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Assessment of The Presence of *sas* Family Genes and Their Relationship with Biofilm Formation among Clinical *Staphylococcus aureus* Isolates

Alka Hasani^{1,2,3}, Leila Dehghani¹, Elghar Soltani¹, Hamed Ebrahimzadeh Leylabadlo^{4*}

¹Infectious and Tropical Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

²Clinical Research Development Unit, Sina Educational, Research and Treatment Center, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran.

³Department of Bacteriology and Virology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran.

⁴Liver and Gastrointestinal Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

***Corresponding Author:** Hamed Ebrahimzadeh Leylabadlo, Ph.D, Liver and Gastrointestinal Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

Phone: +98-914 738 4585
hamedebr7@gmail.com

Abstract

Background: The success of *Staphylococcus aureus* as an important human pathogen is probably due to possession of various virulence determinants. Attachment and biofilm formation is considered the main step in any infection. The present study aimed to determine the presence of *S. aureus* surface (*sas*) genes and their association with biofilm formation and antibiotic resistance.

Methods: *S. aureus* isolates collected were analyzed for biofilm formation using polystyrene microtitre plates. All *S. aureus* isolates were also examined for the determination of *sas* genes by PCR assays and antibiotic susceptibility assay by disk diffusion method.

Results: Biofilm formation assay revealed that 29 *S.aureus* isolates were weak biofilm producers, 57 had moderate biofilm production, while only five isolates showed strong biofilm formation. The biofilm production was not revealed among nine isolates. The frequency of *sas* genes were 95 (88%), 94 (87%), 94 (87%), 92 (85.2%), 98 (90.7%), 93 (86.1%), 97 (89.8%), 87 (80.6%), and 85 (78.7%) for *sasF*, *sasA*, *sasC*, *sasE*, *sasG*, *sasH*, *sasI*, *sasJ*, and *sasK* genes, respectively.

Conclusion: High incidence of biofilm production was noticed in *S.aureus* strains positive for *sas* genes indicating the precise role of them as virulence-associated genes. Moreover, phenotypically weak or moderate biofilm formation can be well managed by antibiotic therapeutics and allow timely elimination of planktonic cells prior biofilm production.

Keywords: *Staphylococcus aureus*, Biofilm, *sas* Genes

Introduction

Staphylococcus aureus is a commensal bacterium colonizing inside the anterior nares of approximately 20% human population while, about of 60% humans are intermittent carriers.^{1, 2} The organism is an important cause of infections related to indwelling medical devices, like cardiovascular shunts, central venous catheters, and artificial joints. Moreover, *S. aureus* has also been implicated in nosocomial infections, particularly bloodstream, pneumonia, and surgical site infections.^{3, 4} The pathogenesis of *S. aureus* is attributed to the concerted effect of biofilm formation, production of toxins, and some extracellular factors, besides the invasive interactions of some strains like adherence and resistance to phagocytosis.^{5, 6}

The ability to make a biofilm is significant matter for any microbe in device-related infections. *S. aureus* biofilm formation begins once cells are attached to an inanimate or host surface, such as fibronectin and fibrinogen.^{7, 8} Adherence to these host proteins is mediated through a group of staphylococcal surface proteins termed MSCRAMMs (Microbial Surface Components Recognizing Adhesive Matrix Molecules).^{9, 10} Surface proteins are classified into four families according to a structure-function analysis and to their different motifs. The predominant type of cell wall-associated protein belongs to LPXTG (Leu-Pro-X-Thr-Gly; wherein X represents any amino acid) family, whose participants are covalently anchored to the membrane through a sortase-dependent mechanism.¹¹ Surface proteins of *S. aureus* have been classified into four families: the MSCRAMM family, the NEAT (iron-regulated surface determinant family) motif family, the three-helical bundle family, and the G5-E repeat family.¹²

