Prevalence of Toxin Genes among the Clinical Isolates of *Staphylococcus aureus* and its Clinical Impact

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ABSTRACT

Introduction: *Staphylococcus aureus* (*S. aureus*) causes a variety of infections, ranging from a mild skin infection to blood stream infections and deep seated infections. As *Stapylococcus aureus* bacteremia (SAB) has the tendency to cause endovascular and metastatic infections, complications can occur at almost all sites of the body. Hence, SAB is associated with increased morbidity and mortality in spite of appropriate antimicrobial treatment. The virulence in *S. aureus* is determined by the presence of adhesins and toxins, which behave like superantigens (SAgs) and leads to a massive release of proinflammatory cytokines causing overwhelming inflammatory response leading to endothelial leakage, hemodynamic shock, multiorgan failure, and possibly death. **Materials and Methods:** One year prospective study conducted in a tertiary care hospital in southern part of India included all patients with SAB. Clinical details were filled according to. All isolates were subjected to polymerase chain reaction (PCR) for enterotoxin profiling. **Results:** A total of 101 patients of SAB were identified which comprises of 61 (60.4%) patients with methicillin-susceptible *S. aureus* (MSSA) and 40 (39.6%) patients with methicillin-resistant *S. aureus* (MRSA). Most common predictors of mortality were prior hospitalization and antibiotic intake, severe organ dysfunction, shock, tachycardia, and leukocytosis. Two-third of the isolates had at least one enterotoxin, most prevalent was *sea*; 28% and 27% (*P* - value = 0.001) MSSA isolates had *seg* and *sei*; whereas, 38.6% (*P* - value < 0.001) of MRSA isolates were found to have *sea*. The most common enterotoxin associated with mortality was *sei*, which comprised of 38% of all mortality. **Conclusion:** In SAB, the significant predictors of mortality were prior hospitalization and antibiotic intake, presence of multiorgan dysfunction, and shock. Although overall significance between the enterotoxin and shock could not be demonstrated, it successfully demonstrated the difference of enterotoxin between MSSA and MRSA.

Key words: Bacteremia, *Staphylococcus aureus,* Toxin profile

INTRODUCTION

Staphylococcus aureus (*S. aureus*) causes a wide range of bacterial infection from minor skin infections such as pimples and boils to deep seated abscesses and life-threatening conditions such as bacteremia, endocarditis, and osteomyelitis. Colonization of the skin and anterior nares is common in up to one-third of the healthy population predisposing to significant infections.[1] *S. aureus* is a significant cause of blood stream infections and *S. aureus* bacteremia (SAB) has the tendency to cause endovascular and metastatic infections which can result in severe complications. In spite of appropriate treatment, these complications are associated with high morbidity and mortality and pose a significant financial burden.[2] SENTRY Antimicrobial Surveillance Program during the period 1997-2002, found that *S. aureus* was the most common cause of nosocomial bacteremia in North America with a prevalence of 26% and the second most common cause of nosocomial bacteremia in Europe.[3] Although gram-negative bacteria accounts for the most common cause of bacteremia in India, *S. aureus* tops the list among gram-positives.[4,5] Since the first outbreaks of methicillin-resistant *S. aureus* (MRSA) in the 1960s, their prevalence has steadily increased in healthcare and community settings and is still prevalent worldwide owing to the resistance to several classes of antibiotics.[6] Various

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studies have reported the prevalence of MRSA in India to be around 34-38% among *S. aureus* isolates from blood.[7]

Community-acquired MRSA (CA-MRSA) commonly presents as skin and soft tissue infections and necrotizing pneumonias; and compared to hospital-acquired (HA-MRSA) they are more susceptible to non-beta-lactam antibiotics like quinolones and trimethoprim-sulfamethoxazole. The *S. aureus* strain producing Panton–Valentine leukocidin (PVL) gene described in 1932 is known to cause necrotizing pneumonias among the young and the immunocompetent individuals and the risk factors varies from overcrowding to colonization.[8] One of the major risk factors for MRSA infection is the colonization with MRSA and a previous study has reported identical nasal isolates in 82% of patients with MRSA bacteremia.[9] The other common risk factors for a HA-MRSA infection included prior hospitalization, antibiotic use, presence of prosthetic devices, and surgical wounds. Comorbidities like diabetes mellitus, malignancy, and nasal colonization; presence of prosthetic device; injection drug use; etc.; are the major risk factors for CA SAB.[10]

