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The role of non-β-lactam antimicrobials and screening for vancomycin resistance in methicillin resistant *Staphylococcus aureus*

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ABSTRACT

The study was carried out to determine the prevalence of MRSA, VRSA and their current antimicrobial susceptibility pattern to various non-β-lactam antimicrobial agents to record the current status of MRSA response to commonly used antistaphylococcal antibiotics in Aligarh, India for a period of two years. A total of 430 *Staphylococcus aureus* strains were isolated from various clinical samples. Two hundred ninety (67.44%) of *S. aureus* were isolated from pus and most of these were from orthopaedics 124 (28.85%) and surgery wards 99 (23.02%). One hundred thirty eight (32.09%) strains were confirmed to be methicillin resistant by both phenotypic and genotypic methods. More than 80% of MRSA strains were multidrug resistant. However, all were uniformly sensitive to vancomycin and linezolid. Levofloxacin was the drug found to be resistant in just 16.47% MRSA strains. Vancomycin is the drug of choice for MRSA treatment. However, regular screening should be done for vancomycin intermediate and vancomycin resistant strains of *Staphylococcus aureus* .We suggest the use of levofloxacin for MRSA treatment since overuse of vancomycin can lead to the development of vancomycin resistance and more importantly it is beyond the scope of poor patients in developing countries like India.

Keywords: *S. aureus,* MRSA, VRSA, screening, non-β-lactam, multidrug resistance

INTRODUCTION

Staphylococcus aureus is an important human pathogen causing pyogenic, disseminated and toxin mediated infections (Oliveira *et al.*, 2001; Enright *et al.*, 2002; Robinson *et al.*, 2003). The spectrum of *S. aureus* infections includes food poisoning, meningitis (Betley *et al.*, 1992; Ish-Horowicz, 1992; Collee *et al.*, 2006), toxic shock syndrome (Betley *et al.*, 1992; Collee *et al.*, 2006), as well as dermatological disorders ranging from minor infections and eczema to blisters and scalded skin syndrome (Winn *et al.*, 2006). Infections caused by *S. aureus* used to respond to β-lactam and related group of antibiotics. However due to development of methicillin resistance amongst *S. aureus* isolates (MRSA); treatment of these infections has become problematic. MRSA is a serious threat to the hospitalised patients globally and it now represents a challenge for public health as community associated infections appear to be on the increase in both adults and children (Layton *et al.*, 1995). Currently treatment options for MRSA infections are limited to very few and expensive drugs like teicoplanin and vancomycin. Already resistant to multiple classes of antibiotics , MRSA had been reported to acquire resistance even to vancomycin (Asadullah *et al.*, 2003), so in the near future the treatment options for MRSA infections are going to shrink further. The development of resistance to multiple antibiotics and control of disease transmission by MRSA isolates in hospitals or communities have been recognised as a major challenge as the bacterial population that expresses the resistance varies according to the environmental conditions (Qureshi *et al.*, 2004). Therefore the knowledge of prevalence of MRSA and VRSA and their antimicrobial profile become necessary in the selection of appropriate empirical therapy of these infections. We determined the prevalence of MRSA, VRSA and their current antimicrobial susceptibility pattern to various non-β-lactam antimicrobial agents to record the current status of MRSA response to commonly used antistaphylococcal antibiotics in western UP, India.

MATERIALS AND METHODS

The present study was conducted in the department of Microbiology, J.N. Medical College and Hospital, AMU, Aligarh during the period from August 2005 to July 2007. Various clinical specimens such as pus, blood, urine, CSF, pleural fluid, peritoneal fluid, ascitic fluid, bile, cervical swab, semen, conjunctival swabs and ear swabs received in the Microbiology laboratory were studied. The samples were cultured on 5–10% sheep blood agar, MacConkey agar, Mannitol salt agar and Robertson's cooked meat broth. Only those specimens from which staphylococci were isolated were included in the study. All the isolates suggestive of *S. aureus* were identified by the standard biochemical procedures (Collee et al, 2006). The methicillin susceptible strain ATCC 25923 was used as a control for the diagnostic procedures. All isolates were maintained in 0.5%–1% semisolid nutrient agar stabs and

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seeded with cork stoppers soaked with hot sterile paraffin until analysed (Colle and Miles, 1989).

Oxacillin disc diffusion test

All the isolates were subjected to oxacillin disc diffusion test using oxacillin 1 µg disc. A 0.5 McFarland turbidity standard suspension of the isolate was made and lawn culture was done on Mueller-Hinton agar (MHA) plates containing 4% NaCl. Plates were incubated at 37 °C for 18 h and zone diameters were measured. An inhibition zone diameter of ≤ 10 mm was reported as methicillin resistant and ≥ 13 mm was taken as methicillin sensitive.