The surface of *S. aureus* cells contains various forms of proteins (up to 24) covalently attached to peptidoglycan through sortases. Some of these proteins were termed with respect to ligands or characteristics with which they were first related.¹² While labels of some proteins remain

secured, however, other protein tags have been changed for instance, SasH was changed to AdsA when its adenosine synthase function was reported.¹³ The novel genes encoding previously uncharacterized Sas proteins have been recognized from genome sequences and among these proteins, the SasG surface protein has been implicated in the biofilm formation, playing a significant role in the bacterial accumulation phase.¹⁴ Published literature depicts that SasG-expressing *S.aureus* strains form peritrichous fibrils of varying density on the cell wall which facilitates biofilm formation independently of intracellular adhesive polysaccharides (PIA).¹⁵ This study was carried out to determine the biofilm-forming capacity by quantitative microtiter plate assay as well as the presence of *sas* genes encoding PIA in *S. aureus* clinical isolates and determine any correlation between biofilm formations.

Material and Methods

Bacterial strains and characterization

This cross-sectional study was carried out from February 2018 to March 2019, and 97 *S. aureus* isolates from various clinical specimens were collected from the patients either admitted to or attended Sina Hospital, one of the University Teaching Hospitals of Tabriz University of Medical Sciences, Iran. Source of specimens comprised of blood, wound, burn, urine, and body fluids. The *S.aureus* isolates were confirmed using conventional biochemical tests including gram staining, coagulase test, DNase test, mannitol salt agar growth, and genotypic PCR test using the *nuc* gene. Duplicate isolates and species other than “*aureus*” were excluded from the investigation. Eighty-seven of the total 97 *S. aureus* isolates were related to inpatients and 10 isolates were collected from various specimens of outpatients. This study was approved by The Ethics Committee of Tabriz University of Medical Sciences, Iran (IR.TBZMED.REC.1394.930).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility assay was performed by disc diffusion method in conformance to the guidelines of the Clinical and Laboratory Standards Institute (CLSI).¹⁶ The following antibiotic disks were used: cefazolin (30µg), ceftiofur (30µg), trimethoprim/sulfamethoxazole (25µg), erythromycin (15µg), gentamicin (10µg), penicillin (10µg), and clindamycin (2µg) (MAST Diagnostics, Merseyside, UK). According to CLSI recommendations, vancomycin E-test and vancomycin screen agar plates containing 6µg/mL vancomycin were used to analyze the vancomycin susceptibility testing of *S. aureus* isolates (17). Ceftiofur disc diffusion test (30µg) and oxacillin screening agar (plates containing 6mg/L of oxacillin and 4% NaCl) assessed the MRSA isolates. The control strains comprised of i) *S. aureus* ATCC® 25923TM, ii) *S. aureus* ATCC® 33591TM, and iii) *S. aureus* ATCC® 29213TM.

In vitro Biofilm Assay

For analyzing biofilm formation by *S. aureus* isolates, polystyrene plates (Greiner Bio-One, Germany) were used according to static conditions as previously described.^{18, 19} First, bacterial suspensions were prepared from overnight culture of *S. aureus* isolates in Trypticase Soy Broth (TSB) (Hi-media, India), matched for equivalent with 10⁷ Colony Forming Unit (CFU)/mL (0.5 McFarland) optical density and then 100 microliters of inoculum was added to each well. The plate was washed with 1x Phosphate Buffered Saline (PBS; pH 7.4) only once after 48 hours incubation at 37°C and 0.1% Crystal Violet (CV) was used to stain the wells for 30 minutes at ambient temperature. Extra CV stain was washed under running tap water and then 200µL of 95% ethanol was added to the wells with CV stained biofilm for solubilizing to measure the absorbance of the supernatant at 750 nm. The biofilm formation process was done in triplicate and the mean biofilm

absorbance optical density (OD) (ELISA auto reader; model 680, Bio-rad) was calculated at 570nm. The bacteria-free well was the negative control. The results of isolates were classified as weak/non-biofilm producers with OD values <0.12, moderate biofilm producers with OD= 0.12–0.24, and strong biofilm producers with OD values >0.24.²⁰