S. aureus produces several virulence factors such as teichoic acids, microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), and capsular polysaccharides which enable adhesion to host mucosal surfaces or implanted devices and secreted virulence factors such as hemolysins, leukocidins, enterotoxins, and exfoliative toxins that aid in invasion and spread of infection.[11] The toxins, such as TSST-1 and enterotoxins, are superantigens (SAgs) which trigger a massive release of proinflammatory cytokines producing an overwhelming inflammatory response that results in endotoxin-like shock which includes endothelial leakage, hemodynamic shock, multiorgan failure, and possibly death.[12] There are about 21 known SAgs currently including the TSST-1, enterotoxins (SEA-E and SEI), enterotoxin like toxin (SelG, H, and J-U)[11,13] and at least 15 exotoxins (SET1–SET15).[14] Since very few studies have been reported on the prevalence of superantigenic enterotoxins among the isolates of *S. aureus* causing bacteremia, this study was conducted to determine the prevalence of enterotoxins among the blood culture isolates and its association with clinical severity and outcome.

MATERIALS AND METHODS

This prospective study was conducted over a period of 1 year from May 2011 to April 2012 at Christian Medical College and Hospital, Vellore, situated in south India. All patients above 12 years of age whose blood culture grew *S. aureus* were enrolled in the study. Patients' demographic data, duration of symptoms, co-morbid illnesses, risk factors for bacteremic illness, organ dysfunction, complications, length of hospital stay, and hospital outcome were collected in preconstructed data abstraction forms. Their treatment details and course in hospital were noted and they were followed-up till discharge from the hospital or death. The blood stream infection was classified as primary when there was no identifiable cause for bacteremia. Catheter-related bacteremia refers to bacteremia secondary to intravenous catheter including the hemodialysis catheter and was not related to any other focus of infection. Secondary blood stream infection was defined as infection due to a secondary focus anywhere in the body except the vascular site. The infection was defined as severe if there was involvement of two or more organ systems.

S. aureus was identified by using the conventional methods, such as Gram's stain and catalase activity. Antimicrobial susceptibility was determined by disc diffusion methods on Müller-Hinton agar according to the Clinical and Laboratory Standards Institute (CLSI) guidelines 2012.

The presence of toxin genes was detected using polymerase chain reaction (PCR). The blood culture isolates of *S. aureus* were subcultured for deoxyribonucleic acid (DNA) extraction. The following steps were carried out for DNA extraction: [14]

- The overnight growth of an isolate from one blood agar plate was suspended in 300 μl of 0.85% NaCl.
- Heated to 70°C for 15 min.
- Centrifuged for 2 min (microfuge) and the supernatant removed.
- Resuspended in 50 μl TE buffer, 10 μl mutanolysin (Sigma # M9901 is 10,000 U, dilute in 3.3 ml of TE buffer to make 3,000 units/ml stock solution, store at −20°C 500 μl aliquots) and 8 μl of hyaluronidase (Sigma # H3506 is 100mg, dilute in 3.3 ml to make 30 mg/ml solution, store at −20°C as 500 μl aliquots) was added.
- Kept in water bath at 37°C for 30 min.
- Heat inactivated at 100°C for 10 min.
- Centrifuged for 4 min in a microfuge and 2.5 μl of supernatant was used.

The extracted Genomic DNA was used as a template for amplification with the primers described in Table 1. Sequences specific for *sea, seb, sec, sed, see, seh, sei, seg*, and *tst 1* encoding SEA, SEB, SEC, SED, SEE, SEH, SEI, SEG, and TSST-1, respectively, were detected by PCR. The thermal conditions were as follows: Pre-denaturation was 95°C for 5 min, denaturation for 1 min at 94°C, annealing for 1 min

Table 1: Staphylococcal toxin-specific oligonucleotide primers and amplicon sizes

