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VANCOMYCIN HETERORESISTANCE, AND INDUCIBLE AND CONSTITUTIVE CLINDAMYCIN RESISTANCE IN STAPHYLOCOCCI.

Microbiology

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ABSTRACT

Vancomycin and clindamycin represent useful options in therapy of staphylococcal infections. High MICs to vancomycin, heteroresistance and inducible clindamycin resistance, often not detected in routine, are valuable in therapeutic decision making for staphylococcal infections. We evaluated clinically significant, non-repetitive 197 *S. aureus* and 67 coagulase negative staphylococci (CoNS) isolates for antimicrobial resistance (AMR), methicillin resistance (oxacillin agar screen, cefoxitin disc test, and oxacillin MIC), vancomycin MICs (agar dilution and broth dilution), vancomycin heteroresistance (BHIA-6 method), and constitutive and inducible clindamycin resistance (D-test, 15mm and 25mm). Methicillin resistance was observed in 80 (40.6%) and 31 (46.3%) isolates of *S. aureus* (MRSA) and CoNS (MRCoNS) respectively. Resistance to erythromycin, clindamycin, aminoglycosides and ciprofloxacin was more common among MRSA than methicillin sensitive *S. aureus* (MSSA) (P value < 0.001). Intermediate resistance to vancomycin was found in 0%, 21.3%, and 1.5% of MSSA, MRSA and CoNS isolates. Vancomycin heteroresistance was found in 0%, 16.3%, and 4.5% of MSSA, MRSA and CoNS isolates. Inducible clindamycin resistance was found in 12%, 22.5%, and 9% of MSSA, MRSA and CoNS isolates. Detection of rampant AMR in staphylococci by various supplementary methods is of importance.

KEYWORDS

Mrsa, Mrcons, Vancomycin Heteroresistance, Visa, Inducible Clindamycin Resistance.

Introduction

Antimicrobial drug resistance in staphylococci is a growing menace, and in fact strains of drug resistant *Staphylococcus aureus* have achieved the superbug status in clinical medicine.[1,2] Drugs like penicillin and cloxacillin once effective are now of no avail in majority of hospital acquired infections due to increasing antimicrobial resistance. Vancomycin is a therapeutic alternative in cases of MRSA infections. However, its efficacy in treating such infections is at best moderate.[3] Therapeutic efficacy of vancomycin especially decreases with increasing MIC values.[4] In addition, a new phenomenon of heteroresistance to vancomycin has been detected in staphylococci which may pose a serious threat against therapy with this agent.[5] Clindamycin (a lincosamide), is used in staphylococcal skin and superficial infections as an alternate in cases of penicillin allergy. It has recently been found to dramatically improve life threatening community acquired methicillin resistant *Staphylococcus aureus* (CAMRSA) infections.[6] Being a protein synthesis inhibitor, it can decrease toxin production in virulent staphylococcal clones.[6,7] However, detection of resistance to this antibiotic is tricky; isolates resistant to macrolides may show false in-vitro sensitivity to clindamycin unless the D-test is applied.[6,8] Treatment with clindamycin in infections caused by such microbes may not be successful.[8] The detection of methicillin resistance and vancomycin resistance in staphylococci is also associated with discrepant results when tested by non-standard methods.[8] This problem is due to the phenomenon of heteroresistance and susceptibility testing to these two antimicrobials must follow the CLSI guidelines for accurate results.[9] Keeping this growing menace of drug resistance in mind, we evaluated clinically significant *S. aureus* and coagulase negative staphylococci (CoNS) isolates by disc diffusion susceptibility testing against common first line and second line antibiotics. Further, constitutive and inducible clindamycin resistance was determined using the D-test (at both 15mm and 25mm disc distances). Methicillin resistance was determined using cefoxitin disc diffusion, oxacillin agar screen and oxacillin MIC testing. Vancomycin MICs were determined in all isolates, and vancomycin heteroresistance was determined using the Brain Heart Infusion Agar-6 method.