DNA Extraction and Detection of Sas Genes

The template DNA was prepared by DNeasy kit (Qiagen Inc.) according to the manufacturer's instructions (21). Nanodrop 1000 (NanoDrop, Wilmington, USA) determined the concentration of the extracted DNA. Finally, 1µL of extracted DNA was added as a template for amplification of *sas* genes. Detection of *sas* genes (*sasA*, *sasC*, *sasE*, *sasF*, *sasG*, *sasH*, *sasI*, *sasJ* and *sasK*) was achieved by PCR amplification using primers as depicted in Table 1. PCR conditions for all nine *sas* (*A*, *C*, *E*, *F*, *G*, *H*, *I*, *J*, and *K*) genes comprised of: 30 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, and final extension at 72°C for 10 min. Aliquots of the reaction mixtures were electrophoresed in 1.5% agarose gel (Yekta Tajhiz Azma, Iran) and stained with SYBR™ Safe DNA Gel Stain (Invitrogen).

Statistical Analysis

Chi-square or fisher's exact test (if needed) was used for statistical analysis with the SPSS program version 20 (SPSS, Chicago, IL, USA). The relationship between *sas* genes and biofilm formation was analyzed by Spearman's rank correlation. P-value ≤0.05 was considered statistically significant.

Results

The patients enrolled in the present study had a mean age of 40.3 ± 24 years and *S. aureus* isolates were obtained from 55 (50.9%) males and 44 (40.7%) females. Most (91.7%) *S. aureus* isolates were resistant to penicillin (Fig. 1). The inhibition zone around the cefoxitin disk recognized 22 (22.68%) *S. aureus* isolates as Methicillin-Resistant *S. aureus* (MRSA). The biofilm production assay was assessed among all 97 *S. aureus* isolates. In total, 88 of 97 (90.7%) *S. aureus* isolates were observed as biofilm producers. The findings of the biofilm formation assay showed that 29 (26.9%) *S. aureus* isolates had weak biofilm production, 57 (52.8%) were moderate biofilm producers, only five (4.6%) isolates provided strong biofilm production. The biofilm production was not observed among nine (8.3%) *S. aureus* isolates.

When the presence of *sas* genes was analyzed among *S. aureus* isolates, *sasF* was observed as the most commonly detected gene [$n=95$; (88%)]. The prevalence of other surface protein genes was as followed: 94 (87%), 94 (87%), 92 (85.2%), 98 (90.7%), 93 (86.1%), 97 (89.8%), 87 (80.6%), and 85 (78.7%) for *sasA*, *sasC*, *sasE*, *sasG*, *sasH*, *sasI*, *sasJ*, and *sasK*, respectively (Fig. 2).

The correlation between *sas* genes, biofilm capability, and antibiotic resistance are shown in Fig. 3. Among biofilm formation, significant correlations were found between moderate biofilm producers with *sasF* ($r = -0.264$, $p\text{-value} = 0.008$). According to Fig. 2, among antibiotic resistance assessment, *sasF* was correlated with cefazolin ($r = 0.220$, $p\text{-value} = 0.028$), cefoxitin ($r = 0.202$, $p\text{-value} = 0.044$), co-trimoxazole ($r = 0.263$, $p\text{-value} = 0.008$), ciprofloxacin ($r = 0.220$, $p\text{-value} = 0.028$), and gentamycin ($r = 0.210$, $p\text{-value} = 0.036$) antibiotic resistance. On the other hand, *sasG* gene had correlation with cefazolin ($r = 0.227$, $p\text{-value} = 0.005$), cefoxitin ($r = 0.261$, $p\text{-value} = 0.009$), ciprofloxacin ($r = 0.277$, $p\text{-value} = 0.005$), and gentamicin ($r = 0.269$, $p\text{-value} = 0.007$) resistance.

The source of *S. aureus* isolates comprised of: blood [n=39; (36.1%)], wound [n=36; (33.3%)], burn wound [n=16; (14.8%)], urine [n=6; (5.6%)], and body fluids other than urine and blood [n=3; (2.8%)]. Table 2 shows the distribution of *sas* genes among various clinical specimens. Among various clinical specimens, *sasH* was significantly associated with *S.aureus* isolates obtained from wound specimens (P= 0.040).

