Characterization of Methicillin Resistant *Staphylococcus aureus* based on its virulence factors and antimicrobial susceptibility profile

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Abstract

Staphylococcus aureus remains as one of the most potent bacterial human pathogen because of its expression of various virulence factors and also due to its property of multidrug resistance. A total of 468 non –duplicate *S.aureus* strains obtained from various clinical specimens were included in the study. Methicillin Resistant *S.aureus* (MRSA) strains were screened for DNase production using DNase agar, hemolytic property of the isolates were detected in 5% sheep blood agar plates, hemagglutination property of the isolates were demonstrated using 1% O group RBC's,slime production was detected using congo red agar medium and biofilm production was quantitatively assayed by microtitre plate method. Antimicrobial susceptibility profile was studied for the MRSA isolates. Out of 468 *S.aureus* strains, 114(24%) strains were detected as MRSA. Among the MRSA strains 99(86%) were positive for DNase, 77(67%) were showed beta hemolysis, 49 (42%) strains were positive for hemagglutination, 70 (61%) were slime producers, most of the strains were biofilm producers, 4(3.5%) were non-adherent, 85(74%) were weakly adherent, 23(20%) were moderately adherent and 2(1.7%) were strongly adherent. About 56%, 60% and 63% of the isolates were resistant to cotrimoxazole, erythromycin and ciprofloxacin respectively and about 22% and 33% of the isolates showed resistance towards clindamycin and gentamicin respectively. All the isolates were sensitive to vancomycin, teicoplanin and linezolid. About 10(8.7%) strains showed high level mupirocin resistance (HLMR) and 1(0.8%) strain showed low level mupirocin resistance (LLMR). Both HLMR and LLMR (100%) strains showed susceptibility to fusidic acid. MRSA infections remain a major threat in both community and nosocomial settings. Therefore a thorough understanding of its virulence mechanisms and regular surveillance of antimicrobial susceptibility pattern will help the clinician to choose appropriate treatment options and to control the emergence of multidrug resistant strains.

Keywords: Methicillin resistant *Staphylococcus aureus*, Virulence factors, Antimicrobial resistance, Biofilm formation.

Introduction

Staphylococcus aureus is one of the leading cause of superficial to life-threatening disseminated infections. Its diverse pathogenic property is due to its various virulence determinants which help the bacteria to richly establish its colonization to invasion and tissue damage by easily evading the host defense mechanisms.(1) Virulence factors of *S.aureus* include the cell- wall associated or cell-surface factors and secreted factors or enzymes. The former includes the bacterial capsule, slime formation and latter includes the secreted enzymes and toxins like lipases, DNase, proteases, hemolysins and some super antigens like toxic shock syndrome toxin and enterotoxin.(2) Treatment options for *S.aureus* infections have been limited due to the emergence of multidrug resistant strains like MRSA. One of the reasons for the emergence of these multidrug resistant strains is due to the ability of the bacteria to form biofilm promoted by the microbial surface recognizing adhesive matrix molecules especially in indwelling medical devices and also in infections osteomyelitis, endocarditis, periodontitis, etc. Furthermore this serves as the major defense mechanism to evade the action of antibiotics.(3)

S.aureus also remains as one of the common colonizer of external nares. Under some circumstances these bacterial strains from external nares can be introduced into sterile body sites leading to serious infection. Hence these strains from the external nares need to be decolonized by some topical agents. Mupirocin remains as one of the common topical antibiotic to decolonize nasal MRSA colonizers and also plays an important role in hospital infection control practice. But repeated use of these antibiotics has resulted in the emergence of high and low level mupirocin resistance leaving limited options to eradicate these colonizers. Thus in such instances fusidic acid remains as yet another alternative agent to help in the decolonization of these colonizers.(4)

Materials and Methods

Collection of *S.aureus* strains: A total of 468 *S.aureus* strains were obtained from various clinical specimens like blood, exudates, urine and respiratory samples obtained from patients who attended various clinical departments of Chettinad hospital and Research Institute, Kelambakkam for some therapeutic process during a period of 2 years from September 2013 - November 2015. This work was carried out in Chettinad Hospital and Research Institute, Department of Microbiology, Kelambakkam. These strains were stocked in 20% glycerol broth and stored at -20^{0}C until used for further analysis.