Gene	GenBank accession no.	Primer	Oligonucleotide sequence (5′-3′)	Size of the amplified product (bp)	References
Sea		SEA-1	TTGGAAACGGTTAAAACGAA	120	[14]
		SEA-2	GAACCTTCCCATCAAAAACA		
Seb		SEB-1	TCGCATCAAACTGACAAACG	478	[14]
		SEB-2	GCAGGTACTCTATAAGTGCC		
Sec		SEC-1	GCATAAAAGCTAGGAATTT	257	[14]
		SEC-2	AAATCGGATTAACATTATCC		
Sed		SED-1	CTAGTTTGGTAATATCTCCT	315	[14]
		SED-2	TAATGCTATATCTTATAGGG		
See		SEE-1	CAAAGAAATGCTTTAAGCAATCTTAGGCCAC	482	[14]
		SEE-2	CTTACCGCCAAAGCTG		
Seg		SEG-1	AATTATGTGAATGCTCAACCCGATC	642	[14]
		SEG-2	AAACTTATATGGAACAAAAGGTACTAGTTC		
Seh		SEH-1	CAATCATCATATGCGAAAGCAG	375	[14]
		SHE-2	CATCTACCCAAACATTAGCACC		
Sei		SEI-1	CTCAAGGTGATATTGGTGTAGG	576	[14]
		SEI-2	AAAAAACTTACAGGCAGTCCATCTC		
Tst		TSST-1	ATGGCAGCATCAGCTTGATA	350	[14]
		TSST-2	TTTCCAATAACCACCCGTTT		

at 55°C, and extension for 1 min at 72°C. PCR products were analyzed by electrophoresis using 2% agarose gel at 100 V for 1.5 h.[15] [Figure 1].

RESULTS

A total of 101 patients with SAB were enrolled in the study during the 1-year study period comprising of 61 (60.4%) patients with methicillin-susceptible *S. aureus* (MSSA) and 40 (39.6%) patients with MRSA. The mean age of patients is 46.7 years and the male:female (M:F) ratio is 2.9:1. Forty-one (40.6%) patients had diabetes mellitus, 21 (20.8%) were hypertensive, and 13 (12.8%) patients had associated chronic kidney disease and rheumatic valvular heart Disease was present in three (2.9%) patients; and 15 (14.8%) patients had other comorbidity, which included malignancy and chronic pulmonary conditions like chronic obstructive airway disease. Twenty-four (23.7%) had more than one associated comorbidity. Primary bacteremia was found in 57 (56.4%) patients; whereas, 44 (43.6%) patients had secondary bacteremia source of which included skin and soft tissue infections like ulcer, abscess, erythroderma, and surgical wound and pneumonia and bone involvement.

The length of hospital stay prior to bacteremia was found to be an average of 5.01 days. Thirty-six (35.6%) patients had prior hospitalization and 22 (21.8%) of them received prior antimicrobial therapy. The overall hospital mortality occurred in 21 patients (20.7%). Among all the patients who died, 11 (52.4%) of them were treated in the intensive care unit (ICU) compared to four (5%) whose outcome was favorable. Complications such as shock, tachycardia, and leukocytosis were seen in 13 (61.9%), 18 (85.7%), and 18 (85.7%) patients, respectively. Metastatic infections were seen among a third (28.6%) of the patients who died and two-thirds (85.7%) had multiorgan dysfunction [Table 2].

The predictors of mortality were explored using logistic regression analysis [Table 2]. The most common predictors of mortality were prior hospitalization and prior intake of antibiotic, severe organ dysfunction, shock, tachycardia, and leukocytosis.

Presence of at least one enterotoxin was found among one-third of the total isolates and 27 (36%) isolates presented with more than one enterotoxin.

Among all the isolates of SAB; *sea*, the most prevalent enterotoxin, was found in 39 (38.6%) isolates followed by *seg* and *sei*, which were found in 28 (27.8%) and 27 (26.8%) isolates respectively. Among patients who died, *sei* gene was found in eight (38.1%) isolates. The other toxins