According to the source, 87 (89.6%) *S.aureus* isolates were collected from inpatients admitted to six different wards (Table 3). The most common source of *S.aureus* isolates was the infectious ward [n=21; 19.4%], followed by burn [n=17; 15.7%], intensive care unit (ICU) [n=17; 15.7%], internal [n=12; 11.1%], skin [n=12; 11.1%], and surgery [n=7; 6.5%]. The distribution of *sas* genes among the various clinical wards of the hospital is depicted in Table 3. The *sasC* and *sasK* genes were the most prevalent genes in patients admitted to the skin ward. Furthermore, *S.aureus* isolates isolated from the various specimens of patients admitted in ICU were statistically associated with the *sasG* gene.

Discussion

The success of *S. aureus* as a human pathogen is probably due to possession of several virulence determinants. *S. aureus* expresses many virulence factors, with an ability to bind to host surfaces and form biofilms to initiate disease. Binding to abiotic surfaces like catheters and implanted devices is one of the most essential virulence traits in *S. aureus*, responsible for chronic or persistent infections.²² In this regard, providing the phenotypic characterization of adhesion and biofilm formation and related genetic elements in different isolates of *S. aureus* might provide a better understanding of the complicated process of biofilm formation and infections caused by this microorganism.²³ Though presence of intracellular adhesion (*ica*) locus incorporating *icaABCD*

genes in *S.aureus* has been related to the catheter-associated and nosocomial infections, however, lacunae exist in the role of *sas* genes in these infections.^{24, 25}

In the current study, all isolates were susceptible to vancomycin. So far, several reports have been published on the presence of vancomycin-resistant *S.aureus* (VRSA) in Iran²⁶ and other countries including Pakistan, USA, Nigeria, and India.²⁷ In the present study, rate of non-susceptibility to penicillin was 91.7%, the result being compatible to another investigation from Iran where all isolates were resistant to penicillin.²⁸

Biofilm formation in microtiter plates is the most generally used method. However, the presence and expression of biofilm genes should be established by using genotypic characterization methods. In our study, 90.7% *S. aureus* clinical isolates produced biofilm and a high prevalence of *sas* genes was a distinct feature among them. Significant correlation was found between the presence of *sasF* harboring *S.aureus* isolates and moderate biofilm production. Among different clinical specimens, *sasH* gene was significantly associated with *S.aureus* isolates obtained from wound specimens. A study investigated the presence of *sas* genes and biofilm formation among *S. aureus* clinical isolates and found that *sasG* plays an essential role in the bacterial accumulation phase, but is not implicated in the initial phase of biofilm formation.¹⁴ Another study confirmed the presence of *sasC* gene in high number of clinical *S.aureus* isolates and the possible significance of this gene in colonization and infection.²⁹ In another study, *sasG*, *sasH* *sasK* and *sasE* genes were found to occur at low frequency (53%, 68% and less than 44% respectively).¹⁴ Contrary to the above investigation, in the present study the presence of *sasG*, *sasH* *sasK* and *sasE* genes was observed in 90.7%, 86.1%, 78.7% and 85.2% *S.aureus* isolates respectively. Another research correlated the presence of *sasG* and *sasH* genes with invasive disease.³⁰ The current study is

compatible with another investigation whereby the frequency of *sasA*, *sasI*, and *sasJ* was observed in 100%, 96% and 97% *S.aureus* isolates respectively.¹⁴ To our knowledge, this is the first study from Iran assessing the prevalence of *sas* genes among biofilm producing *S.aureus* strains.

It seems from the study that simultaneous presence of antibiotic resistance, biofilm formation ability, and *sas* genes among *S. aureus* isolates may lead to the emergence of strains with high pathogenicity and high resistance thereby increasing the risk of failure of antibiotic treatment and the possibility of recurrence of infections caused by this organism. Future research study is required on more number on *S.aureus* isolates to confirm these results. The results of this study indicate the high prevalence of *sas* genes in the *S.aureus* isolates. However, for investigating their relationship with biofilm formation and antibiotic resistance, more accurate evaluation of their expression and production is a necessity under laboratory and living conditions. In addition, the relationship between their presence from diverse sources requires the use of precise molecular epidemiological methods.

