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Characteristics of Clinically Significant Invasive *Staphylococcus aureus* Infections in a Tertiary Care Centre

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Abstract

The purpose of this study was to evaluate the antibiotic susceptibility of clinically significant *Staphylococcus aureus* and its association with biofilm production. The antibiotic resistance pattern and biofilm production by *S. aureus* isolated from invasive sites such as deep tissue and bone, deep seated pus, blood and other sterile body fluids were studied. The prevalence of multidrug resistant strains and the associated risk factors and co-morbidities were noted. Samples were subjected to antibiotic susceptibility testing using modified Kirby-Bauer disc diffusion method and biofilm production was detected by using microtiter plate assay. Of the total 80 clinically significant invasive *S. aureus* strains, resistance to penicillin was observed in 70(88.6%) isolates and 38 (47.5%) isolates were resistant to cephalothin. Resistance to erythromycin was observed in 42(52.5%) isolates and 14(17.5%) isolates were resistant to clindamycin. Resistance to ciprofloxacin was 79.5%(n=63). Resistance to rifampicin was observed in 1 isolate (2.1%) and 1 isolate (1.3%) was resistant to teicoplanin. All the isolates were sensitive to vancomycin, tigecycline and linezolid. Out of the 80 *S. aureus* strains, 21 (26.3%) strains were biofilm producers and 59(73.75%) strains were non-biofilm producers. Among the biofilm producers, resistance to penicillin (57.14%), cephalothin (57.1%) and ciprofloxacin (57.14%) was higher compared to non-biofilm producers. (penicillin 45.7%, cephalothin 35.6% and ciprofloxacin 38.9%,). The rate of MRSA isolated from invasive infections was high (41.25%). We conclude that MRSA and biofilm-producing strains exhibit higher resistance to antibiotics and hence beta-lactams may not be a good empirical antibiotic of choice, especially in biofilm producers. Clindamycin may be an effective alternative substitute to vancomycin forin MSSA and MRSA treatment. Since the patients improved after appropriate antibiotic treatment, we support the role of an early start of appropriate and adequate antibiotic therapy for better patient outcome. We conclude that *S. aureus* strains exhibited a high resistance to penicillin, β-lactam, macrolide and fluoroquinolones. The rate of MRSA was found to be 41.25%. MRSA and biofilm producing strains exhibit higher resistance to antibiotics. The high prevalence of MDRSA was high (53.75%), which could potentially pose beas a threat to public health, antibiotic use and patient outcome.

Keywords: Invasive, *Staphylococcus aureus*, MRSA, Antibiogram, Biofilm production, Resistance

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INTRODUCTION

Staphylococcus aureus is a commensal as well as an opportunistic pathogen responsible for infections that range from mild skin and soft tissue infection to severe sepsis, toxic shock syndrome and pneumonia[1]. It is one of the most frequently isolated pathogens in major causes of nosocomial infections (E.g: surgical site infections, pneumonia) and community-acquired infections (skin and soft tissue infections, blood stream infections)[2].

Invasive *Staphylococcus aureus* are pathogenic isolates from sites such as pleural fluid, synovial fluid, cerebrospinal fluid, deep-seated abscesses or bone and blood[3].

Methicillin-Resistant *Staphylococcus aureus* (MRSA) is a major pathogen in nosocomial infections, causing serious problems in hospitals worldwide[4]. MRSA is of significance due to its resistance to β-lactam antibiotics and its association with multidrug resistance in *Staphylococcus aureus*[5].

MRSA is classified into two types, Community-Acquired MRSA(CA-MRSA) and Healthcare-Associated MRSA (HA-MRSA). CA-MRSA can be defined as the MRSA infection in a person who has not yet recently been hospitalised or has not undergone a medical procedure. HA-MRSA can be defined as the MRSA infection in which the *Staphylococcus aureus* strain is resistant to several antibiotics and was recovered from patients who frequently visit healthcare facilities[6].