Material and Methods

Bacterial strains: 197 consecutive clinically significant *S. aureus* isolates and 67 CoNS isolates were tested. Only invasive isolates (blood, CSF, sterile body fluids, sputum, BAL, and pus samples) were included in the study. A standardized inoculum (0.5 McFarland, 1.5 X 10^8 cfu/ ml) was prepared from 4-5 isolated staphylococcal colonies of *S. aureus* and CoNS isolates using the direct colony suspension method. Antimicrobial discs were applied to the Mueller-Hinton agar plates within 15 minutes of inoculation. Disc diffusion antimicrobial susceptibility of the isolates was performed for these isolates following CLSI guidelines against the following antimicrobials: gentamicin (10µg), amikacin (30µg), ciprofloxacin (5µg), cefotaxime (30µg), netilmicin (30µg) and vancomycin (30µg). *S. auerus* ATCC 25923 was used for quality control assessment.[8]

Oxacillin agar screen and cefoxitin disc (30µg) diffusion test was put up for detecting methicillin resistance in these isolates. MHA plates containing 2% NaCl, and 6 µg/ml oxacillin was inoculated in an area of 10-15 mm with a cotton swab dipped in 0.5 McFarland suspension (1.5 X 10^8 cfu/ml) of staphylococcal isolates and incubated for 24 hours at 35°C. The plates were examined using transmitted light and presence of more than one colony or a light film of growth signified methicillin resistance by this method. For the cefoxitin disc diffusion test, CLSI guideline was followed to make the interpretation of methicillin resistance (≤ 21mm zone size for *S. aureus* and ≤ 24mm zone size for CoNS). Further, all staphylococcal isolates were tested by agar dilution MIC against oxacillin (Ranbaxy Pvt. Ltd., India). Agar dilution MIC of these isolates was done on Muller Hinton agar plates containing 2% NaCl, and appropriate antibiotic concentrations. Plates were incubated for 24 hours at 35°C, and the results interpreted according to CLSI guidelines.[8]

Agar dilution and broth dilution vancomycin MICs were tested for all the isolates irrespective of methicillin resistance. The isolates were further screened for heteroresistance against vancomycin. Brain heart infusion agar (BHIA) plate containing 6 µg/ml vancomycin was inoculated with 10µl of a 0.5 McFarland suspension (1.5 X 10^8 cfu/ml) of staphylococcal isolates and incubated for 24 hours at 35°C. The plates were examined using transmitted light and presence of more than one colony signified heteroresistance. [5,8]

Two double disc approximation tests (D-test), one at a distance of 15mm and another at a distance of 25mm, were performed by applying a clindamycin disc (2µg) next to a erythromycin disc (15 µg) at distances of 15 mm and 25 mm. *S. aureus* ATCC BAA-977 (iMLS$_B$) and *S. aureus* ATCC BAA-976 (cMLS$_B$) were used daily as controls for the test. Organisms that show flattening of the zone around the clindamycin disc adjacent to the erythromycin disc were reported as strains with inducible resistance to clindamycin. *S. aureus* ATCC 25923 was used for routine quality control assessment of erythromycin and clindamycin discs.[8]

Results

Out of the 197 *S. aureus* isolates tested, 80 (40.6%) were resistant to methicillin by the oxacillin agar screen (OAS) method. All of these 80 OAS resistant *S. aureus* isolates were also oxacillin resistant by cefoxitin disc diffusion method, further all of these MRSA isolates had MIC ≥ 4 µg/ml to oxacillin. The MICs of oxacillin to *S. aureus* were 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32 and ≥ 64 µg/ml in 23, 9, 15, 32, 38, 21, 22, 15, 15, and 7 isolates respectively. Among the 67 CoNS isolates, 36 were resistant by the OAS method. However, the MIC results did not corresponded to the OAS results in 5 isolates; only 31 of the 36 isolates positive by OAS had oxacillin MIC ≥ 0.5 µg/ml (ie; were resistant according to CLSI guidelines). The other 5 isolates positive on agar screen, and another 31 isolates negative by OAS had MIC values ≤ 0.25 µg/ml, sensitive by CLSI guidelines. Further, the cefoxitin disc diffusion test detected 32 strains as methicillin resistant, however only 31 were resistant as per the MIC values to oxacillin (≥ 0.5 µg/ml). All isolates sensitive as per the cefoxitin disc test were also sensitive as per the MIC values (≤ 0.25 µg/ml). The MICs of oxacillin to CoNS were 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, and ≥ 32 µg/ml in 7, 15, 14, 5, 7, 7, 6, 2, 3, and 1 isolates respectively. Taking oxacillin MIC values as the gold standard, the sensitivity and specificity of OAS was 100% and 100%, and 100% and 86.1% for *S. aureus* and CoNS isolates respectively. Similarly the sensitivity and specificity of the cefoxitin disc test was 100% and 100%, and 100% and 97.2% for *S. aureus* and CoNS isolates respectively. Thus, our staphylococcal isolates were categorized as MRSA (n=80), MSSA (n=117), and CoNS (n=67) [methicillin resistant (MRCoNS) (n=31), and methicillin sensitive (MSCoNS) (n=36)] (Table 1).**Legends to Table 1**: S=Sensitive, I = Intermediate susceptible, R=Resistant NA=Not applicable (as per CLSI guidelines), *P value < 0.001

Table 1: Antimicrobial susceptibility (disc diffusion, CLSI guidelines) results among staphylococcal isolates.