MIC determination of oxacillin

MIC was determined by agar dilution test. 10 different dilutions of oxacillin were selected such that the concentrations that allowed determination of MIC breakpoints defining susceptible (≤ 2 µg/mL) (CLSI, 2004) and resistant (≥ 4 µg/mL) (CLSI, 2004) values were included. Lowest concentration at which the growth was inhibited by 80% or more was recorded as MIC.

PCR amplification for mec A and fem B genes

Multiplex PCR (Geha *et al.*, 1994) was carried out on all the *S. aureus* strains found methicillin resistant on MIC determination. All the MRSA strains were for the mec A and femB genes using the following oligonucleotides sequence.mec A1-5' GTA GAA ATG ACT GAA CGT CCG ATA A-3', mec A2-5' CCA ATT CCA CAT TGT TTC CGT CTA A-3', fem B1-5' TTA CAG AGT TAA CTG TTA CC-3', fem B2-5' ATA CAA ATC CAG CAC GCT CT-3'. A 50 µL PCR reaction mixture consisted of 45 µL of mastermix containing PCR buffer (1X), d NTP mix (0.2 mM of each), primer (0.5 µM), Taq DNA polymerase (0.25 U), and $MgCl_2$ (1.5 mM) with 5 µL of template DNA. Cycling parameters of PCR were set as follows: hot start 94 °C for 4 min followed by 35 cycles of melting at 94 °C for 45 s, annealing at 50 °C for 45 s, and extension at 72 °C for 1 min. Analysis of amplified products was done by gel electrophoresis. Amplicons of 310 bp were consistent with mec A and of 651 bp with fem B gene amplification.

Screening of vancomycin resistance

Himartshu Method

Overnight grown cultures were adjusted to 0.5 McFarland approximately 1.5 x 10^8 cfu/mL and 10 µL spots was inoculated on BHI agar containing 4 µg/mL of vancomycin. The plates were incubated at 35 °C for 24–48 h. Appearance of any growth indicated vancomycin resistance (Wootton *et al.*, 2001).

CDC Method

Overnight grown cultures were adjusted to 0.5 Mc-Farland approximately 1.5 x 10^8 cfu/mL and diluted 100 times to get an inoculum of approximately 1.5 x 10^6 cfu/mL. Ten µL spots of the cultures were inoculated on BHI agar containing 6 µg/mL of vancomycin. The plates were incubated at 35 °C for 24–48 h. Appearance of any growth indicated vancomycin resistance (Tenover *et al.*, 2001).

Method by Tenover et al. (2001)

Overnight grown cultures were adjusted to 0.5 Mc-Farland 1.5 x 10^8 cfu/mL and diluted 100 times to get an inoculum of approximately 1.5 x 10^6. Ten µL of both the above mentioned inoculums were spot inoculated on MHA agar containing 5 µg/mL of vancomycin. The plates were incubated at 35 °C for 24–48 h. Appearance of any growth indicated vancomycin resistance (Huber *et al*., 1999).

MIC determination of vancomycin

Vancomycin MICs for the clinical isolates were determined by agar dilution method in Mueller Hinton agar according to the protocol of National committee for Clinical Laboratory Standards (NCCLS). Eight different dilutions of van-comycin were selected such that the concentrations that allowed determination of MIC breakpoints defining susceptible (≤ 4 µg/mL) (Tenover *et al*., 2001), intermediate (8–16 µg/mL) (Tenover *et al*., 2001) and resistant (≥ 32 µg/mL) (Tenover *et al*., 2001) values were included. Lowest concentration at which the growth was inhibited by 80% or more was recorded as MIC.

Antibiotic susceptibility testing of all the MRSA strains was done using Kirby Bauer's disk diffusion method (CLSI, 2000) for the following non-β-lactam antimicrobial agents: amikacin 30 µg, ciprofloxacin 5 µg, clindamycin 2 µg, cotrimoxazole 25 µg, erythromycin 15 µg, gatifloxacin 5 µg, gentamycin 10 µg, levofloxacin 5 µg, linezolid 30 µg, ofloxacin 5 µg, sparfloxacin 5 µg, tobramycin 10 µg, vancomycin 30 µg.