Biofilm is defined as clusters of bacteria, lodged in an extracellular polysaccharide matrix[7,9]. Biofilm formation can lead to an uncompromising infection and poor outcome[7]. The clinical importance of biofilm infections induced by biofilm producing pathogens relies on in the fact that bacteria in biofilms aid the resistance the action of pathogens to antimicrobial compounds by forming a protective sheath around them and persist despite sustained host defence and hence (Ref) infections caused by biofilm-producing microorganisms express prolonged hard-to- treat infections[7]. Biofilm formation facilitates increases the mutation rates and thus helps in the spread of antibiotic resistance[8]. (Ref)Biofilm infections are initially mild but later may act as reservoirs of infection through sloughing[8]. Such biofilm producing bacteria may be transported to other sterile body sites of the carrier or transmitted to other patients with inbuilt medical devices or immunocompromised state. This would complicate the treatment options, especially in resource limited settings where assays for the biofilm detection are unavailable. Besides, standard *in vitro* antibiotic susceptibility tests may not be predictive of the therapeutic outcome of biofilm associated infections[8].

Staphylococcus aureus and *Staphylococcus epidermidis* are common biofilm producers. Biofilm formation helps the attachment of microorganisms to biomaterial and protects them from host immune response[9]. Biofilm producing strains tend to exhibit resistance to antibiotics, disinfectant and germicides[10]. Hence it is considered to be a virulence factor.

Hence the ability of a strain to produce biofilm needs to be detected in order to give effective treatment due to increasing trends of resistance to antibiotics that is observed in *S. aureus* strains.

MATERIALS AND METHODS

A total of 80 clinically significant *Staphylococcus aureus* samples were collected from Kasturba Medical College Hospital (Ambedkar Circle and Attavara), a tertiary care center situated in Mangalore, Karnataka, over a period of 6 months from November 2018-April 2019 from invasive sites such as deep tissue, bone, deep seated pus, blood and other sterile body fluids of patients from the hospitals

Collection and processing of specimen

The received clinical samples were inoculated on 5% sheep blood agar, chocolate agar and MacConkey's agar and incubated for 24 hrs at 37°C. *Staphylococcus aureus* strains were identified using standard biochemical tests.

Antibiotic susceptibility test for all clinically significant *Staphylococcus aureus* isolates was performed by modified Kirby-Bauer disc diffusion method on Mueller Hinton Agar (MHA).Antibiotics that was used are penicillin(1µg), cephalothin(30µg), cotrimoxazole (25µg), erythromycin (15µg), clindamycin (2µg), ciprofloxacin (5µg), tetracycline (30µg), linezolid (30µg), vancomycin(30µg), teicoplanin (30µg), tigecycline(15µg), rifampicin (5µg) and gentamicin (10µg)[5]. The results were interpreted according to the Clinical Laboratory Standards Institute (CLSI)

guidelines[11]. *Staphylococcus aureus* ATCC 25923 was the control strain used[5].

Detection of Methicillin resistance

The *S. aureus* strains were screened for resistance to methicillin by using a cefoxitin(30µg) disk on Mueller–Hinton agar plates. The zone of inhibition was determined after 16–18 h incubation at 37°C. Zone size was interpreted as per CLSI criteria: susceptible ≥22 mm; resistant ≤21 mm[11].

Detection of Vancomycin susceptibility using E-Strips

All the MRSA strains were tested for Vancomycin susceptibility using Vancomycin E-test® strips (BioMerieux) and interpreted according to CLSI guidelines[11].

Detection of Inducible clindamycin resistance

D- test was conducted to test the resistance to erythromycin and clindamycin according to CLSI guidelines[11].

Detection of biofilm production

Biofilm assay was done by using microtiter wells[10].

The bacterial colony was inoculated into Brain-Heart Infusion (BHI) broth and incubated at 37°C 24 hours. The cultures were further diluted using fresh medium and adjusted to 0.5 MacFarlands Standard. 200µl of these diluted suspensions were transferred into 96-well polystyrene microtiter plate in triplicate and incubated at 35°C for 24 hours[10]. Sterile broth was used as blank. The plates were then tapped gently. The contents of the wells were emptied. 200µl of phosphate buffer saline (PBS) was used to wash the wells. Wells were washed 3-4 times to remove free floating bacteria. 100µl of 2% Sodium acetate was added as a fixative. 0.1% crystal violet was used to stain the wells. Deionised water was used to wash off the excess stain. 33% of Glacial Acetic Acid was added to individual wells and optical density (OD)value of the stained adherent biofilm was read using a micro ELISA autoreader at a wavelength of 570nm.An OD value of more than 0.240 was considered as strong biofilm producers, 0.120-0.240 as moderate biofilm producers and less than 0.120 as non-biofilm producers[10].