	MSSA (n=117)			MRSA (n=80)***			CoNS (n=67)***		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Erythromycin	94 (80.3)	0	23 (19.7)*	24 (30)	0	56 (70)*	36 (53.7)	1(1.5)	30 (44.8)
Clindamycin	108 (92.3)	0	9 (7.7)*	41 (51.3)	0	39 (48.8)*	42 (62.7)	0	25 (37.3)
Gentamicin	101(86.3)	1 (0.9)	15 (12.8)*	28 (35)	0	52 (65)*	42 (62.7)	0	25 (37.3)
Amikacin	112 (95.7)	1 (0.9)	4 (3.4)*	49 (61.3)	1 (1.3)	30 (37.5)*	49 (73.1)	0	18 (26.9)
Cefotaxime	95 (81.2)	2 (1.7)	20 (17.1)*	0	0	80 (100)*	27 (40.3)	0	40 (59.7)
Ciprofloxacin	58 (49.6)	0	59 (50.4)*	1 (1.3)	0	79 (98.8)*	31 (46.3)	0	36 (53.7)
Netilmicin	103 (88)	0	14 (12)*	37 (46.3)	3 (3.8)	40 (50)*	42 (62.7)	0	25 (37.3)
Oxacillin	NA	NA	NA	NA	NA	NA	36 (53.7)	0	31 (46.3)
Vancomycin	117 (100)	NA	0	80 (100)	NA	0	66 (98.5)****	NA	0

** All methicillin resistant *S. aureus* isolates identified by agar screen testing were further tested by agar dilution MIC and found to have equivalent results.

*** 36 isolates were agar screen positive and 32 were methicillin resistant by cefoxitin disc diffusion test, however upon MIC testing only 31 isolates had MIC ≥ 0.5 µg/ml. Thus only 31 isolates were considered to be methicillin resistant according CLSI guidelines.

**** One CoNS isolate had zone diameter of 14 mm against the vancomycin disc. As per CLSI guidelines no interpretation is given, except for further testing by MIC. MIC of this isolate to vancomycin was 8 µg/ml

Resistance against erythromycin, clindamycin, gentamicin, amikacin, ciprofloxacin and netilmicin was significantly more common among MRSA than MSSA (P value < 0.001) (Table 1). Resistance to cefotaxime (100%) and ciprofloxacin (98.8%) was absolute among the MRSA isolates (Table 1). Among CoNS isolates resistance was high against cefotaxime (59.7%), ciprofloxacin (53.7%), and erythromycin (44.8%) (Table 1).

All MSSA isolates had vancomycin MIC ≤ 1µg/ml, thus were all vancomycin sensitive (breakpoint 4 µg/ml) (Table 2). Of the MRSA isolates, 17 (21.3%) were intermediate resistant to vancomycin by MIC values (between 4 and 8 µg/ml), and the rest sensitive. Among CoNS isolates, 66 (98.5%) had MIC to vancomycin ≤ 4µg/ml, and thus were sensitive according to CLSI guidelines (breakpoint 8 µg/ml), and one isolate was intermediate sensitive (Table 2). Among the MSSA, MRSA, and CoNS, 0, 13 (16.3%), and 3 (4.5%) isolates turned out to be heteroresistant by BHIA-6 method.

