Avicenna J Clin Microb Infec. 2017 May; 4(2):e46049. doi: 10.5812/ajcmi.46049.

Published online 2017 April 23. **Research Article**

Genotype Diversity and Evaluation of Biofilm Formation in *sasX* Positive Methicillin-Resistant *Staphylococcus aureus*

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Received 2017 January 30; **Revised** 2017 March 02; **Accepted** 2017 March 29.

Abstract

Objectives: *Staphylococcus aureus* surface protein X (*sasX*) is a newly described protein that has different roles in virulence, such as biofilm formatin, as a bacterial approach for pathogenesis. *SasX* gene was initially found in eastern Asian countries, yet, studiese have shown spreading of this gene to other strains. Because there is no report on this gene in Iran, the current study aimed at determining the prevalence, genotype diversity, and evaluation of biofilm formation in *sasX* positive methicillin-resistant *Staphylococcus aureus* (MRSA).

Methods: This experimental study was conducted on strains isolated from inpatients during 2014 to 2015. Cultivation, identification, and confirmation of MRSA isolates were performed. The evaluation of biofilm production, *sasX* gene detection, and Random amplified polymorphic DNA (RAPD)-polymerase chain reaction (PCR) were done. Data collection and comparison were performed by SPSS and gelcompare softwares.

Results: Overall, 140 isolates (77.8%) formed a biofilm and 23 (12.6%) isolates were *sasX* positive. Amongst 23 *sasX*$^+$ strains, 21 (91.3%) isolates created a biofilm. The RAPD-PCR analysis of 23 *sasX* positive isolates showed 19 RAPD types with 4 common types (CT) and 15 single types (ST).

Conclusions: Frequency of *sasX* gene was somewhat similar to another study in eastern countries and the ability of biofilm formation in *sasX*$^+$ strains compared to all of MRSA isolates were not significant (P value > 0.05) and a correlation was not found between RAPD type and biofilm grade.

Keywords: Biofilm, Clonal Diversity, Methicillin Resistant *Staphylococcus aureus*

1. Background

Staphylococcus aureus is an opportunistic pathogen that is responsible for a large number of hospital- and community-acquired infections. Methicillin resistant *S. aureus* are now distributed through the world and have become a major concern with the treatment of patients (1). *Staphylococcus aureus* have many different virulence factors, including Cell Wall-Anchored (CWA) proteins that have a vital role in pathogenesis and survival of *S. aureus* in the environment. Some of the CWA proteins are MSCRAMMs, which have a crucial role in colonization and binding to host extracellular matrix (ECM) (2, 3). Many previous studies have suggested that CWA proteins such as ClfB, FnBPs, SasC, protein A, and sasG participate in biofilm formation, which is one of the bacterial approaches for pathogenesis and resistance against antibiotics (4-7). One of the MSCRAMMs is a newly described protein named as *S. aureus* surface protein X (*sasX*). The *sasX* gene is located at the 3' end region of a 127.2-kb ΦSPβ-like prophage, and encodes a secreted, surface-anchored protein and has a key role in colonizing and pathogenesis. Primarily, *sasX* was found in ST239 clones, yet, it has spread to other clones. Based on recent studies, this protein significantly enhances nasal colonization, lung disease, abscess formation and immune evasion in animal models, and promotes inter-cellular aggregation of *S. aureus* (8). Furthermore, immunization with recombinant *SasX* leads to protection against *S. aureus* colonization in the same model (9).

2. Objectives

Given the importance of this gene in the pathogenesis and increase of spreading between different clones and no reports about this gene in Iran, the determination of the prevalence of *sasX*$^+$ strains, genotype diversity, and evaluation of biofilm formation from different clinical sources were performed.

3. Methods

3.1. Clinical Samples and Bacterial cultivation

In the current experimental study, clinical samples were collected from different clinical sources from several hospitals of Iran University of Medical Science (IUMS) in Tehran during 2014 to 2015. The samples were cultivated on blood agar and phenotypic identification of suspicious isolates was performed by gram staining, catalase, coagulase and DNaes activity, and susceptibility to cefoxitine was determined by disk diffusion method based on clinical and lavratory standards institute (CLSI) 2015 protocols.