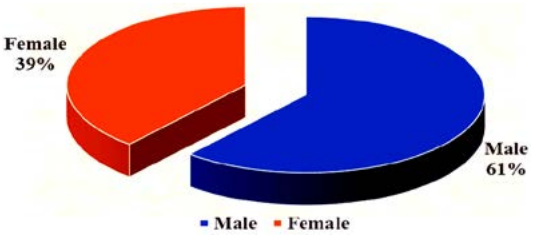

Fig. 1. Gender distribution

Data collection

Data, including age, sex, duration of hospital stay, history of chronic disease, history of instrumentation or device implantation(the risk factors and co-morbidities) collected from the medical records department were assessed. Bacteriological culture from other sites will be noted to trace the probable source of sepsis. The treatment, outcome in the patients were followed up.

Statistical analysis

p-value was calculated by ANOVA using SPSS v.25 statistical analysis software (IBM Corporation New York, USA). Biofilm formation, drug resistance was compared by Pearson's chi-square test.

RESULTS

Out of the total 80, clinically significant invasive *Staphylococcus aureus* isolates majority were skin and soft tissue infections (n=64,80%) followed by bloodstream infections (n=12,15%) and bone infections (n=4, 5%). The study group belonged to the age < 1yr up to 80 yrs. The mean age was 46.8 and as shown in Fig. 1, 31(38.8%) were females and 49(61.3%) were males.

Table 1. Antibiotic resistance pattern in *Staphylococcus aureus*

Antibiotics tested	Number of resistant isolates (%)
Penicillin	70(88.6%)
Cephalothin	38 (47.5%)
Co-trimoxazole	12(15%)
Erythromycin	42(52.5%)
Clindamycin	14(17.5%)
Cefoxitin	33(41.2%)
Ciprofloxacin	63(79.5%)
Tetracycline	4(5.7%)
Linezolid	0(0)
Vancomycin	0
Teicoplanin	1(1.3%)
Tigecycline	0
Rifampicin	1(2.1%)
Gentamicin	18(23.4%)

Table 2. Distribution of MLS_B phenotypes in MRSA and MSSA

Oganisms	HA-MRSA	CA-MRSA	MSSA	Total
$iMLS_B$	0(0%)	2(7.14%)	5(10.63)	7(8.8%)
$cMLS_B$	0(0%)	3(10.7%)	3(6.38)	6(7.5%)
MS_B	4(80%)	14(50%)	17(36.17%)	35(43.8%)
CL	0	0	0	0
NR	1(20%)	9(32.1%)	22(46.8%)	32(40%)
TOTAL	5(100%)	28(100%)	47(100%)	80(100%)

Table 3. Risk factors and Co-morbidities observed in patients with *Staphylococcus aureus* invasive infections

Risk factors	Number	Percentage (%)
Urinary Tract Infection	13	16.3
Skin and Soft Tissue Infection (SST)	10	12.5
Respiratory Tract Infection	10	12.5
Bone Infection	19	23.8
CNS	7	8.8
Cardiac	6	7.5
GIT	7	8.8
Dialysis	2	2.5
Diabetes mellitus	38	47.5
Hypertension	29	36.3
Immunocompromised	11	13.7
Priorsurgery/ Hospitalisation	43	53.8
Neutropenia	2	2.5
Hypalbuminaemia	7	8.8
Blood transfusion	7	8.8

The resistance pattern of 80 *Staphylococcus aureus* isolates is shown in table 1. All the isolates were sensitive to vancomycin, tigecycline and linezolid. Vancomycin MIC was noted and found to be in the range from 0.5 to 2µg/ml. Of the 33 MRSA isolates,39.4%(n=13) exhibited vancomycin MIC of 0.5µg/ml, 33.3% (n=11) exhibited a MIC of 1µg/ml,12.1%(n=4) exhibited a MIC of 1.5 µg/ml and 15.2%(n=5) MIC of 2 µg/ml.

