RESEARCH ARTICLE

sodA and *gap* Genes as Markers for The Identification of *Staphylococcus capitis*

Rininta Firdaus[1,2]*, Hege Hartz Jartun[2], Miriam Khider[2]

[1]Faculty of Pharmacy, Universitas Pancasila, Jl. Raya Lenteng Agung No.56-80, Jagakarsa, Jakarta 12640, Indonesia
[2]Department of Clinical and Molecular Medicine, Norwegian University of Science and Technology, Høgskoleringen 1, Trondheim, Norway

*Corresponding author. E-mail: rininta.firdaus@gmail.com

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Abstract

BACKGROUND: Rapid and accurate identification of *Staphylococcus capitis* is required to provide a better prognosis for endocarditis patients and tackle the emergence of multidrug resistant strains of the bacteria in hospitals. The current study was aimed to develop polymerase chain reaction (PCR) assay for specific identification of *S. capitis* using *sodA* and *gap* genes as markers.

METHODS: Five sequences of *sodA* and sixteen sequences of *gap* registered in GeneBank were analysed using bioinformatic tools. PCR primers were designed based on the conserved and specific regions of *sodA* and *gap*. Four clinical isolates of *S. capitis* (named no. 56-59) and six reference strains of coagulase-negative staphylococci (CoNS) species including *S. epidermidis* ATCC 35984, *S. epidermidis* 48951I/09, *S. lugdunensis* 44987/09, *S. sciuri* 109645I/08, *S. warneri* 135612/09, *S. hominis* 114202/08 were used to validate the conventional PCR system.

RESULTS: The current PCR system only amplified the DNA template of *S. capitis*. Current primers specifically targeted *S. capitis* as the agarose images only showed bands from *S. capitis* samples.

CONCLUSION: The *sodA* and *gap* genes might serve as effective markers for identification of *S. capitis* using conventional PCR. The PCR assay in the current study was able to identify five clinical isolates of *S. capitis* accurately without mispriming, misamplification and mis-identification.

KEYWORDS: *Staphylococcus, S. capitis, sodA, gap*, PCR

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Introduction

Staphylococcus capitis, a coagulase-negative species (CoNS) of staphylococci, has similar morphology to *S. aureus*, but the species lack clumping factors and deoxyribonuclease.(1) Although CoNS are constituents of the normal skin flora of healthy individuals (2), the bacteria have a tendency to colonise and produce biofilm on prosthetic implants such as intravascular lines or cardiac valves (1). The CoNS may be introduced to the medical devices during the device placement, venepuncture, or through breaks in the mucous membrane or skin. CoNS infection mainly leads to prosthetic valve endocarditis, but it possibly causes native valve endocarditis in immunocompromised patients (<10%).(3) About 5% of reported CoNS pathogenic isolates were *S. capitis*. The bacteria has been reported to cause clinical manifestations including endocarditis, pneumonia, urinary tract infection, catheter-related bacteraemia and cellulitis.(4-8)

Recently, *S. capitis* has emerged to become a worldwide multidrug-resistant clone responsible for nosocomial late-onset sepsis (LOS) in neonatal intensive care units (NICUs).(9) Multiresistant *S. capitis* LOS cases

have been reported in several countries including Australia, Belgium, and United Kingdom. A distinguished methicillin-resistant *S. capitis* strain named NRCS-A was isolated from several NICUs in France.(10) The strain shows a multidrug resistance profile, including resistance to vancomycin, the first-line antimicrobial agent for LOS. Such a failure to the antibiotic treatment of LOS increase the morbidity of premature neonates in NICUs.

Rapid identification of *S. capitis* in the clinical laboratory is required for differentiation to other staphylococcal and CoNS species, thus specific treatment and initial prevention of dissemination could be performed promptly. Conventional benchtop phenotypic characterisation and biochemical based commercial test kits including fatty acid analysis are commonly used for *Staphylococcus* species identification. However, the accuracy from these identification techniques may differ to various degrees. PCR assay may provide a better alternative for its higher specificities and sensitivities. The 16S-23S rDNA intergenic space region (11), *16S rRNA* (12-14), *hsp60* (15-17), *sodA* (18), *tuf* (19), *rpoB* (20, 21), *dnaJ* (22), *gap* (23) were previously used as target genes for identification of *Staphylococcus* species.

