3 strains of *S. saccharolyticus* with 100% query cover and >77% identity • 19 strain of *S. aureus* with 65% query cover and 67% identity

The specificity of the genes was tested bioinformatically using Blastn software.

aureus, *Staphylococcus haemolyticus (S. haemolyticus)*, *Staphylococcus saprophyticus (S. saprophyticus)*, *Staphylococcus hominis (S. hominis)*, *Staphylococcus equorum (S. equorum)*, *Staphylococcus simulans (S. simulans)*, *Staphylococcus lugdunensis (S. lugdunensis)*, and *Staphylococcus schleiferi (S. schleiferi)*.

serp0107, which is a putative transcriptional regulator gene, was introduced by Liu *et al* in 2006 as the species-specific diagnostic target to detect *S. epidermidis*. The sequence of this gene with 882 *bp* length was obtained from the (nt 91276–92157) of *S. epidermidis* RP62a (GenBank Accession No. CP000029) [18]. Our assessment of this sequence using the BLASTN search tool showed that a highly similar sequence is also present in *S. saccharolyticus* and *S. capitis*. Also, similar points to the *serp0107* gene are found in other *Staphylococcus* species, including *S. caprae*, *S. haemolyticus*, *Staphylococcus warneri* (*S. warneri)*, *S. hominis*, and *Staphylococcus argenteus* (*S. argenteus)* leads to a lack of complete specificity of this gene for the detection of *S. epidermidis*.

S. epidermidis surface protein C (*SesC*) gene was introduced by Khodaparast *et al* in 2016 as a specific diagnostic target of *S. epidermidis*. SesC protein is expressed more in *S. epidermidis* biofilm-associated cells than planktonic ones. Also, this target is appropriate to design various NAATs due to the suitable length (2031 *bp*) [19]. Nevertheless, our evaluation of this gene showed that similar sequences are found in three other species of the *Staphylococcus* genus, which reduces its specificity. The highest similarity was seen for the *S. saccharolyticus* with 83-100% query cover and >69% identity, followed by *S. capitis* and *S. caprae*.

Finally, the last introduced species-specific gene for this bacterium was the *ecpB* gene, encoding the Staphostatin A protein. This 318 *bp* gene was initially introduced to differentiate *S. aureus* and *S. epidermidis* [20]. However, the high similarity with a sequence in *S. aureus* with 65% query cover and 67% identity makes it unspecific. The assessment of available sequences in NCBI also showed that this gene is also found in *S. saccharolyticus* with 100% query cover and >77% identity.

According to the results obtained using the BLASTN search tool, the Se400 sequence is completely specific to the *S. epidermidis* and is not found in any other micro-organisms, unlike the other sequences having nonspecific regions in several points. Therefore, using this sequence seems to solve the unavailability of a completely specific target for *S. epidermidis*.

In this study, the specificity of the Se400 target sequence was confirmed by PCR. The specificity of Se400 in all *S. epidermidis* strains highlighted both the efficacy of the comparative genomic analysis for finding possible targets and the significance of experimental research. Also, the Se400-PCR test was able to detect very low levels of genomic DNA template. One of our limitations was the small number of bacterial strains tested, and it is recommended that further research be conducted with a large sample size. Future studies might include comparing the previously known target gene with Se400 sequence in clinical samples.

Conclusion

In conclusion, *S. epidermidis*-specific target sequences were identified using a new comparative genomics method for finding species-specific nucleotide sequences. Many nucleotide targets were assessed, and a target sequence was applied to design a PCR test to detect *S. epidermidis* in clinical samples. Further surveys are being planned to include more bacterial strains for the evaluation of the particular targets. Unique tar-

gets may be found using this method for the detection of any micro-organism, for which a genome sequence is available.

Conflict of Interest

None declared.

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