Antibiotic resistance observed in MRSA was higher compared to MSSA and the distribution is as shown in Fig. 2.

Methicillin resistance (MRSA) was observed in 33 (41.3%) isolates and 47(58.8%) isolates were MSSA. Of the 33 MRSA isolates, 5(15.2%) were HA-MRSA infections and 28(84.8%) were CA-MRSA infections.

The distribution of MLS_B phenotypes in MRSA and MSSA is shown in Table 2.

Twenty-one (26.3%) out of the 80 *S. aureus* strains were biofilm producers and 59(73.75%) strains were non-biofilm producers. Of the 21 biofilm producers, 19(23.8%) strains produced moderate biofilm and 2(2.5%) strains produced strong biofilm. The two strains that exhibited strong biofilm production were isolated from samples that belonged to the extremities of age i.e <1 and 80 years old.

Twelve out of the 21 biofilm producing strains were MRSA and 9 were MSSA. The distribution of biofilm in MRSA and MSSA is as shown in Fig. 2. The p-value is 0.222 and is not significant.

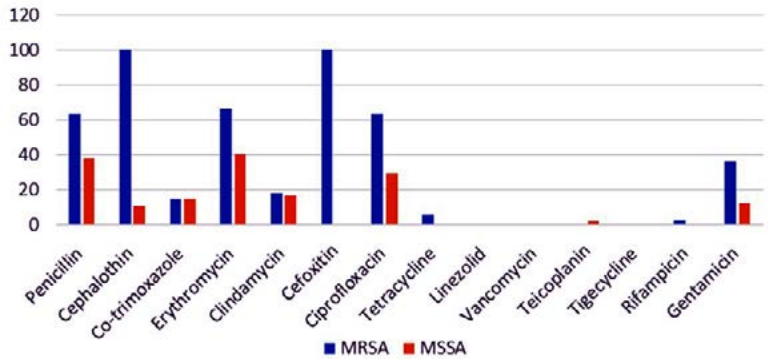

Fig. 2. Antibiotic resistance pattern

Table 4. Empirical antibiotics used in the various infections studied

Antibiotics given		Infections			
		Skin & soft tissue	Blood	Bone	Total
Amoxicillin-clavulanic acid	Count	14	3	0	17
	%	25.9%	30.0%	0.0%	25.4%
Clindamycin	Count	19	4	1	24
	%	35.2%	40.0%	33.3%	35.8%
Vancomycin	Count	5	1	0	6
	%	9.3%	10.0%	0.0%	9.0%
Linezolid	Count	7	0	2	9
	%	13.0%	0.0%	66.7%	13.4%
Cefuroxime	Count	3	0	0	3
	%	5.6%	0.0%	0.0%	4.5%
Ciprofloxacin	Count	2	0	0	2
	%	3.7%	0.0%	0.0%	3.0%
Others	Count	4	2	0	6
	%	7.4%	20.0%	0.0%	9.0%
Total	Count	54	10	3	67
	%	100.0%	100.0%	100.0%	100.0%

The association of MRSA with biofilm production and antibiotic resistance pattern in biofilm and non-biofilm producers are as shown in the Fig. 3 and 4 respectively. Among the 21 biofilm producers, 12(57.1%) isolates, were resistant to penicillin and 12(57.1%) were resistant to cephalothin . Among the 59 non-biofilm producers, 27 (45.7%) isolates were resistant to penicillin and 21 (35.6%) were resistant to cephalothin. Thus higher percentages of beta lactam resistance were observed in biofilm producers.