This study was aimed to profile *sodA* and *gap* genes as markers for specific differentiation of *S. capitis* from other CoNS species. The *sodA* and *gap* genes encode superoxide dismutase A and glyceraldehyde-3-phosphate dehydrogenase, respectively. Both enzymes reduce oxidative stress and maintain the sustenance of the pathogen inside the host. Among *Staphylococcus* species, the DNA sequence similarities of *sodA* and *gap* are 78% and 60%, respectively.(23) The DNA sequence similarities of *sodA* and *gap* were found to be lower than those of *16S rRNA* (~97%) (23), *tuf* (89%) (19), *rpoB* (~86%) (23), *hsp60* (~82%) (23) and *dnaJ* (78%) (22). Here, we developed the PCR methods for these two genes and validated the results with four clinical isolates of *S. capitis* and other six CoNS strains. The developed PCR assay of *sodA* and *gap* in current study may complement other species-level PCR identification of *S. capitis* and an initial approach for methicillin-resistance typing.

Methods

Sample

The DNA sequences of *sodA* and *gap* of *S. capitis* were collected from GenBank (Rockville Pike, USA, https://www.ncbi.nlm.nih.gov/). Five sequences of *sodA* and 16 sequences of *gap* were downloaded in FASTA format (txt files) and then further analysed for primer design.

Four isolated clinical strains of *S. capitis* collected from Øya St. Olavs Hospital (Trondheim, Norway) named no. 56, 57, 58, and 59 were used to validate the PCR assay. The isolates were collected retrospectively from NICU infants in 2008 regardless of their methicillin resistance in the microbiological records. Identification of bacterial isolates was initially performed by Gram staining, catalase and benzidine tests, and the determination of anaerobic acid production from glucose. In addition, a simple scheme was performed according to previous reference method. (24,25) *S. capitis* was characterized as Gram positive, coagulase negative, urease positive, pyrrolidonyl aminopeptidase (PYR) positive, trehalose negative, mannitol positive, and susceptible to novobiocin. The bacterial isolates were also identified as *S. capitis* by amplification of *tuf* gene as previously described.(19)

Six strains from other CoNS species were also used in this study, including *S. epidermidis* ATCC 35984, *S. epidermidis* 48951I/09, *S. lugdunensis* 44987/09, *S. sciuri* 109645I/08, *S. warneri* 135612/09 and *S. hominis* 114202/08. All strains were cultured in trypticase soy agar and incubated 37°C for 24 hours. Bacterial cells were harvested in sterile saline for DNA extraction.

DNA Sequence Analysis

Multiple alignments by Clustal Omega (European Bioinformatics Institute, Hinxton, UK, https://www.ebi.ac.uk/Tools/msa/clustalo/) were each performed for *S. capitis sodA* and *gap* gene sequences to look for the highest similarity area and avoid single nucleotide polymorphisms (SNPs). The particular area were aligned to sequences from other *Staphylococcus* species. Specific regions of *S. capitis* in *sodA* and *gap* sequences were analysed for PCR primer design. Alignments were performed using basic local alignment sequence type (BLAST) online (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

PCR Primer Design

PCR primers were design based on the specific area analysed from the multiple alignments. Primers were generated by OLIGO Primer Analysis Software (Molecular Biology Insights Inc., Colorado, USA) with preferred values (length 18-22 nucelotides, GC content: 40-60%, melting temperature (T_m) 50-60°C, T_m difference between primers <5°C, T_m difference between primers and PCR products <22°C). Primer candidates were tested by BLAST to determine sensitivity and specificity. The

selected primers were produced by Oligo Sigma (Oslo, Norway).

PCR Assay

The number of bacteria (2×10^9 cells) was determined by measuring the optical density (OD_{600nm}). DNeasy® Blood and Tissue Kit (Qiagen, Oslo, Norway) was utilised to extract the total DNA. The quality of DNA samples was verified by measuring the absorption ratio using a Nanodrop ND-1000 spectrophotometer at 260/280 nm wavelength (NanoDrop Technologies, Oslo, Norway).

The concentration of DNA template was 1:10 dilution. The PCR reaction includes 5 µL of 10×PCR buffer with $MgCl_2$ (Thermo Fisher Scientific, Oslo, Norway), 2.5 µL of dNTP (1 mM), 0.2 µL of AmpliTaqGold (Thermo Fisher Scientific, Oslo, Norway), 2 µL of each PCR primer (each 100 ng μL^{-1}), 36.3 µL of ddH_2O, and 2 µL of DNA template. PCR was performed using GeneAmp PCR System 9700 (Applied Biosystem, Oslo, Norway). The optimised PCR condition used in current study were initial denaturation 95°C for 10 minutes followed by 35 cycles denaturation 94°C for 60 seconds, annealing 58°C for 60 seconds, and extension 72°C for 30 seconds. Extra elongation step were also used at 72°C for 7 minutes and 10°C for an indefinite time.