Table 2: Baseline characteristics of patients with the outcome

Patients' characteristics	Death (*n* = 21)	Alive (*n* = 80)	*P* - value	Odds ratio (95% CI)
Male	16 (76.2)	59 (73.8)	1.000	—
Female	5 (23.8)	21 (26.2)	1.000	—
MRSA	8 (38.1)	32 (40.0)	1.000	—
MSSA	13 (61.9)	48 (60.0)	1.000	—
LHPB (mean)	2.43 days	5.69 days		—
PH	15 (71.4)	21 (26.2)	<0.001	7.1 (2.4–20.5)
PA	11 (52.4)	11 (13.8)	<0.001	6.9 (2.4–20.1)
Comorbidity				
DM	8 (38.1)	33 (41.2)	0.810	—
HTN	8 (38.1)	13 (16.2)	0.037	—
RHD	2 (9.5)	1 (1.2)	0.109	—
CKD	1 (4.8)	12 (15)	0.292	—
Others[a]	1 (4.8)	14 (17.5)	0.185	—
No risk factors	6 (28.6)	30 (37.5)	0.610	—
Single risk factor	9 (42.9)	32 (40)	0.808	—
2 or more risk factors	6 (28.6)	18 (22.5)	0.572	—
Primary source of infection	13 (61.9)	44 (55)	0.628	—
ICU admission	11 (52.4)	4 (5.0)	<0.001	20.9 (5.5-78.3)
Leukocytosis	18 (85.7)	44 (55)	0.012	5.0 (1.3-17.9)
Metastatic infection	6 (28.6)	6 (7.5)	0.016	4.9 (1.4-17.4)
More than 2 organ dysfunction	18 (85.7)	7 (8.8)	<0.001	0.02 (0.0-0.07)
Shock	13 (61.9)	4 (5.0)	<0.001	2.3 (1.4-19.6)
Tachycardia	18 (85.7)	33 (41.2)	<0.001	8.5 (2.3-31.4)

LHPB: Length of hospitalization prior to bacteremia, PH: Prior hospitalizations, PA: Prior antimicrobials, DM: Diabetes mellitus type 2, HTN: Hypertension, RHD: Rheumatic heart disease, CKD: Chronic kidney disease, MRSA: Methicillin-resistant *Staphylococcus aureus*, MSSA: Methicillin-susceptible *Staphylococcus aureus*, ICU: Intensive care unit, CI: Confidence interval, [a]Others like malignancy and chronic obstructive airway disease

found in the isolates of those patients who died were *sea* and *seg* genes, which were present in seven (33.3%) isolate [Figure 2].

On comparison of the toxins among the MRSA and MSSA isolates, most prevalent enterotoxin among the MRSA isolates was found to be *sea* (*P* - value < 0.001) and among the MSSA isolates *seg* (*P* - value = 0.001) and *sei* (*P* - value < 0.001) predominated [Figure 3].

DISCUSSION

The blood stream infections caused by *S. aureus* is steadily increasing and is an important cause of morbidity and mortality, and it clearly represents a significant burden on the healthcare systems. In this study, we tried to determine the prevalence of different enterotoxins among the isolates of *S. aureus* obtained from the blood cultures of patients admitted in our hospital. Of the 101 patients we studied, 61 patients grew MSSA in their blood culture and 40 patients were found to have MRSA. The most prevalent toxin genes among all the isolates of SAB, were the *sea* gene (39, 38%) closely followed by *seg* (28, 27.7%) and *sei* (27, 26.7%). In contrast to our study, Nhan *et al*., demonstrated *sec* (15.1%) and *seb* (10.2%) genes to be the most prevalent toxin genes in their study.[16] In our study, the significant predictors of mortality were previous hospitalization, prior antibiotic intake, more than two organ dysfunction, and presence of shock. Other factors leading to mortality were tachycardia, leukocytosis, admission into ICU, and metastatic infections. Previously studies have reported older

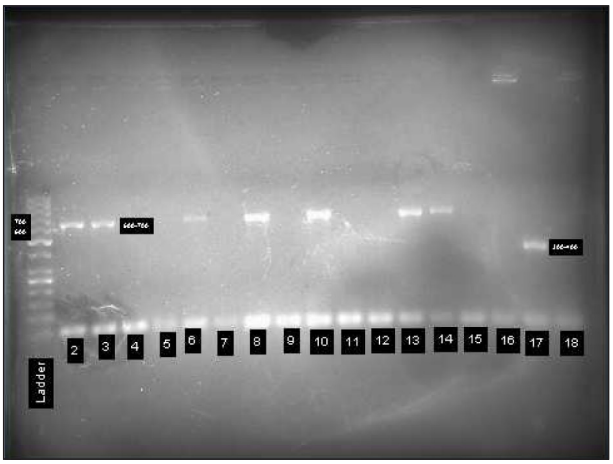

Figure 1: Picture of the PCR product on electrophoresis using 2% agarose gel. PCR: Polymerase chain reaction

