Emergence of methicillin resistant *Staphylococcus aureus* (MRSA) isolates from north India harboring a novel *sasX* gene: further analyzing its role in biofilm formation

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Received 22 December 2016
Accepted 30 April 2017

ABSTRACT

Objective: The present study was aimed to identify and characterize *sasX* gene in methicillin resistant *Staphylococcus aureus* (MRSA) isolates from north east India and further analysing its role in biofilm formation and pathogenesis.

Methods: A total of 150 laboratory adopted *S.aureus* isolates were investigated for antimicrobial susceptibility patterns using 8 commercially used antibiotics of different groups. The MRSA strains identified were further screened for the presence of *sasX* gene by using polymerase chain reaction (PCR). The comparison of *sasX*$^+$ and *sasX*$^-$ MRSA isolates were further studied for biofilm production by in vitro methods: Tube Method (TM) and microtiter Plate assay (adhesion assay).

Results: Out of 150 isolates, 24 were found resistant to methicillin among which 8 strains were found harbouring the *sasX* gene. All these isolates were also found highly resistant to Oxacillin, Methicillin and Penicillin antibiotics. The formation of bacterial aggregates and promotion of biofilm formation was observed much higher in *sasX*$^+$ MRSA isolates as compared to sasX$^-$MRSA isolates.

Conclusion: The overall results signifies that the *sasX* gene more likely facilitates biofilm production, promotes cell adhesion and helps in immune evasion mechanisms, thus is a major risk factor which needs to be further investigated. This is probably the first study from India reporting the isolation and characterization of *sasX* gene in MRSA isolates.

KEY WORDS: *Staphylococcus aureus*, MRSA, *sasX* gene, Biofilm

Introduction

Staphylococcus aureus is a major human pathogen that causes frequent and often severe infections throughout the world. Most importantly, *S. aureus* has become increasingly resistant to methicillin, an antibiotic of first choice against *S. aureus* infections. Methicillin is a penicillinase-resistant penicillin derivative that was developed as a response to the global spread of the penicillinase gene among *S. aureus* strains, which started in the 1950s [1]. However, methicillin-resistant *S. aureus* was detected only about one year after the introduction of methicillin into clinical use. Now a day's many countries report methicillin resistance rates among invasive hospital isolates of *S. aureus* that reach and exceed 50%. More recently, MRSA has also appeared in the community (community-associated MRSA, CA-MRSA), posing an additional threat to public health systems. Recently Li et al., found an evidence of the acquisition of a new virulence determinant through horizontal gene transfer. This determinant can be triggered for the occurrence of new successful clones and identified as *Staphylococcal* surface protein *SasX* which is to be epidemiologically connected to a successful spreading clone of the MRSA sequence type 239 (ST239) [5]. *SasX* was first identified in TW20 strain from an MRSA outbreak in an intensive-care unit in the UK. Whole-genome sequencing revealed that it is located in a mobile genetic element at the 3' end of a 127.2 kb ΦSPβ like prophage, which lacks other virulence-associated genes [2].

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The *sasX*-containing element which was initially restricted to ST239 spread to other sequence types. This behavior indicates towards an increased mobilization capacity of this element within the hospitals worldwide [3]. *SasX*, a LPXTG surface-anchoring motif containing protein, was expressed on the bacterial cells surface and it has an impact on the colonization capacity and pathogenicity of *S. aureus*. *SasX is* believed to play a key role in MRSA colonization

and pathogenesis, thereby increasing the possibilities of nasal colonization, lung diseases, abscess formations and biofilm formations. These formations are bacterial communities enclosed in a hydrated extracellular matrix, which may consist of proteins, polysaccharides, nucleic acids, or combinations of these molecules promoting mechanisms of immune evasion [4]. *SasX* increases interbacterial aggregation and biofilm formation, which leads to decrease phagocytosis and increases survival in human blood and provide resistance to human neutrophils [5].The prevalence of *SasX* among Asian and international MRSA clones is not known but believed that this may increase further. The present study was designed to identify and characterize *SasX* gene in MRSA isolates and its role in biofilm formation.

Materials and methods

IRB/ IEC Approval

The present study was approved by institutional ethical committee of Shoolini University, Solan, Himachal Pradesh, India under Registration Number: SUIEC/13/32.