3.2. Detection of nucA, mecA, and sasX

Detection of *nucA*, *mecA*, and *sasX* genes was investigated by Multiplex-Polymerase Chain Reaction (M-PCR) in the Master cycler gradient instrument (Eppendorf, Germany). DNA was extracted from the isolates, which were grown overnight on brain heart infusion (Merck Co., Germany) agar plates, by the Genomic DNA Purification Kit (Yekta Tajhz Azma Co, Iran). The used primers are listed in Table 1. One microliter of prepared DNA (0.5 μg) was added to a final volume of 25 μL of PCR master containing 11 μL of 2 $\times$ MasterMix (Yekta Tajhz Azma Co, Iran), including 1 $\times$ PCR buffer, 1.5 mmol/l $MgCl_2$, 0.15 mmol/L dNTPs, and 1.25 IU Taq DNA polymerase, (Yekta Tajhz Azma Co, Iran) 0.9 μL of each of the 3 primer pairs, and 7.6 μL of sterile distilled water. DNA amplification was performed with the PCR cycling conditions as follows: initial denaturation at 95°C for 7 minutes, followed by 40 cycles of amplification (denaturation at 94°C for 45 seconds, annealing at 56°C for 40 seconds, and extension at 72°C for 1 minute), with a final extension step at 72°C for 5 minutes. The amplified products were visualized by electrophoresis on 1.5% agarose gel stained with Gel Red.

Table 1. Oligonucleotides Used in This Study

Gene	Sequence	Size, bp	Ref
nucA	F: 5′-GCGATTGATGGTGATACGGTT-3′	270	10
	R: 5′-AGCCAAGCCTTGACGAACTAAAGC-3′		
mecA	F: 5′-CCTAGTAAAGCTCCGGAA-3′	314	11
	R: 5′-CTAGTCCATTCGGTCCA-3′		
sasX	F:5′-AGAATTAGAAGTACGTCTAAATGC-3′	522	6
	R:5′-GCTGATTATGTAAATGACTCAAATG-3′		

3.3. Random amplified polymorphic DNA (RAPD)-Polymerase Chain Reaction (PCR)

Random amplified polymorphic DNA (RAPD)-polymerase chain reaction (PCR) was carried out by 3 primers as shown in Table 2. In brief, amplification reactions were performed in a final volume of 25 μL containing 1x amplification buffer (Yekta Tajhz Azma Co, Iran), 20 ng DNA, 1.6 pmol of oligonucleotides, 0.25 mM of each deoxynucleotide triphosphate (dATP, dTTP, dCTP, and dGTP), 2.5 mM $MgCl_2$, and 2.5 U of Taq polymerase (Yekta Tajhz Azma Co, Iran). The amplification was performed in Eppendorf thermocycler (Eppendorf, Germany) and programmed for initial denaturation cycle at 94°C for 3 minutes followed by 40 cycles of 94°C for 1 minute, 35°C for 1 minute, and 72°C for 2 minutes, and final extension step at 72°C for 10 minutes (10). The amplified fragments were separated by agarose gel electrophoresis on a 1.5% 1x Tris/Borate/EDTA (TBE) for 3 hours at 60 V and stained with safe stain for determining the best genotype profile and greater reliability, the experiments were repeated twice.

Table 2. Oligonucleotides of Random Amplified Polymorphic DNA (RAPD)-Polymerase Chain Reaction (PCR) Used in This Study

Primer	Sequence	Tm, °Ca	Ref
M13 S63	5′-GAAACAGCTATGACCATG-3′	34	12
B0043-17	GCGATCCCCA	34	13
S33	CAGCACCCAC	34	14

3.4. Biofilm Assay

Biofilm assays were performed according to microtiter plate assay (11). Briefly, the isolates were grown in TSB (Merck Co., Germany) 1% Glc at 37°C for 18 hours. Cultures were diluted 1: 100 in TSB-1% Glc and 200 μL was inoculated into each well. The microtitre plate was incubated at 37°C for 20 hours (3). Supernatants were removed from each well and the remaining material were gently washed 3 times with sterile phosphate-buffered saline (PBS), then dried and fixed at 65°C for 1 hour. Finally, the plates were stained with crystal violet (Gram-stain), gently washed twice, and 100 μL of 70% (vol/vol) ethanol and 10% isopropyl alcohol (vol/vol) was added (8). The absorbance was read in a microplate reader at 570 nm. Biofilm formation was scored as follows: non-biofilm forming (A570 < 1); +, weak (1< A570<2); ++, moderate (2< A570 < 3); +++, strong (A570 > 3).

4. Results

4.1. Samples and Bacterial Cultivation

Overall, 325 isolates were collected from all of the samples, including patients' normal flora, blood, urine, other body fluids, wound discharge, sputum, diabetic foot ulcer, trachea, and abscesses. Amongst all of the clinical samples (the details of samples are shown in Table 3), 186 phenotypical MRSA strains (57.2%) were isolated.