PCR product was separated by gel electrophoresis in 2% agarose gel using SYBR Safe (Thermo Fisher Scientific, Oslo, Norway) as gel stain. After immersing the gel with 1% Tris-acetate-EDTA (TAE) buffer; samples containing 1×DNA loading dye were loaded alongside 1 kb Plus DNA Ladder (Invitrogen, Oslo, Norway). The gel was run at 80 V for 45 minutes and visualized using Gel Doc XR plus (Biorad, Watford, UK). The quantification of visualised PCR products was adopted from previous study.(26) Band intensities were quantified using Image J software (National Institutes of Health, Madison, USA). Each band density was compared to the ladder density (relative density).

Results

DNA Sequence Analysis

Specificity of a gene is determined by DNA sequence similarities of targeted and non-targeted genes. The sequence similarities between five sequences of *sodA* genes were 99-100% (Supplement 1), while the sequence similarities between sixteen sequences of *gap* genes were 97-100% (Supplement 2). The SNP areas were avoided for the primer design. The highly conserved area were then aligned to other *Staphylococcus* species using BLAST. The specific area for *sodA* and *gap* are presented in Supplement 3 and Supplement 4, respectively.

Primer Design

The PCR primers targeted for specific regions of *sodA* and *gap* were designed semi-automatic (manually) using OLIGO software (Table 1). All primers were consist of 20 nucleotides. The GC content and T_m of the primers were on the range of 45-56% and 49.7-59.4°C, respectively. The Tm difference among primers of each gene were less than 5°C. Hairpins, mispriming, and self-dimerization were not detected.

PCR Assay

The primer application in PCR assay of *sodA* and *gap* for the identification of *S. capitis* was performed with conventional GeneAmp PCR System 9700 (Applied Biosystem, USA). The primers were validated using several isolates of *S. capitis* (named no. 56-59), and referral strains from other *Staphylococcus* species (*S. epidermidis*, *S. lugdunensis*, *S. sciuri, S. warneri* and *S. hominis*). Figure 1 and Figure 2 show the agarose images from PCR assays of *sodA* and *gap*, respectively. Bands appeared only from *S. capitis* isolates. Primers were specific to strains of *S. capitis* and these primers did not generate any products from other *Staphylococcus* strains used in current study. Still, more

Table 1. PCR primers sequences targeted *sodA* and *gap* gene for *S. capitis* identification. The primers were generated by OLIGO software (Molecular Biology Insights, Colorado, USA).

Gene		Primer Sequence	Length	T_m	GC-content	PCR Product
sodA	Forward	CTCAGCAGTTGAAGGAACAG	20	57.8°C	56%	275 bp
	Reverse	CTAACCATGCCCAACCAGAT	20	59.4°C	50%	
gap	Forward	ATCGATGGTGGATTCCGTGT	20	51.8 °C	50%	270 bp
	Reverse	AGAAGCACCTGATACAACTG	20	49.7°C	45%	

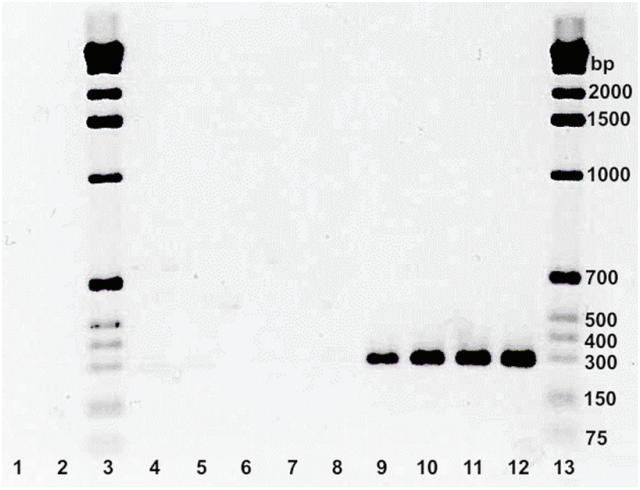

Figure 1. Agarose gel image of PCR product from identification assay of *sodA* gene. Lane 1: negative control (ddH_2O); Lane 2: *S. Epidermidis* ATCC 35984; Lane 3: 1 kb Plus DNA Ladder; Lane 4: *S. epidermidis* 48951I/09; Lane 5: *S. lugdunensis* 44987/09; Lane 6: *S. sciuri* 109645I/08; Lane 7: *S. warneri* 135612/09; Lane 8: *S. hominis* 114202/08; Lane 9: *S. capitis* isolate no. 56; Lane 10: *S. capitis* isolate no. 57; Lane 11: *S. capitis* isolate no. 58; Lane 12: *S. capitis* isolate no. 59; Lane 13: 1 kb Plus DNA Ladder.

samples from other *Staphylococcus* strains are needed to validate the specificity of current primers.