Sample collection and isolation

A total of 150 laboratory adopted isolates were screened for the presence of *S.aureus*. The isolates were inoculated in mannitol salt agar (Hi Media, India) to obtain the growth of suspected *S. aureus*. Plates were incubated for 24 hours at 37°C. Cultures were identified as *Staphylococci* by classic microbiological methods: Gram's stain, catalase and coagulase activity on rabbit plasma (Table-1).

Table 1. Phenotypic Characterization of *Staphylococcus aureus* isolated from various sources (Total=150).

S. No.	Infection site	Culture Characteristics	Biochemical Characteristics			
		Mannitol Salt Agar	Gram's reaction	Catalase	Coagulase	DNase
		Yes	No	Yes	No	Yes
1	Pus	69	-	69	-	69
2	Blood	21	-	21	-	21
3	Skin and soft tissue	17	-	17	-	17
4	Nasal	19	-	19	-	19
5	Urine	24	-	24	-	24

Antibiotic susceptibility test

The susceptibility of all the 150 *S.aureus* isolates towards Oxacillin (1mcg), methicillin (5mcg), Erythromycin (15mcg), Tetracycline (30mcg), Cotrimoxazole (25mcg), Ciprofloxacin (5mcg), Clindamycin (2mcg) and Penicillin (10u) was checked by Bauer-Kirby disc diffusion assay. The results were interpreted in accordance with Clinical Laboratory Standards Institute guidelines (CLSI, 2006). The following assay was carried out in triplicates on Mueller-Hinton agar supplemented with 4% NaCl.

DNA extraction

The DNA extraction was carried out as described by Sambrook et al., with slight modifications [6]. Briefly, the overnight grown bacterial cultures were centrifuged and the pellets thus obtained were suspended in lysis buffer (PBS containing 0.5% SDS, 100mg/ml proteinase K) and incubated at 37°C for 1 hour. Further, an equal volume of phenol: chloroform (1:1) was added to the cell suspension, vortexed and centrifuged for 5 minutes at 10,000 rpm. The aqueous phase was collected and transferred to a fresh tube. The DNA was precipitated by adding 60 ml of 3 M sodium chloride and three volumes of absolute alcohol. After overnight incubation at -20°C, the DNA pellet was washed twice with 99% cold ethanol, air-dried and suspended in 500 µl of TE buffer (10 mMTris-HCI (pH 8) and 1 mM EDTA (pH 8).

PCR of *sasX* gene

A 522-bp fragment of the s*asX* gene was amplified by using specific primers previously reported by Li et al., [5]. The PCR cycle followed for an amplification of *sasX* gene was: 95ºC for 30s of initial denaturation; followed by 34 cycles for 30s at 95ºC and 30 sec for 50ºC and 72ºC for 2 min for final extension. Amplicons were electrophoresed on 1.0% Agarose Gel (1% agarose, 1× Tris-buffered EDTA; 90 V for 90 min) containing 1µg/ml ethidium bromide and visualized in a gel documentation system (Gel-doc-It2 imager).

Sequencing

Three amplicons out of 8 were subjected for DNA sequencing by Sanger-Dideoxy sequencing method. After sequencing, consensus sequences were generated from the forward and reverse sequence data for each isolate by using Codon Code Aligner software. The sequences were submitted to the gene bank with accession nos.

Biofilm formation in *sasX*$^+$ MRSA isolates

The formation of biofilm by *S. aureus* isolates was performed using TM (tube method) and microtiter dish assay.

Tube method

This quantitative assay was performed as described by Christensen et al., [7]. Briefly, the glass tubes containing 3 mL of trypticase soy broth were inoculated with a loopful of pure culture of a MRSA strain grown overnight on blood agar plate. After 48 h incubation at 37°C, the content of each tube was decanted and the tubes were stained with 1% safranin for 7 min. followed by washing with distilled water for 5 min. A positive result was indicated by the presence of an adherent film of stained material on the inner surface of the tube. Presence of stained material at the liquid-air interface was not regarded as indicative of slime production. The tubes containing trypticase soy broth only were taken as negative controls. The results were scored visually as 0-absent, 1-weak, 2-moderate, 3-strong.