Table 2: Minimum Inhibitory Concentration (MIC) of staphylococcal isolates to vancomycin

MIC values (µg/ml)	<0.125	0.125	0.25	0.5	1	2	4	8
MSSA (n=117)	11 (9.4)	40 (34.2)	40 (34.2)	20 (17.1)	6 (5.1)	0	0	0
MRSA (n=80)	0	3 (3.8)	7 (8.8)	4 (5)	27 (33.8)	22 (27.5)	16 (20)	1 (1.3)
CoNS (n=67)	2 (3)	9 (13.4)	7 (10.4)	6 (9)	14 (20.9)	23 (34.3)	5 (7.5)	1 (1.5)

Legends to Table 2: MSSA = Methicillin sensitive *Staphylococcus aureus*, MRSA = Methicillin sensitive *Staphylococcus aureus*, CoNS = Coagulase negative staphylococci

Compared to MSSA, MRSA isolates showed significantly greater inducible (12% *vs.* 22.5%) and constitutive (6% *vs.* 45%) clindamycin resistance. In the CoNS isolates 9% and 34% isolates showed inducible and constitutive clindamycin resistance respectively. There were in all 40 staphylococcal isolates (16 MSSA, 18 MRSA, and 6 CoNS) with inducible clindamycin resistance, all of them were D-test positive at both 15mm and 25mm disc distances. Among the MSSA, MRSA, and CoNS, 2 (1.7%), 3 (3.8%), and 2 (3%), isolates were resistant to clindamycin despite being erythromycin sensitive.

Table 3: Resistance to Erythromycin and clindamycin among staphylo coccal isolates.

	Sensitive to both E and C (%)	iMLS$_B$ (%)	cMLS$_B$ (%)	MS$_B$ (%)	Sensitive to E, Resistant to Cd (%)
MSSA (n=117)	92(78.6)*	16 (12)**	7 (6)*	0	2 (1.7)
MRSA (n=80)	21(26.3)*	18 (22.5)**	36 (45)*	2(2.5)	3 (3.8)
CoNS (n=67)	28(50.9)	6 (9)	23 (34.3)	2(3)	2(3)

Legends to Table 3: E= Erythromycin, C=Clindamycin, MS$_B$= Erythromycin resistant only (no inducible or constitutive resistance to clindamycin), iMLS$_B$=inducible clindamycin resistance (D-test positive), cMLS$_B$= constitutive clindamycin resistance, * P value < 0.001, **P value < 0.05

Discussion

Systemic antimicrobial agents useful against *S. aureus* include staphylococcal penicillinase resistant penicillins (methicillin, nafcillin, oxacillin, cloxacillin and others), glycopeptides (vancomycin and teicoplanin), fluoroquinolones, aminoglycosides (amikacin, gentamicin, and netilmicin), tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole, and macrolide-lincosamide-streptogramin group of compounds.[3] Despite the multitude of effective therapies, not one can be used in an infected patient without the threat of antimicrobial resistance looming large on the mind of the clinician. In effect *S. aureus* has developed resistance to virtually all classes of antimicrobial agents in clinical use.[3] The problem is compounded manifold when one appreciates the multiple-resistance phenotypes in many of these strains, especially those of MRSA. Linezolid, a new alternative is available but suffers from major disadvantages; viz. it is only bacteriostatic and has serious adverse effects of anemia and thrombocytopenia. [3,10] In case of CoNS, the problem of antimicrobial resistance is even more acute. More than 80% of CoNS isolates are at present resistant to methicillin, and a majority of these strains are also resistant to aminoglycosides, fluoroquinolones, and macrolide-lincosamide group of drugs.[11] Reduced susceptibility to glycopeptides has also been reported frequently among CoNS isolates.[12,13]

Results of our study indeed confirm this gloomy scenario; a majority of our strains (both *S. aureus* and CoNS) were resistant to methicillin. Multiple resistance phenotypes (methicillin along with fluoroquinolones, aminoglycosides, macrolides, and clindamycin) of MRSA and MRCoNS were frequently observed in this study. Of grave significance is the high resistance being reported against the fluoroquinolone, ciprofloxacin (50.4% in MSSA, 98.8% in MRSA, and 53.7% in CoNS).

Numerous methods are available for detection of methicillin resistance in staphylococci. These include MIC breakpoints, oxacillin and cefoxitin disc diffusion tests, oxacillin agar screen assays, PBP 2a' detection using latex agglutination, and *mecA* gene detection.[3,8,11] Of these, *mecA* gene detection is considered gold standard [8] but was not possible in our study due to cost consideration. Oxacillin disc diffusion has previously been found to be an unreliable method for detection of resistance because of the heteroresistance phenomenon.[9] CLSI guidelines for accurate determination of methicillin susceptibility status include MIC determination and cefoxitin disc diffusion. We carried out the MIC determination test and compared it with the oxacillin agar screen method, a routinely used method due to the ease of testing. We found the oxacillin agar screen to be a valid method for detection of methicillin resistance in *S. aureus* isolates (100% sensitivity and specificity). However, the procedure was not accurate in determining the oxacillin susceptibility status in the CoNS isolates (86.1% specificity). Cefoxitin disc diffusion test however performed equally well among both the *S. aureus* as well as CoNS isolates (100% and 97.2% specificity respectively). Only one discrepant result was noted in a CoNS isolate with this method.