The band densities are presented in Table 2. Area densities lower than 500 were considered as background. In general, the area densities of visualised bands in all gels were between 9702 and 15861. *S. capitis* isolate no. 56 had the lowest area of *sodA* and the highest area of *gap*. In contrast, *S. capitis* isolate no. 59 had the highest area of *sodA* and the lowest area of *gap*. *S. capitis* isolate no. 57 and 58 had relative similar area densities in both *sodA* and *gap* gels.

Discussion

In current study, the *sodA* and *gap* genes were used as markers for identification of *S. capitis* because the genes' sequences are well-conserved within the species, yet contain variable domains to discriminate between close-related species. The other gene, *tuf*, has been used as a marker for a regular identification of *S. capitis* in the hospital laboratory in regards to the previous published methods.(10,19,23) However, the amplification of *tuf* was unable to discriminate *S. epidermidis* strains entirely probably due to the close relationship between *S. capitis* and *S. epidermidis* as shown in *tuf*-based phylogenetic tree (bootstrap values of 64%) generated in previous study.(23) Thus, development of PCR system using *sodA* and *gap* as markers would complement the established tuf-based assay.

The *sodA* and *gap* had been used as markers for identification of *Staphylococcus* species. A pair of general primers had been proposed to detect the internal fragment of *sodA* which represents approximately 83% of all sequence variants of *sodA*.(18) Partial gene sequencing of *gap* have been developed in previous study as a molecular tool for taxonomical analysis of *Staphylococcus* species.(23) While previous studies were aimed to detect *Staphylococcus* species and CoNS strains (23), the PCR system in current study was designed to specifically identify *S. capitis*.

Phylogenetic trees of *Staphylococcus* species had been generated in previous study based on *sodA* and *gap* genes. (23) The phylogenetic tree of *sodA* showed that *S. capitis* was in the same cluster with *S. caprae*, *S. warneri* and *S. epidermidis*, while the phylogenetic tree of gap showed a close relationship between *S. capitis, S. warneri, S. epidermidis* and *S. aureus*. Current PCR system have been validated using isolate strains of *S. capitis*, and reference strains of other *Staphylococcus* species including *S. warneri, S. epidermidis, S. lugdunensis, S. sciuri* and *S. hominis*. The system so far have been able to distinguish *S. capitis* from close related species *S. warneri* and *S. epidermidis*, also from distant *Staphylococcus* species *S. lugdunensis*, *S. sciuri* and *S. hominis* . Future study validating the PCR

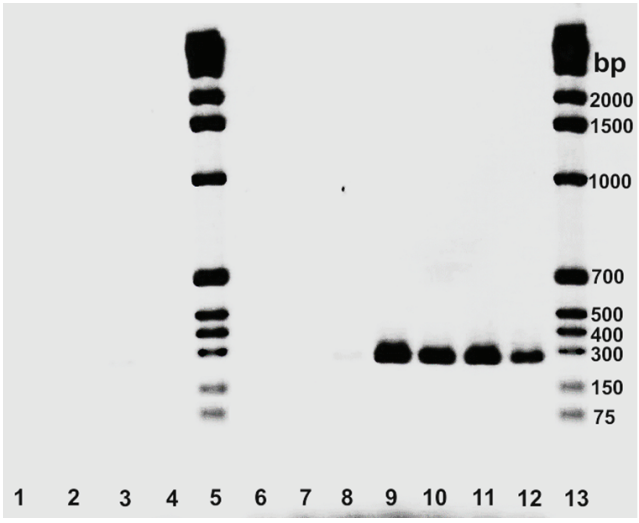

Figure 2. Agarose gel image of PCR product from identification assay of *gap* gene. Lane 1: negative control (ddH_2O); Lane 2: *S. Epidermidis* ATCC 35984; Lane 3: 1 kb Plus DNA Ladder; Lane 4: *S. epidermidis* 48951I/09; Lane 5: *S. lugdunensis* 44987/09; Lane 6: *S. sciuri* 109645I/08; Lane 7: *S. warneri* 135612/09; Lane 8: *S. hominis* 114202/08; Lane 9: *S. capitis* isolate no. 56; Lane 10: *S. capitis* isolate no. 57; Lane 11: *S. capitis* isolate no. 58; Lane 12: *S. capitis* isolate no. 59; Lane 13: 1 kb Plus DNA Ladder.