Conclusion

In conclusion, the high prevalence of biofilm formation among majority of *S.aureus* isolates positive for *sas* genes, shows these genes play role as virulence markers in *S. aureus* infections. Moreover, the presence of *sas* genes in antibiotics resistant *S.aureus* isolates may lead to the emergence of strains with high pathogenicity and resistance.

Ethical Issues

The present study was supported by Research Center of Infectious and Tropical Diseases (Grant No. 9408) and the manuscript was written based on a dataset of Master of Science thesis registered at Tabriz University of Medical Science. The authors would like to thank the staff of Microbiology division of Sina hospital for their collaboration. The Ethics Commission of Tabriz University of Medical Sciences approved this study (Number: TBZMED.REC.1394.930)

Author Contributions

AH: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing - original draft. **LD:** Data curation, Resources. **ES:** Software, Validation, Visualization. **HEL:** Data curation, Formal analysis, Investigation, review & editing.

Conflict of Interest

No conflict of interest existed.

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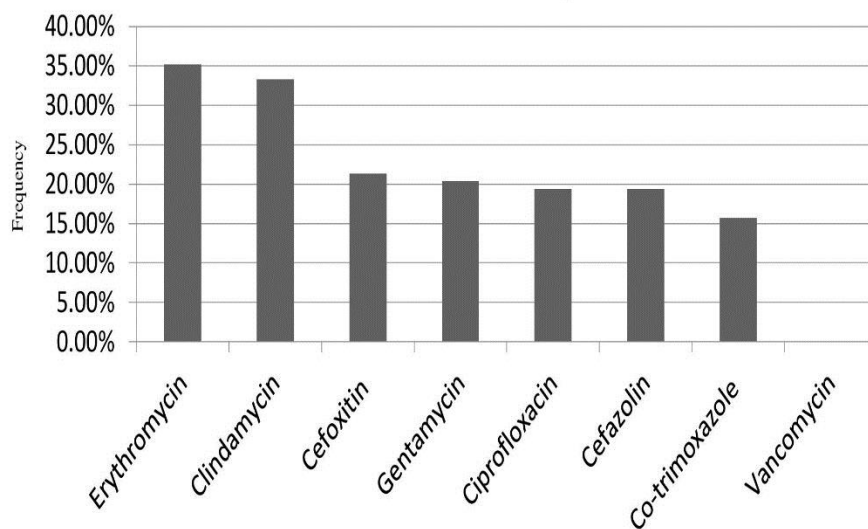


Fig 1. Antibiotic resistance patterns of *S. aureus* isolates according to the disk diffusion assay.