Identification of *S.aureus* strains: *S.aureus* strains from various clinical specimens were identified by standard microbiological procedures. Beta hemolytic and creamy yellow pigmented bacterial colonies on blood agar were subjected to routine identification procedures which includes, Gram stain (Gram positive cocci in clusters), catalase test (positive), tube coagulase test (positive)and mannitol fermentation (mannitol fermenting and non-motile).[5,6]

Detection of MRSA: All the 468 *S.aureus* strains were screened for methicillin resistance using 6µg oxacillin screen agar. Briefly, 10µl of 0.5 McFarland bacterial suspension was spot inoculated in Muller Hinton agar containing 6µg oxacillin and 4% Nacl. The plates were incubated at 35^0C for 18-24 h and inspected for any growth after incubation. These isolates were also subjected to Kirby Bauer disc diffusion method using 30µg cefoxitin disc which remains as a surrogate marker to identify mecA mediated resistance. Lawn culture was made in MHA plates using 0.5 McFarland bacterial suspension and the plates were incubated at 35^0C overnight. After incubation, plates were observed for zone of inhibition. The strains were considered as MRSA if the zone of inhibition was ≤21mm and considered as methicillin susceptible (MSSA) if the zone of inhibition is ≥22mm.[7]

Detection of DNase: This test was carried out by using DNase agar (Himedia Laboratories Ltd.). The test strains were spot inoculated in DNase agar and incubated at 37^0C for 24 hours. The bacterial strains were considered positive for DNase if clearing zone around the spotted colony is observed after pouring 1N HCl.[8]

Detection of beta hemolysin: MRSA strains were spot inoculated onto 5% sheep blood agar and incubated at 37^0C overnight. After overnight incubation the plates were kept at 4^0C to observe hot –cold type of hemolysis produced by beta hemolysin.[9]

Hemagglutination: Hemagglutination test was carried out in 96 well round bottom microtitre plate. Bacterial colony was suspended in phosphate buffer saline (PBS) and turbidity was adjusted to 1.0 McFarland standard. Then, each bacterial strain suspension was subjected to two serial dilutions in such a way that the final volume reaches to 50µl. Then equal volume of 1% O group RBC's suspended in PBS was added. After proper shaking, the microtitre plates were incubated in room temperature for 2 hours and observed for hemagglutination.[10]

Slime formation: Slime formation was detected in MRSA strains using congo red agar (CRA) containing brain heart infusion broth, 5% sucrose, agar and 0.08% congo red. Strains were inoculated and plates were incubated at 37^0C overnight. MRSA strains that grew as dry black colonies with crystalline consistency were considered as slime producers and those that produced pink colonies were considered as negative for slime formation.[10]

Biofilm assay: Briefly, the strains were inoculated in BHIB and incubated overnight. Then 200µl of the bacterial suspension was transferred to 96 wells flat bottomed microtitre plate containing 200µl of BHIB containing 1% glucose and the test was carried out in duplicates. After 24 hours of incubation, the plates were removed and quantified for overall growth at OD_{550}. Then the plates were washed three times in distilled water using plate washer. The biofilm were fixed by incubating the washed plate at 60^0C for 1h. The biofilm were stained using 0.06% crystal violet for 5 minutes and washed thrice with distilled water using plate washer. Quantification of biofilm was done by eluting the bound crystal violet with 30% acetic acid and OD_{550} was measured.[11] The cut off OD was calculated as three standard deviations above the mean OD of the negative control. Strains were non-adherent if the $OD \leq OD_c$; strains were weakly adherent if the $OD_c < OD \leq 2x\ OD_c$; strains were moderately adherent if $2x\ OD_c < OD \leq 4x\ OD_c$ and strongly adherent if $4x\ OD_c < OD$.[12]

Detection of HLMR and LLMR: All the MRSA isolates were tested for high and low level mupirocin resistance by agar dilution method according to CLSI guidelines. *Staphylococcus aureus*- ATCC 25923 was used as control strain.

Results

Out of 468 non-duplicate *S.aureus* strains included in the study, 114(21%) of the strains were detected as MRSA. Among these MRSA isolates, 3 isolates (1.7%) were from urine, 3 isolates (2.6%) were from respiratory samples, 7(6.1%) were blood isolates and 101(89%) were from exudates samples.

Among the MRSA strains 99 (86%) were positive for DNase, 77(67%) were showed beta hemolysis, 49(42%) strains were positive for hemagglutination, 70(61%) were slime producers, most of the strains were biofilm producers, 4(3.5%) were non-adherent, 85(74%) were weakly adherent, 23(20%) were moderately adherent and 2(1.7%) were strongly adherent. (Table 1 & 2)

About 56%, 60% and 63% of the isolates were resistant to cotrimoxazole, erythromycin and ciprofloxacin respectively and about 22% and 33% of the isolates showed resistance towards clindamycin and gentamicin respectively. All the isolates were sensitive to vancomycin, teicoplanin and linezolid. About 10(8.7%) strains showed high level mupirocin resistance (HLMR) and 1(0.8%) strain showed low level mupirocin resistance (LLMR). Both HLMR and LLMR (100%) strains showed susceptibility to fusidic acid. (Table 3 & 4)

Table 1: Distribution of various virulence factors among MRSA strains

S. No.	Virulence factors	No. of isolates (%) n=114
1	DNase	99(86)
2	Beta hemolysis	77(67)
3	Hemagglutination	49(42)
4	Slime formation	70(61)
5	Biofilm formation	110(96)
5a.	Non-adherent	4(3.5)
5b.	Weakly adherent	85(74)
5c.	Moderately adherent	23(20)
5d.	Strongly adherent	2(1.7)