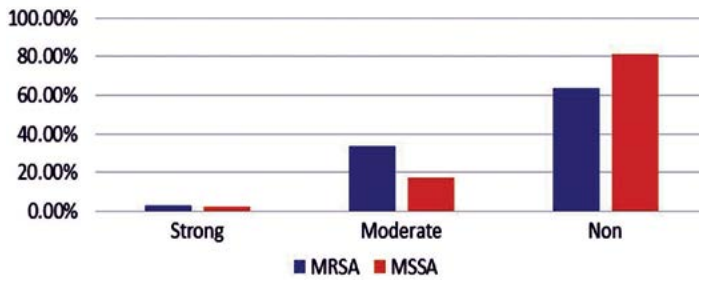

Fig. 3. Biofilm distribution in MRSA and MSSA

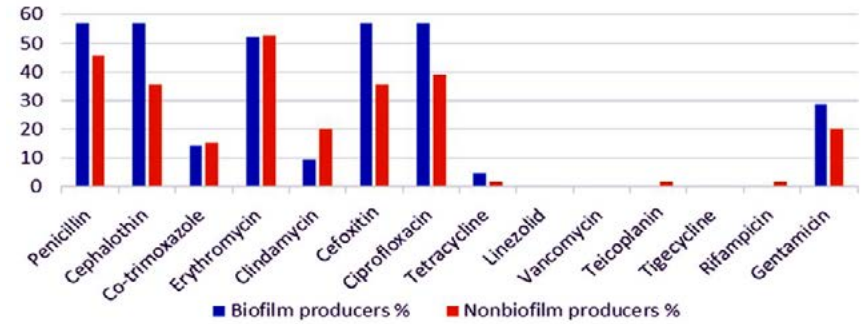

Fig. 4. Antibiotic resistance in Biofilm and Non-biofilm producers

In this study, 43 isolates (53.75%) were Multidrug-Resistant *Staphylococcus aureus* (MDRSA). Extensively Drug Resistant *Staphylococcus aureus* (XDRSA) was not found in our study.

The predominant co-morbidities observed are shown in Table 3. The list of antibiotics used in the empirical therapy are shown in Table 4.

Out of the total 80 patients with *Staphylococcus aureus* invasive infections, 73(91.3%) patients improved with antibiotic treatment. There was no significant improvement in 5(6.3%) patients. Mortality was observed in 2(2.5%) of the patients.

DISCUSSION

The obtained results revealed high rates of resistance to penicillin (88.6%), β-lactam (53.2%), macrolide (52.5%) and fluoroquinolone (79.5%). A multicentre study in 2016, by Mendem *et al.,* showed higher resistance to penicillin (97.64%), macrolides (56%), β-lactams (61%) and fluoroquinolones (50%)[12].

S.aureus strains were susceptible to linezolid and vancomycin. This is in par with a study by Belbase *et al.*, published in 2017, in which 100% susceptibility was observed towards vancomycin and linezolid thus making it the antibiotic of choice in MRSA infections[13].

Results revealed a low rate of resistance to clindamycin (17.5%) in contrast to the study by Al-Zoubi *et al.*, where the isolates showed a clindamycin resistance rate of 81.8%[14]. Only 1 isolate (2.1%) exhibited resistance to rifampicin which supports the results obtained from a previous study from Kuwait Hospitals where an insignificant increase in rate of resistance to rifampicin from 0.1% to 1.6% , during the period of 2011-2015 was recorded[15].

Our study revealed higher percentages of antibiotic resistance in MRSA compared to MSSA. This is in par with a study by Saba *et al* in the year 2017, where MRSA was found to be more resistant in comparison to MSSA[16].

Among the 33 MRSA isolates, the majority (84.8%) were CA-MRSA and the rest as compared to (15.2%) were are HA-MRSA. A study by Huang *et al.,* demonstrated a higher measure of CA-MRSA (45%) among MRSA in their setup[17].

This study revealed 26.25% of the *Staphylococcus aureus* isolates as biofilm producers, classified into 12 MRSA and 9 MSSA. Biofilm production was observed to be higher in MRSA (p=0.222), Signifying no association between biofilm production and MRSA). Higher percentages of resistance to penicillin, beta lactams and ciprofloxacin were observed among the biofilm producers. This was similar to results observed in a study by Khan *et al.,* in the year 2011, wherein biofilm production was higher in MRSA and resistance to all groups of antibiotics were exhibited by biofilm producers[18].