Table 3. Prevalence of *sasX*$^+$ Positive Isolates in Normal Flora and Clinical Samples[a]

Sample Type	Number of Samples	MRSA Prevalence	*SasX*+ MRSA Prevalence
Normal flora	83 (25.5)	60 (33)	7 (30.6)
Blood	59 (18.1)	22 (12)	3 (13.04)
Urine	58 (17.8)	35 (19)	2 (8.89)
Wound	33 (10.1)	37 (21)	4 (17.03)
Sputum	27 (8.3)	15 (8)	1 (4.35)
Diabetic Foot Ulcer	9 (2.8)	2 (1)	1 (4.35)
Trachea	13 (4)	2 (1)	1 (4.35)
Abscess	33 (10.1)	5 (3)	3 (13.04)
Other body Fluids	10 (3.1)	4 (2)	1 (4.35)
Total number	325 (100)	182 (100)	23 (100)

[a]Values are expressed as No. (%).

4.2. Datection of nucA, mecA, and sasX

Molecular assays showed that 182 isolates were *nuc*$^+$ and *mecA*$^+$(the details of isolates are shown in Table 3). For detecting *sasX* and evaluation of biofilm formation, *nucA*$^+$ and *mecA*$^+$ strains were considered, 23 (12.6%) of which were *sasX* positive. Prevalence of *sasX* positive isolates is shown in Table 3.

4.3. Random amplified polymorphic DNA (RAPD)-Polymerase Chain Reaction (PCR)

The analysis of 23 *sasX* positive isolates showed 19 RAPD types with 4 common types (CT) covering 8 (34.7%) isolates with 95% similarity and 15 Single Types (ST) covering 15 (65.3%) isolates. Between these types, CT O and Q strains were isolated from the same source (normal flora and wound, respectively), yet, only strains that belonged to CT O were able to form strong biofilms (Figure 1).

4.4. Biofilm Assay

Biofilm formation experiments revealed that 140 isolates (77.7%) were able to form biofilm, including weak (12.9%), medium (34.3%), and strong (52.8%) categories (the details are shown in Table 4). Amongst 23 *sasX*$^+$ strains, 21 (91.3%) isolates were able to form a biofilm. The details of biofilm formation of *sasX*$^+$ strains are listed in Table 5.

5. Discussion

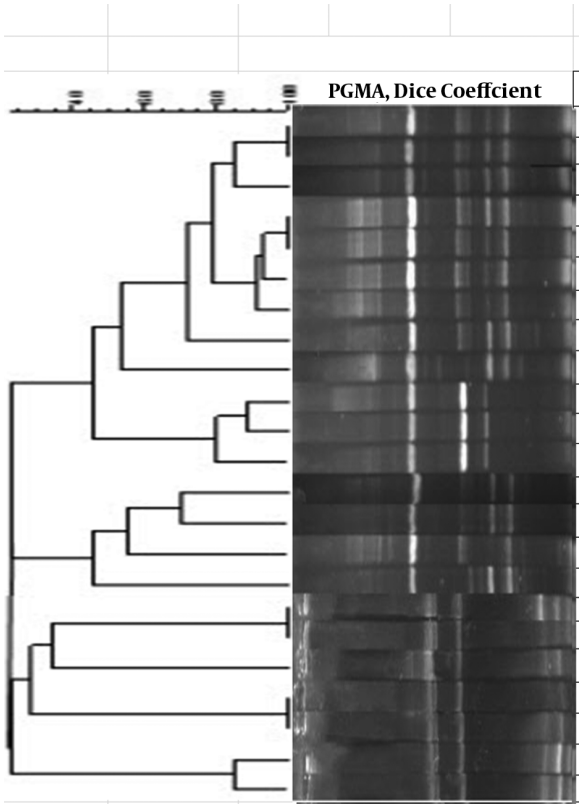
Staphylococcus aureus are widespread bacteria that can cause different infectious diseases, including superficial, invasive, and life-threatening infections. Furtheremore, MRSA is prevalent in hospitals and the community and has become a major concern around the world.

Many different virulence factors, such as surface proteins, are involved in the pathogenesis of these bacteria. One of these important proteins is *sasX* that has many different roles in this process, including biofilm formation, which helps the bacteria in producing micro colonies and adhesion on the surfaces and bacterial resistance against unexpected conditions (11). Regarding recent studies, *SasX* had an important role in the pathogenesis of the asian population and ST239 colon (8) and based on its importance, even new studiese have focused on the role of *SasX* protein in immunization and vaccinaton (9). Furthermore, recent studies have shown that the *sasX* gene is spreading to other colonies and species (8, 12). On the other hand, in Iran there has been no study on the prevalence of *sasX* gene and ability of biofilm formation in *sasX*$^+$ positive strains from different clinical sources.