Table 2. The band densities of *sodA* and *gap* genes. Band densities of sodA (Figure 1) and gap (Figure 2) were analysed and quantified using Image J software (National Institutes of Health, Madison, USA).

Sample	*sodA*		*gap*	
	Area Density	Relative Density to Marker	Area Density	Relative Density to Marker
ddH_2O	236.849	0.184	120.950	0.032
S. epidermidis ATCC 35984	29.536	0.023	267.607	0.071
S. epidermidis 48951I/09	336.506	0.262	174.950	0.047
S. lugdunensis 44987/09	56.950	0.044	145.536	0.039
S. sciuri 109645I/08	65.778	0.051	228.607	0.061
S. warneri 135612/09	101.950	0.079	466.577	0.124
S. hominis 114202/08	409.335	0.319	35.950	0.010
S. capitis isolate no. 56	9790.418	7.624	11686.520	3.105
S. capitis isolate no. 57	13288.370	10.348	13233.290	3.516
S. capitis isolate no. 58	14011.850	10.911	13389.890	3.557
S. capitis isolate no. 59	15859.083	12.349	9702.338	2.578

system with strains of *S. caprae* and *S. auerus* would fill the gaps of current findings. Validation of the primers in real-time PCR would also needed for further study.

The *sodA* and *gap* genes from *S. capitis* have sequence similarities less than 90% to *S. warneri* and *S. epidermidis*. Mispriming may easily occur if the PCR primers are not specific. To ensure the sensitivity of the PCR system, the primers should target the conserved regions of the gene and avoid SNPs area. However, the primers should also target the unique area specific to *S. capitis* to differentiate it to other *Staphylococcus* species. Thus, the particular area match to these two criteria were carefully analysed and further processed by the OLIGO software with specific preferences. The analysis using OLIGO did not reveal any 'run' and 'repeat' with T_m difference not more than 3°C. The primer-dimer bonds were not detected in the designed primers. The analysis using BLAST again showed that the primers were specific, and no mismatch was detected with the non-targeted DNA.

The PCR condition had been optimised to choose the effective concentration of template DNA (Supplement 5). The undiluted concentration caused tailing on the gel, therefore 10-fold dilution was considered as the optimum concentration. The annealing temperature also had been optimised from 50-62°C (Supplement 6). The optimum temperature was achieved at 58°C to avoid artefacts.

The visualised agarose gels showed bands only from *S. capitis* samples (isolate no. 56-59). A single band of ~300 bp appeared from each *S. capitis* isolate. The variation of band densities was occurred from PCR products of isolates no. 56 and 59, as the two isolates showed the lowest and highest area densities for *sodA* and *gap*. It would be tempted to state that strong expression in *sodA* might contribute to the thin band appearance on *gap*. However, such variation might happen due to different loading conditions of the sample. Still, the primers of the assays was specifically amplify the targeted gene sequence. Future study on defining the sequence of each band was needed to fully answer the diverse densities.

The *dnaJ* and *hsp60* are other potential markers for identification of *S. capitis*.(22,23) The *dnaJ* has mean similarity 77.6% among *Staphylococcus* species and had been reported showing remarkable discrimination except for the most similar pairs such as *S. condimenti* and *S. carnosus* (90.9 %), *S. intermedius* and *S. delphini* (92.3 %), and *S. pulvereri* and *S. vitulinus* (99.3 %).(22) The *hsp60* has mean similarity 82% among *Staphylococcus* species. (23) Although the mean similarity is higher than *dnaJ*, *sodA* and *gap*, the *hsp60* had been reported as an effective marker to identify specifically *S. capitis* subsp. ureolyticus as well as *S. sciuri, S. caseolyticus, S. hominis, S. warneri, S. hyicus* and *S. haemolyticus* (17).

The developed PCR assay in current study provides higher specificities and sensitivities than conventional benchtop phenotypic characterisation and biochemical tests for species-level identification of *S. capitis*. The

amplification of *sodA* and *gap* may complement the *tuf*-based PCR assay. The PCR identification would become an initial step for methicillin-resistance typing of clinical isolates of *S. capitis*.

Conclusion

The *sodA* and *gap* genes might serve as effective markers for identification of *S. capitis* using conventional PCR. The PCR assay in the current study was able to identify five clinical isolates of *S. capitis* accurately without mispriming, misamplification and mis-identification.

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