The high prevalence of MDRSA (53.75%) observed in our study is in par, which supports the results obtained from a study in 2015 by Bhattacharya *et al.,* where the prevalence of MDRSA was high (57%)[5].

Regarding the current study,The common empirical antibiotics used in these 80 patients were, clindamycin (35.8%,n=24), amoxicillin-clavulanic acid (25.4%,n=17), linezolid (13.4%, n=9) and vancomycin (9.0%,n=6). We observed that β-lactams alone were not used as empirical therapy in any of these patients, instead a combination of β-lactam inhibitor antibiotics like amoxicillin-clavulanic acid were used to treat in 25.9%(n=14) of the SSTI.

Clindamycin was used to treat in 35.2%(n=19) of the SST infections, 40%(n=4) of the bloodstream infections and 33.3%(n=1) of bone infections. Macrolides such as erythromycin are inactive against MRSA, due to development of resistance among the strains and hence not recommended for the treatment of SST infections. Clindamycin is a choice, is often administered as a first-line antibiotic to treat SST infections and for MSSA osteomyelitis[19]. Considering the low rate of clindamycin resistance observed in our study, clindamycin could be used as an effective empirical as well as therapeutic alternative to vancomycin, thus limiting vancomycin use, which would prevent the emergence of VISA and VRSA. Also, clindamycin is effective on MRSA as well as MSSA.

In our study, vancomycin was used to treat SST infections (9.3%) and bloodstream infections (10%). Vancomycin and other gylcopeptides are commonly used in the eradication of *S. aureus* infections. vancomycin has always been preferred for treatment of MRSA infections. A

study by Rayner *et al.,* indicates that vancomycin is effectively used to treat MRSA infection, but not MSSA infections[19].

Linezolid was used to treat bone infections (66.7%) and SST infections (13%). According to the study in 2012 by Watkins *et al.*, linezolid was evaluated and considered to be an alternate and effective treatment to MRSA infections due to the appearance of strains that exhibited reduced vancomycin susceptibility[20].

From the empirical therapy administered to 67 patients, the antibiotics were changed in 3 patients, in one patient, amoxicillin-clavulanic acid was changed to clindamycin as it was a bone related infection and for the other two patients, clindamycin was changed to linezolid for one as it was an MRSA isolate that was resistant to clindamycin and in the other, vancomycin was changed to clindamycin as it was MSSA infection and significant improvements were observed in the patients after administration .

Mortality observed in our study though low (2.5%, n=2), were secondary to Staphylococcal bacteremia who had not received empirical antibiotic therapy. This supports the role of antibiotic therapy in better patient outcome.

CONCLUSION

The rate of MRSA isolated from the invasive infections in our study was high (41.25%). Our study supports the view that MRSA exhibited higher resistance to antibiotics. Biofilm producers showed higher percentages of resistance to penicillin, cephalothin and ciprofloxacin compared to non-biofilm producers. Hence, we conclude that MRSA and biofilm producing strains exhibit higher resistance to antibiotics and hence beta-lactams may not be a good empirical antibiotic of choice especially in biofilm producers.

The prevalence of MDRSA in our study was high (53.75%), which could be a threat to the antibiotic use and patient outcome. Owing to the lower rate of resistance to clindamycin, we conclude that clindamycin may be an effective alternative substitute to vancomycin in MRSA treatment.

It was observed that patients improved after appropriate antibiotic treatment. The mortality, though low (2.5%), was observed in those who had not received empirical antibiotic therapy. This supports the role of an early start of appropriate and adequate antibiotic therapy for better patient outcome.

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AUTHORS' CONTRIBUTION

GVP: collected the data, performed the experiment and has gathered the literature and drafted the manuscript. ABK has supervised the work, analyzed the data, reviewed the manuscript. SB has supervised the work, analyzed the data and reviewed the manuscript.

CONFLICT OF INTEREST

All authors declare that there is no conflict of interest.

FUNDING

None.

DATA AVAILABILITY

All data sets generated or analysed during the study are included in the manuscript and is saved as Excel sheets .

ETHICS STATEMENT

The above study has obtained ethical clearance from the Institutional ethical committee.

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