RESULTS

A total of 430 *S. aureus* were isolated from various clinical specimens. The majority of *S. aureus* strains were isolated from pus 290 (67.44%) followed by urine 62 (14.42%) and blood 49 (11.39%) (Table 1) and around 50% of these were from the orthopaedics 124 (28.85%) and the surgery wards 99 (23.02%) (Table 2). Out of 430 *S. aureus* strains 143 (33.25%) were found to be methicillin resistant on phenotypic detection by oxacillin disc diffusion test. However, on genotypic detection with the help of multiplex PCR 138 (32.09%) strains had both *mec* A (310 bp) and *fem* B (651 bp) and were confirmed to be methicillin resistant. MIC of all the MRSA isolates was more than 4 µg/mL but none was greater than 256 µg/mL. Prevalence of MRSA was highest amongst the orthopaedics 64 (46.38%) and the surgery 49 (35.50%) wards (Table 2). Out of 138 MRSA strains 112 (81.16%) were MDR-MRSA (resistant to > 3 non-β-lactam antibiotics belonging to different groups) and only 26 (18.84%) strains were non-MDR-MRSA (Table 3). Among the multidrug-resistant isolates maximum 21 (15.22%)

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Table 1: Isolation rate of *S. aureus* in relation to specimen (n=430)

Specimen	No. of isolates (%)
Pus (P)	290 (67.44)
Blood (B)	49 (11.39)
Endocervical swab (Cx)	1 (0.23)
Ear swab (E)	2 (0.47)
Urine (U)	62 (14.42)
CSF (CSF)	7 (1.63)
Conjunctival swab (Cj)	2 (0.47)
Drain tip (Dt)	1 (0.23)
Foley's tip (Ft)	2 (0.47)
Fluids (Pleural, ascitic, peritoneal)	0 (0.00)
Sputum and throat swab	1 (0.23)
Seminal fluid	2 (0.47)
Catheter tip	11 (2.5)
Umblical tip	1 (0.23)
Total	**262 (100)**

Table 2: Isolation rate of *S. aureus* from various wards in hospital

Hospital wards	Non- MRSA	MRSA	*S. aureus*
	N (%)	N (%)	N
Orthopaedics	66 (53.23)	58 (46.77)	124
Surgery	64 (64.65)	35 (33.35)	99
Paediatrics	27 (72.97)	10 (27.03)	37
Gynaecology	29 (75.32)	9 (23.68)	38
Medicine	15 (75.00)	5 (25.00)	20
ICU	6 (54.55)	4 (45.45)	11
ENT	12 (70.59)	5 (29.41)	17
TB and Chest	10 (100.00)	0 (0.00)	10
Ophthalmology	9 (90.00)	1 (10.00)	10
Skin	20 (95.24)	1 (4.76)	21
Plastic surgery (Burn)	15 (65.22)	8 (34.78)	23
Nursery	19 (95)	1 (5)	20
Total	**292 (67.91)**	**138 (32.09)**	**430**

Table 3: Pattern of resistance of MRSA isolates to other drugs (n = 138)

No. of other drugs		No. of MRSA isolates (%)
n MDR	0	0 (0.00)
	1	6 (4.35)
	2	8 (5.80)
	3	12 (8.69)
	4	9 (6.52)
MDR	5	21 (15.22)
	6	16 (11.59)
	7	9 (6.52)
	8	20 (14.49)
	9	17 (12.32)
	10	14 (10.14)
	11	6 (3.62)
	12	0 (0.00)
Total		**138 (100.00)**

were resistant to five antibiotics. Maximum resistance was shown to cotrimoxazole (89.41%) followed by clindamycin (83.53%). Among the quinolones ciprofloxacin was found to be at the bottom of the list with maximum resistance of 81.17%, followed by gatifloxacin (45.88%), sparfloxacin (38.88%), and ofloxacin (23.53%). Levofloxacin was found to be the best amongst the flouroquinolones with just 16.47% resistance. Amikacin was better at 60.00% resistance among the aminoglycosides with gentamicin showing resistance to 69.41% and tobramicin to 71.00% strains. Linezolid and vancomycin were the only two drugs to which all the strains of MRSA were uniformly sensitive. On screening for vancomycin resistance it was found that 11 (7.97%) strains of MRSA were resistant to vancomycin by the Himartshu method, and 8 (5.7%) by the method by Tenover and co-workers. However CDC method detected no vancomycin intermediate (VISA) or vancomycin resistant (VRSA) strains of *S. aureus* which was in correlation with MIC of vancomycin by the agar dilution method. The MIC for vancomycin of all the MRSA strains was found to be less than 4 µg/mL.