Adherence assay on abiotic surface

The MRSA strains were grown overnight in trypticase soya broth (TSB) containing 1% glucose in polystyrene tissue culture plates and incubated at 37°C for 48 hours under aerobic conditions. After incubation, the planktonic cells were washed ten times with deionized water. The adhering bacterial cell in each well was fixed with 2mL of absolute methanol for 20 min. The plate was then emptied and left to dry overnight. The bacterial cells which got adhered to the surface were stained with safranin. The adhered cells got stained on treatment with 0.1% safranin for 15 min and the excess stain was rinsed off by washing the plates thrice with distilled water. The plates were then kept overnight for drying. After the plates were air dried the safranin dye bound to the adherent cells were resolublized on treatment with 1ml of 95% ethanol. The OD of each well was taken at 490nm (A_{490}) using spectrophotometer (UV Vis spectrophotometer 117).The wells containing sterile TSB was taken as a negative control. The adherence capabilities of the test strains were classified into the following four categories: non-adherent weakly, moderately, or strongly adherent, based upon the ODs of bacterial films. The cut-off optical density (ODc) for the microtiter-plate was defined as three standard deviations above the mean OD of the negative control. Strains were classified as follows:

OD ≤ ODc non-adherent

ODc< OD ≤ 2 x ODc weakly adherent

2 x ODc< OD ≤ 4 x ODc moderately adherent

4 x ODc< OD strongly adherent

All the tests were carried out in triplicates and their mean was calculated and considered as final result.

Statistical analysis

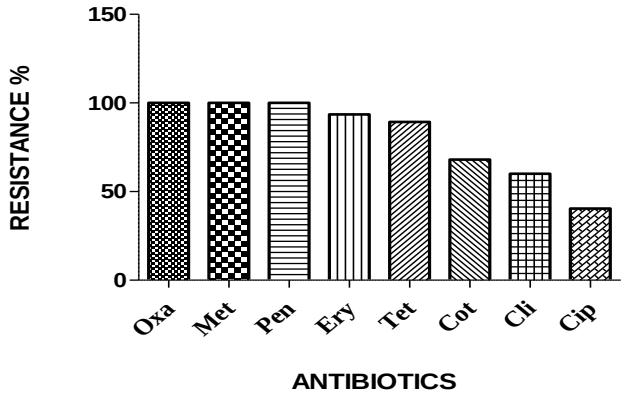
Statistical analysis of the results was expressed as Mean ± Standard Error (S.E.M.) by using graph pad prism version5 (Table 2).

Results

Antibiotic resistance profiles of *Staphylococci* isolates

The antimicrobial resistance profile of all the 150 *S.aureus* isolates against 8 antimicrobial agents of different groups is shown in figure 1. Out of 150 isolates, 24 isolates were found positive for MRSA. The majority of strains were found resistant to Oxacillin (100%), Methicillin (100%) & Penicillin (100%) followed by erythromycin (93.6%), Tetracycline (89.3%), Cotrimoxazole (68%), Clindamycin (63.8%) and least with Ciprofloxacin (40.4%).

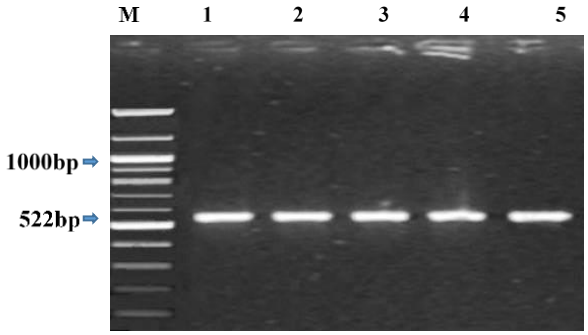
Figure 1. Resistant Pattern of *S. aureus* against various antibiotics.



PCR screening of *sasX* gene

The detection of the presence of *sasX* in all the 24 MRSA isolates was carried out by PCR using specific primers. Only 8 MRSA isolates (33.3%) were found harboring *SasX* gene (522bp) upon PCR amplification (Figure-2).

Figure 2. PCR amplified product of s*asX* gene (522 bp). Ladder [L] - 100 base pair. Lane 1-928, lane 2 - 910, lane 3 - 2, lane 4 - JCSC, lane 5 - CA-97.



Biofilm formation in *sasX*$^+$ and *sasX*$^-$MRSA isolates

All the 8 MRSA isolates were subjected to biofilm formation on antibiotic surface. The result revealed that *sasX*$^+$ isolates were strong biofilm producer in comparison to *sasX*$^-$ isolates (Figure 3;Table-2).