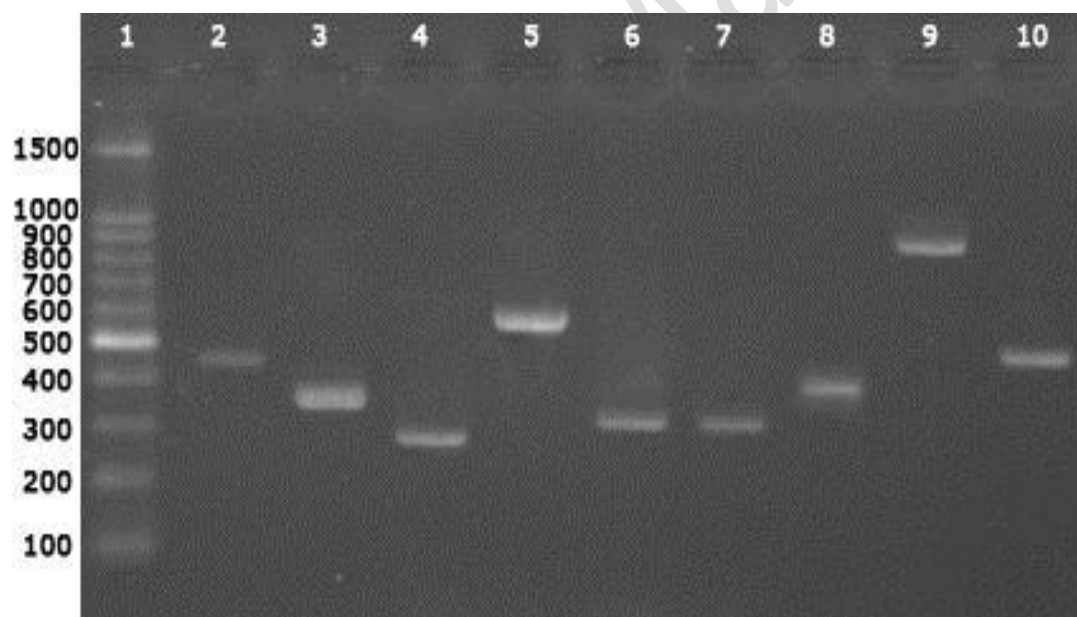


Fig 2. Screening of *S. aureus* isolates for the presence of *sas* genes by PCR methods. Lanes are respectively: **1:** ladder: Ladder 100 bp; **2:** *sasF*; **3:** *sasI*; **4:** *sasA*; **5:** *sasJ*; **6:** *sasE*; **7:** *sasH*; **8:** *sasC*; **9:** *sasK*; **10:** *sasG*.

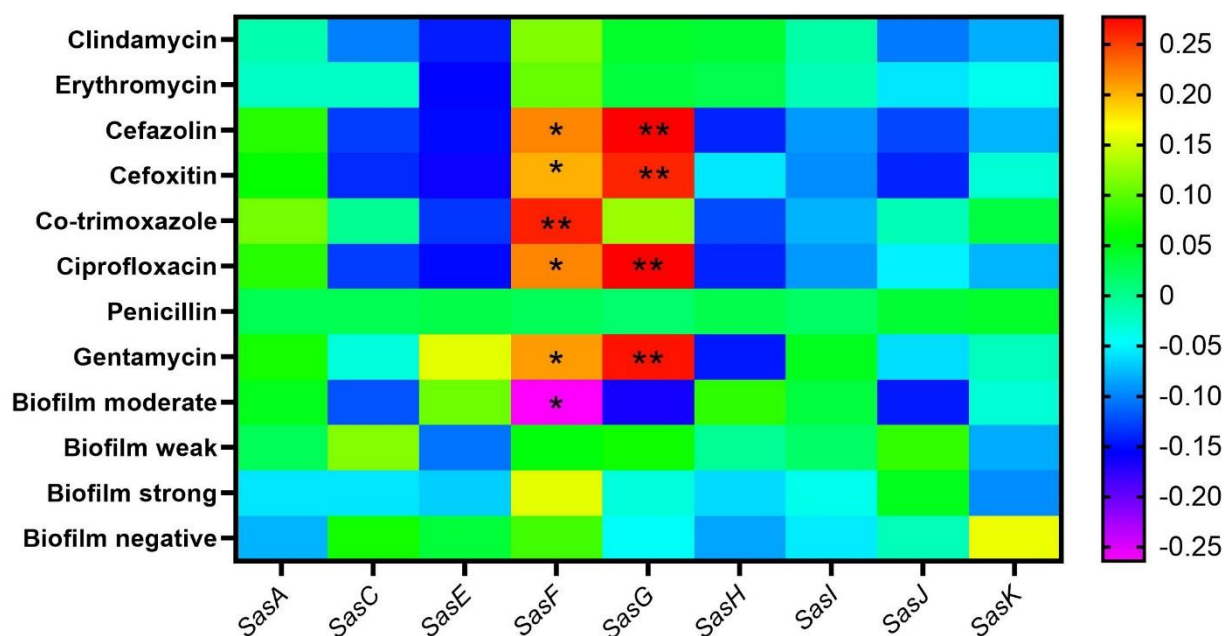


Fig 3. Correlation between *Sas* genes, biofilm capability and the antibiotic resistance. Correlation was tested using Spearman's rank test. Spearman rho value are shown the color of each correlation test corresponding as heatmaps. *p-value<0.05, **p-value<0.01