In this study, 186 MRSA strains were identified phenotypicaly and finally 180 isolates were genetically confirmed by the PCR method. The frequency of MRSA isolates in this study was 56%. Our findings are similar to a previous study from Iran (13). Amongst 180 confirmed MRSA strains, 23 isolates (12.6%) were *sasX* positive. The results were in agreement with that of Li et al. (12). Li et al. reported that the prevalence of *sasX* gene in *S. aureus* isolated from adult patients increased significantly from 2003 to 2011 from 19% to 31% (8). Soumya et al. showed that amongst 40 Staphylococcus epidermidis isolates, only 1 isolate was *sasX*$^+$ (11). In this study, a significant correlation was not found between clinical samples and *sasX* positive isolates.

The results of the current study showed that between 23 *sasX* positive isolates, 19 RAPD types with 4 common types (CT) and 15 single types (ST) were observed that covered 8 (34.7%) and 15 (65.3%) isolates, respectively. Between these types, CT O and Q strains were isolated from the same source (Normal flora and wound respectively), yet, only strains that belonged to CT O were able to form a strong

Figure 1. Dendrogram Cluster Analysis of Random Amplified Polymorphic DNA (RAPD)-Polymerase Chain Reaction (PCR) Data for 23 *sasX* Positive Isolates



RAPD Type	Sample	Hospital	Bioflim Grade
A	Trachea	A	No Bioflim
A	Blood	B	Strong
B	Normal Flora	B	Strong
C	Abscess	D	Strong
C	Normal Flora	C	Medium
D	Normal Flora	C	Strong
E	Normal Flora	C	Strong
F	Body Fluid	E	Weak
G	Sputum	B	Strong
H	Abscess	A	Strong
I	Urine	D	Medium
J	Urine	D	Strong
K	Blood	E	Strong
L	Blood	A	String
M	Diabetic Foot Ulcer	B	No Bioflim
N	Abscess	D	Strong
U	Normal Flora	H	Stro ng
O	Normal Flora	B	Strong
P	Normal Flora	B	Strong
Q	Wound	A	Medium
Q	Wound	D	Weak
R	Wound	E	Strong
S	Wound	E	Strong

Patterns, sample source, hospital, and biofilm grade were indicated

Table 4. Biofilm Grade in Methicillin-Resistant *Staphylococcus aureus* Isolates From Different Clinical Samples

Clinical Samples	Biofilm Grade			
	Weak	Medium	Strong	Total[a]
Normal Flora	7	16	24	47 (33.57)
Blood	3	6	10	19 (13.57)
Urine	2	11	15	28 (20)
Wound	3	11	15	29 (20.71)
Sputum	2	4	6	12 (8.57)
Diabetic Foot ulcer	0	0	0	0
Trachea	0	0	0	0
Abscess	0	0	3	3 (2.14)
Other body fluid	1	0	1	2 (1.42)
Total	18 (12.9)	48 (34.3)	74 (52.8)	140 (100)

[a]Values are expressed as No. (%).

biofilm. The findings did not show any correlation between each of the RAPD profiles and biofilm grade.

In the present study, 140 isolates (77.7%) created a biofilm. Previous studies from Iran have reported that

Table 5. Biofilm Grade in *sasX*$^{+}$ Strains Isolated from Different Clinical Samples

Clinical samples	Biofilm Grade			
	Weak	Medium	Strong	Total
Normal flora	0	1	6	7
Blood	0	0	3	3
Urine	0	1	1	2
Wound	1	1	2	4
Sputum	0	0	1	1
Diabetic Foot Ulcer	0	0	0	0
Trachea	0	0	0	0
Abscesses	0	0	3	3
Body Fluids	1	0	0	1
Total[a]	2 (9.5)	3 (14.3)	16 (76.2)	21 (100)

[a] Values are expressed as No. (%).

100% of MRSA isolates could form a biofilm (14, 15). The findings showed that the number of biofilm isolates from the normal flora were more than other clinical samples, which agrees with some previous studies (16, 17). The categories of biofilm amongst all of the clinical samples were weak (12.9%), medium (34.3%) and strong (52.8%). Amongst 23 *sasX*$^{+}$ strains, 21 (91.3%) isolates created a biofilm; 2 (9.6%), 3 (14.3%), and 16 (76.1%) isolates formed weak, medium and strong biofilms, respectively. Overall, the findings did not show any significant difference amongst biofilm-producing MRSA isolates and *sasX* positive isolates, yet, most of the *sasX* positive isolates created strong and medium profile.

5. Conclusions

Frequency of *sasX* gene was approximately similar to another study in eastern countries and the ability of biofilm formation in *sasX*+ strains compared to all of MRSA isolates was not significant ($P > 0.05$).

Acknowledgments

This study was supported by the microbiology department, medicne faculty, Iran University of Medical Science, Tehran, Iran. The authors are thankful to the head of the department of Microbiology.

Footnote

Funding/Support: This study was supported by Iran University of Medical Science, Tehran, Iran.

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