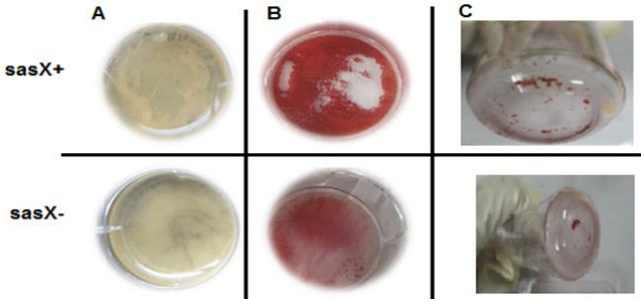
Discussion

MRSA is a phenomenon for which molecular underpinnings are not fully apprehended till date and is leading cause of severe infections in hospitals resulting into epidemics. Recently, Li et al., suggested that the sasX gene plays a significant role in disseminating MRSA epidemics across Asia [5].

Table 2. Screening of *sasX*+ isolates for detection of biofilm and the overall results of the tube and microtiter-plate test.

Categories	Number of isolates	Mean OD value of Microtiter plate	Tube Method
No adherence (0)	-	0.56±0.005	0
Weak adherence (+)	NM-15, NM-20	0.39±0.50 0.45±0.62	1
Moderate adherence (++)	NM-6, NM-8, NM-23, NM-13	1.527±0.58 1.698±0.60 1.798±0.68 2.102±0.69	2
Strong adherence (+++)	NM-3, NM-5	2.64±0.19 2.99±0.21	3

Figure 3. Showing biofilm formation by MRSA isolates. (A) Showing the biofilm formed by *sasX*$^+$ and *sasX*$^-$in a flat-bottom microtiter plate (B) Biofilm stained with safranin. (C) Tube with biofilm in TSB after staining.



In addition, they also suggested that the presence of *sasX* gene in *S. aureus* potentially increased its virulence capacity by boosting the bacterial defense mechanism, particularly in the ST239 clones. In addition, *sasX* also facilitates interbacterial aggregation and biofilm formation and leads to reduced phagocytosis and increased survival in human blood and resistance to human neutrophils [5]. Their findings suggests that the *sasX* is likely a driving force behind the MRSA epidemic in Asia, increasing the bacteria's ability to spread as well as its deadliness once enters the body. Based on these epidemiological and functional evidences of the *sasX* gene contribution in virulence and biofilm formation in MRSA, the present study was designed to identify and characterize *sasX* gene in MRSA isolates and further investigating its role in biofilm formation. Twenty four clinical MRSA isolates were tested for the presence of the *sasX* gene among which eight isolates (32%) were found to

harbor *sasX* gene. Similarly Li et al., reported the 21–39% frequency of the *sasX* gene among MRSA isolates from China [5]. However, Song et al., reported a little higher (36.7%) prevalence of the *sasX* gene among MRSA isolates from inpatients at a teaching hospital in Shanghai [8].

Further, a comparison in biofilm production by *sasX*$^+$ and *sasX*$^-$ isolates was performed by two conventional methods, namely TM (tube method) and microtiter dish assay. The findings suggest that the MRSA isolates harboring *sasX*$^+$ isolates were strong biofilm producer as compared to *sasX*$^-$ isolates**.** In addition, the adherence assay of *sasX*$^+$ and *sasX*$^-$ isolates on PBMCs revealed that the number of viable human PBMCs was less in those incubated with *sasX*$^+$ as compared to *sasX*$^-$ strains. The increased survival in human blood and higher lysis of human PBMCs by MRSA isolates harboring *sasX*$^+$ gene is a sign of their prolonged survival after phagocytosis. This overall analysis suggests that the *sasX* may facilitate intercellular aggregation thereby leading to a significant enhancement of *S. aureus* immune evasion mechanisms. However, more detailed studies are needed to clearly understand the pathogenesis and mechanisms induced by *sasX* bearing MRSA isolates in biofilm formation and evading immune system.

Our data provides insights of *sasX* gene in MRSA isolates that will aid in developing an effective strategy for infection control. To the best of our literature search, it is the first study reporting the emergence of *sasX* gene among MRSA isolates in India. Further a large-scale investigation of the prevalence and continual monitoring of the MRSA isolates harboring *sasX* gene in Indian population is needed.

Conflict of Interest

I declare that we have no conflict of interest.

Acknowledgements

We acknowledge Shoolini University, Solan, Himachal Pradesh, India, for providing us the necessary equipment and chemicals for completion of the study.

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