Abbreviations: **P:** Penicillin; **E:** Erythromycin; **CC:** Clindamycin; **FOX:** Cefoxitin; **GEN:** Gentamicin; **CIP:** Ciprofloxacin; **CZ:** Cefazolin; **CO:** Co-trimoxazole; **VAN:** Vancomycin

Table 1. The primers used for amplification in this study

Primer Name	Sequence (5'-3')	Reference
<i>sasA</i> -primer-F	GCATGACCAGCAAGCTTTTG	(14)
<i>sasA</i> -primer-R	GTTGAAGCTACAGATTTAGTATC	
<i>sasC</i> -primer-F	GCCAGATGCTGAAAAACG	(14)
<i>sasC</i> -primer-R	CGCATCTTCATCAACCATTC	
<i>sasE</i> -primer-F	GCAACAGAAGCTACGAACG	(14)
<i>sasE</i> -primer-R	GATTGTTCTAGTATCCGC	
<i>sasF</i> -primer-F	CGAGCTAATGAGTTGGATAGC	(14)
<i>sasF</i> -primer-R	GCTGGATCTTCCGATGTATC	
<i>sasG</i> -primer-F	GGGAACTCAACAAGAGGCAG	(14)
<i>sasG</i> -primer-R	CAGAACGAGCTTTTCTAACC	
<i>sasH</i> -primer-F	GTGTAATGGGATTATGGCAAG	(14)
<i>sasH</i> -primer-R	CGTTGCTGTGTGAGTTGG	
<i>sasI</i> -primer-F	CGTATCACAAAGCTAAAACG	(14)
<i>sasI</i> -primer-R	CCTTTGTTTTCATCACTAC	
<i>sasJ</i> -primer-F	GTTAAAGCCCCTAAGGAAAC	(14)
<i>sasJ</i> -primer-R	GGCAACTTTTGTACCTTC	
<i>sasK</i> -primer-F	CAGTTAGACAACCGGATG	(14)
<i>sasK</i> -primer-R	TAAATAAAGCCATTATTGCTG	

Table 2. Distribution of *sas* genes among various clinical specimens

Investigated genes	Clinical Specimens				
	Blood	Wound	Burn wound	Urine	Body fluids (other than blood and urine)
<i>sasA</i> (n=94)	36	32	15	6	3
<i>sasC</i> (n=94)	36	35	16	5	2
<i>sasE</i> (n=92)	37	30	16	6	3
<i>sasF</i> (n=95)	37	35	16	4	3
<i>sasG</i> (n=98)	39	36	15	5	3
<i>sasH</i> (n=93)	35	36 P=0.04	15	5	2
<i>sasI</i> (n=97)	39	33	16	6	3
<i>sasJ</i> (n=87)	34	31	16	3	3
<i>sasK</i> (n=85)	33	32	14	3	3

Table 3. Distribution of *sas* genes of *S. aureus* isolates among various clinical wards

Clinical Wards							
	Burn	Infectious	Internal	ICU	Skin	Surgical	Outpatient
<i>sasA</i> (n=94)	16	21	12	15	12	6	8
<i>sasC</i> (n=94)	16	20	12	17	9	6	10
<i>sasE</i> (n=92)	16	18	12	16	11	7	8
<i>sasF</i> (n=95)	14	21	12	15	12	6	10
<i>sasG</i> (n=98)	17	21	12	16	12	7	10
<i>sasH</i> (n=93)	16	21	10	15	10	7	10
<i>sasI</i> (n=97)	17	20	11	17	11	7	10
<i>sasJ</i> (n=87)	14	19	11	14	11	6	8
<i>sasK</i> (n=85)	16	20	10	15	6	7	9