Vancomycin resistance has already emerged at various centers in India.[14,15,16] Previously all staphylococci at our center have been reported to be vancomycin sensitive. However, in this study we document 21.3% of MRSA and 1.5% of CoNS to have intermediate susceptibility to vancomycin. Recent reports also suggest that vancomycin therapy may be ineffective in the treatment of patients whose *S. aureus* isolates have MIC between 1 µg/ml and 2 µg/ml.[4] We documented 22 of our 80 MRSA to have MIC value of 2 µg/ml. The clinical implications of these results are obvious as vancomycin is the first line of therapy against MRSA and complicated MRCoNS infections at our center. Heteroresistance to vancomycin is also a diagnostic and therapeutic challenge.[5,14] Documentation of 16.3% and 4.5% heteroresistance in MRSA and CoNS respectively is a matter of great concern. The method we followed was a simple screen for detection of h-VISA strains. This method was first proposed by Hiramatsu *et al.*, 1997 and has been approved by CLSI since 2007. However confirmation of h-VISA requires performing a population analysis profile (PAP) of each of the isolates which is a tedious procedure without automation.

Macrolides including erythromycin, as well lincosamides are protein synthesis inhibitors acting on the same 50S subunit of ribosome. Macrolide resistant staphylococci may be phenotypically divided into three groups based on their susceptibility to clindamycin; isolates resistant to macrolides only (MS_B), isolates with constitutive resistance to clindamycin ($cMLS_B$), isolates with inducible resistance to clindamycin ($iMLS_B$).[17] Erythromycin resistant isolates with inducible or constitutive resistance to clindamycin ($cMLS_B$ or $iMLS_B$, together MLS_B) should be reported resistant to all macrolides, lincosamides (clindamycin) and type B streptogramins.[8] Resistance to macrolides (erythromycin) can be due to the *msrA* gene (efflux pump) or due to methylation of the 23S RNA transcript of the *erm* gene.[17] A change in the conformation of the superstructure of the 23S ribosomal subunit is responsible for MLS_B resistance. Efficacy of clindamycin in infections due to $iMLS_B$ isolates is controversial. According to CLSI guidelines, D-test positive isolates are to be reported as "resistant". A comment may be added stating "The isolate is presumed to be resistant to clindamycin based on the detection of inducible resistance. Clindamycin may still be effective in some patients".[8] Reports from various parts of India indicate that $iMLS_B$ may be common in *S. aureus*. [17,18,19,20,21] The situation in our center seems to be similar in that 12%, 22.5%, and 9% of MSSA, MRSA, and CoNS strains were found to be D-test positive and would have been falsely reported sensitive in routine testing.

Apart from the D-test, other methods of $iMLS_B$ detection include the broth dilution assay, agar dilution technique and the PCR assays for the *ermTR* variant *A* (*ermA*) gene, *ermB* gene and the *ermC* gene. These methods are either very cumbersome and are expensive in comparison to the D-test. Most laboratories use the simple, inexpensive D-test for reporting such isolates; however the distance between the two discs is kept undefined in the CLSI method between 15mm and 25mm.[8,17] Unlike a recent report we did not find the test results to be variable in our strains if conducted 15mm or 25mm.[17]

Surprisingly, we detected a few isolates which were erythromycin sensitive but clindamycin resistant. This may be due to presence of either of *lnuF or* $Vga(A)_{LC}$ genes which determine isolated clindamycin resistance in absence of erythromycin resistance. The product of *lnu* genes hydrolyse lincosamides specifically, while the *Vga* genes encode for ABC transporter proteins which actively pump out lincosamides (efflux mechanism).[22] Limited such instances of staphylococcal erythromycin sensitivity but clindamycin resistance has been reported from India. Lincosamides and macrolides though act on the same 23S RNA subunit of the ribosome, but are definitely two different classes of antibiotics. Hence erythromycin sensitive does not mean clindamycin sensitive and vice-versa.

Conclusions

Multiple antimicrobial resistance is rampant in staphylococci and its detection by various methods is of urgent importance to microbiology laboratories.

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Conflicts of Interest: Nil.

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