DISCUSSION

MRSA is a major pathogen causing significant mortality and morbidity in the hospitals as well as among the communities. Many of the MRSA are becoming multidrug resistant and are susceptible only to glycopeptides antibiotics such as vancomycin (Mehta *et al.*, 1998). Low level resistance even to vancomycin is emerging at present (Asadullah *et al.*, 2003). The prolonged hospital stay, indiscriminate use of antibiotics, receipt of antibiotics before coming to the hospital etc. are the possible predisposing factors of MRSA emergence (Anupurba *et al.*, 2003). The prevalence of MRSA in our hospital was found to be 32.44%. Similar rate of MRSA isolation had also been reported from other parts of India (Anbumani *et al.*, 2006; Dar *et al.*, 2006; Gopalakrishnan *et al.*, 2006; Oberoi and Varghese, 2006). However, a higher rate (of around 50%) of MRSA prevalence is reported in some studies (Anupurba *et al.*, 2003; Vidhani, 2001; Krishnan, 2002). The high prevalence of MRSA in these studies could be due to several factors like indiscriminate use of antibiotics and unethical treatment before coming to the hospital.

In the present study maximum isolates of *S. aureus* were from pus (77.87%). This justifies the fact that *S. aureus* is a known cause of wound infections. Rate of MRSA isolation was also highest among the pus samples especially from the orthopaedics, surgery wards and the ICU. This might be because the patients in these wards are usually with open wounds and are debilitated. They undergo multiple interventions in the hospital which further increases the risk of MRSA infection due to multiple people involved as well as prolonged stay in the wards. Along with these factors, the patient is usually on multiple antibiotics. On antimicrobial sensitivity testing it was found the MRSA isolates were resistant to multiple groups of antibiotics. Around 80% of the strains were resistant to more than three non-β-lactam antibiotics and were

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multidrug resistant. However all the strains were uniformly sensitive to linezolid and vancomycin. Such an incidence of multidrug resistance had also been reported in other studies from North India (Vidhani, 2001; Dar *et al.*, 2006). Among the flouroquinolones ciprofloxacin was reported to have highest resistance of 81.17%. Similar to our findings, a high resistance of 90–98% had been reported for ciprofloxacin in other studies (Pulimood *et al.*, 1996; Qureshi *et al.*, 2004). Among the aminoglycosides gentamicin had been reported to be resistant in 63.6% isolates by Rajaduraipandi and co-workers (2006), similar to our findings. However some authors had quoted up to 97.8% resistance to gentamicin which is higher compared to ours (Qureshi *et al.*, 2004). Linezolid and vancomycin were the only drugs to which all the strains were sensitive. However some authors had reported emerging vancomycin resistance with strains showing intermediate [VRSA] and complete resistance to vancomycin [VISA] (Asadullah *et al.*, 2003; Gopalakrishnan, 2006). At present the proportion of MRSA with reduced susceptibility [VISA] to vancomycin is well known. Vancomycin resistance can be difficult to detect in clinical microbiology laboratories since sensitivity testing by disc diffusion method may sometimes misclassify intermediate susceptible isolates as fully susceptible. MIC determination by agar and broth dilution methods is considered the gold standard (Srinivasan *et al.*, 2002) but it is beyond the scope of most of the micro-biology laboratories especially in the developing countries like India. The screening methods can be used for early and accurate detection of vancomycin resistance. Among the three screening methods evaluated in our study CDC method was found to be the most accurate with maximum correlation with the MIC of vancomycin detected by agar dilution method. Himartshu method and the method by Tenover and co-workers (2001) were found to be false positive in 11 (7.97%) and 8 (5.7%) isolates respectively. We recommend the use of the CDC method for regular screening for vancomycin resistance in all the microbiology laboratories. However, confirmation of vancomycin resistance should be done by the determination of MIC in all the isolates suspected to have VISA or VRSA. But overuse of vancomycin can promote the emergence of its resistance and more importantly in developing countries like India poor patients cannot afford treatment with these costly drugs. Another limiting factor for vancomycin use is the high rate of adverse affects caused by the same. We suggest that no antimicrobial should be used on regular basis for empirical treatment because of the rapidly changing pattern of drug resistance. So we recommend that proper screening of antimicrobial sensitivity pattern of *Staphylococcus* species should be done within each hospital on annual basis and empirical antimicrobial therapy should be modified accordingly. The next respon-sibility goes to the clinicians who should exercise caution in their use of vancomycin or any antimicrobial and help to prevent the spread of drug resistance by the rationale and proper use of antimicrobials.

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