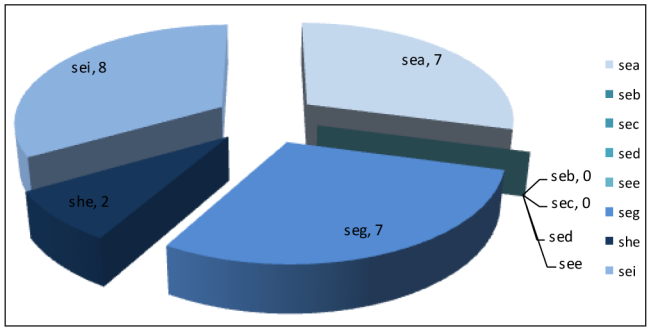

Figure 2: Enterotoxins' profile among patients who died

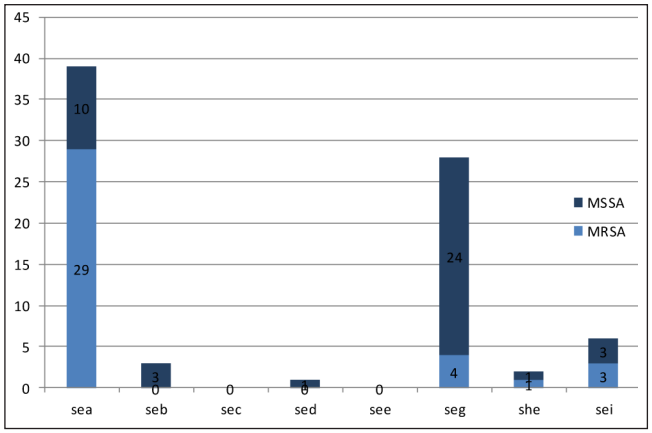

Figure 3: Comparison of enterotoxin profile between MRSA and MSSA. MRSA: Methicillin-resistant *Staphylococcus aureus*, MSSA: Methicillin-susceptible *Staphylococcus aureus*

age, source of bacteremia, presence of shock, severity of illness, SAB caused by MRSA, and inadequate treatment as risk factors for mortality.[17,18]

Different virulence factors have been associated with severity of infections but their implications in causing septic shock are not well studied. In spite of being deficient in lipopolysaccharide (LPS), which is responsible for causing septic shock in gram-negative bacteria, *S. aureus* can produce SAgs which can directly trigger an inflammatory response through the adaptive immune system.[19] Animal models have well-demonstrated the superantigenic property of *S. aureus*, but human studies are lacking. Two-thirds of the isolates in the present study have at least one of the SAgs which is similar to reports by Becker *et al*., and Ferry *et al*.[20,21]

Among the 40 patients with MRSA bacteremia, seven patients had septic shock and 29 (72.5%) isolates showed the presence of *sea* genes. Overall prevalence of shock did not differ among patients with MSSA (58.8%) and MRSA (41.2%). The *sea* gene has been earlier reported to be significantly seen in invasive isolates.[22] A similar study in French ICUs observed that *sea* producing *S. aureus* isolates had an increased predilection of producing septic shock.[23] Among the MSSA isolates, *seg* (85.7%) and *sei* (88.9%) genes were the most prevalent toxin and 10 (58.8%) patients presented with shock. Genes like *seg* and *sei* have been studied as part of *egc* locus is seen more among the MSSA isolates. The presence of these genes makes the isolate less immunogenic which produces an early and a mild TH2 response which counteracts the TH1 response limiting the excessive release of TH1 cytokines that leads to shock.[24]

In conclusion, our study on patients of SAB found that the significant predictors of mortality among patients of SAB were prior hospitalization, prior intake of antibiotics, multiorgan dysfunction, and presence of shock. The enterotoxin profile in our study showed *sea genes* to be the most prevalent enterotoxin among the clinical isolates of MRSA and *sei* and *seg* genes among the MSSA isolates. Although overall significance of these toxins in patients who presented with septic shock could not be demonstrated, it was successful in demonstrating the difference in the toxin profile among the MSSA and the MRSA isolates. Our study was limited by the smaller number of isolates with the presence of toxins' genes and being a tertiary level referral hospital our patients have exposure to antibiotics prior to hospitalization, which may alter the enterotoxin profile of the bacteria.

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