Table 2: Distribution of virulence factors of MRSA among isolates obtained from various clinical specimens

Virulence factors	Blood isolates (%) n=7	Exudate isolates (%) n=101	Respiratory isolates (%) n=3	Urine isolates (%) n=3
Slime formation	3 (42)	64(63)	3(100)	0(0)
DNase	5(71)	90(89)	3(100)	1(33)
Beta hemolysis	6(85)	67(66)	2(66)	2(66)
Hem agglutination	1(14)	39(38)	2(66)	0
Biofilm formation	7(100)	97(96)	3(100)	3(100)
Weakly adherent biofilm producers	5(71)	76(78)	2(66)	2(66)
Moderately adherent biofilm producers	2(28)	19(20)	1(33)	1(33)
Strongly adherent biofilm producers	0	2(2)	0	0

Table 3: Prevalence of HLMR and LLMR among MRSA isolates

Type of Mupirocin resistance	No. of isolates (%) n=114
High level Mupirocin Resistance (HLMR)	10(8.7%)*
Low level Mupirocin Resistance (LLMR)	1(0.8%)*

*All the HLMR and LLMR isolates were susceptible to fusidic acid

Table 4: Distribution of LLMR and HLMR isolates among various clinical specimen

Type of Specimen	HLMR isolates (%) n=10	LLMR isolates (%) n=1
Blood	1 (10)	0
Urine	0	0
Respiratory	0	0
Exudate	9(90)	1(100)

Discussion

The present study gives MRSA prevalence rate of 21%. Indian Network for Surveillance of Antimicrobial Resistance (INSAR) gives a MRSA prevalence rate of 41% which is higher when compared to our study.[13]

In our study, 14% of the MRSA isolates were negative for DNase.[14] Another study by Kateete David P et al., gives 25% negativity for DNase test.[15] Hemolysins play a major role in host cell damage. In the present study 67% of the MRSA isolates showed beta hemolysis which was higher when compared to the study done by Desouky, et al.[16] Another study by Suheyla et. al., shows 58.9% of *S.aureus* strains were found to be hemolytic.[10] *S.aureus* strains that possess hemagglutination property have the ability to adhere to prosthetic devices to establish infection. About 42% of MRSA strains were found to possess hemagglutinating property.[17] This was high when compared to the study done by Mark. et al., which shows that only 13% of *S.aureus* strains were positive for hemagglutination.[18]

Slime producing strains of *S.aureus* has the ability to form intact biofilm and also have higher rate of colonization in host tissues.[19] About 70% of the MRSA strains were found to be slime producers. This report was consistent with another study done by Podbielska et al., in which 69% of *S.aureus* strains were slime producers.[20]

Strains of *S.aureus* which has the property of biofilm formation confer antibiotic resistance and colonize most of the indwelling medical devices.[21] In our study most of the MRSA strains were biofilm producers. Only 3.5% of the strains were found to be non-adherent. About 74% were weakly adherent, 20%

were moderately adherent and 1.7% were found to be strongly adherent. A biofilm prevalence study in MRSA done by Maryam Rezaei, et al., reports that 100% of the MRSA isolates were biofilm producers out of which 15.4%, 19.2% and 65.4% were strong, medium and weak respectively. This report was concordant with our study results on biofilm formation.[22] These biofilm producing MRSA isolates were found to confer high degree of resistance to various antibiotics.[23]

In our study the antibiotics resistance pattern of MRSA isolates includes 63%, 60% and 56% for ciprofloxacin, erythromycin and co-trimoxazole respectively. INSAR report gives a prevalence rate of 79%, 70% and 55% for ciprofloxacin, erythromycin and co-trimoxazole respectively. Similarly the resistance rate for gentamicin, clindamycin includes 33% and 22% respectively. INSAR gives a prevalence rate of 58% for gentamicin and 46% for clindamycin which was higher when compared to our reports.[13] Asian Network for Surveillance of resistant Pathogens (ANSORP) gives a prevalence rate of 77% for ciprofloxacin, 78% for gentamicin, 64% for clindamycin, 90% for erythromycin and 43% for cotrimoxazole.[24] All the MRSA isolates were sensitive to vancomycin, teicoplanin and linezolid.

About 10% of the MRSA strains were HLMR and 0.8% of the MRSA strains were LLMR. Another study by Chaturvedi et al., gives a mupirocin prevalence rate of 15%.[25] All the mupirocin resistant isolates were found to be susceptible to fusidic acid. Another study by Solmaz et al., gives 100% fusidic susceptibility towards mupirocin resistant MRSA isolates.[26]

Conclusion

S.aureus has become the major life- threatening human pathogen due to its ubiquitous virulence properties and antibiotic resistance pattern. Therefore, a better understanding of its virulence mechanisms and associated properties that confer antibiotic resistance is needed in order to overcome the burden of limited options to treat infections caused by highly virulent multidrug resistant *S.aureus* strains both in the hospital and community settings.

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