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A STUDY OF VANCOMYCIN INTERMEDIATE RESISTANCE AND HETERORESISTANCE IN CLINICAL ISOLATES OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* AT A TERTIARY CARE HOSPITAL

Rinkal Kansal, Vishal Sharma * and Shilpa Arora

Department of Microbiology, Guru Gobind Singh Medical College & Hospital, Faridkot - 151203, Punjab, India.

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Correspondence to Author:
Dr. Vishal Sharma

Professor & Head,
Department of Microbiology,
Guru Gobind Singh Medical College & Hospital, Faridkot - 151203,
Punjab, India.

E-mail: vsgmcs@gmail.com

ABSTRACT: Background: Methicillin resistant *Staphylococcus aureus* (MRSA) is known to cause various infections ranging from minor skin and soft tissue infections to devastating septicaemia and endocarditis. The only drug that is currently most effective against MRSA infections is a glycopeptide, vancomycin. Due to continued exposure and injudicious use of this novel drug, cases of reduced susceptibility to vancomycin are appearing which could be complete resistance (VRSA), intermediate resistance (VISA) or heterogeneous resistance (hVISA). **Aims & Objectives:** To study: The antimicrobial susceptibility pattern of MRSA to commonly used antibiotics and vancomycin, teicoplanin and linezolid. The proportion of VISA and hVISA among MRSA isolates. **Material & Methods:** The study was conducted in Microbiology department of GGSMCH, Faridkot in which 150 MRSA isolates were processed as per standard microbiological protocols. Vancomycin susceptibility was studied by 3 methods: vancomycin screen agar using 6 µg/mL of vancomycin, micro broth-dilution and Epsilometer test using Hi-media Vancomycin-Teicoplanin graded E-strip. **Results:** Of 11726 specimens received, *Staphylococcus aureus (S. aureus)* was isolated from 230 specimens. 85.22% (196) were methicillin resistant (MRSA). 150 consecutive MRSA isolates were considered for this study. Maximum resistance was observed against ampicillin (100%), followed by ciprofloxacin (88%), erythromycin (79.33%), cotrimoxazole (64.67%), clindamycin (52.1%) and amikacin (51.3%). No MRSA isolate was resistant to teicoplanin, rifampicin and linezolid and also did not show any resistance to vancomycin (hVISA, VISA, VRSA). **Conclusion:** There is high prevalence of MRSA in our hospital and all isolates showed high level of resistance to multiple classes of antibiotics except linezolid, rifampicin, and glycopeptides, thereby limiting the therapeutic options. This indicates that there is an urgent need of implementation of effective infection control practices.

INTRODUCTION: *S. aureus* is ubiquitous in the environment and it colonizes the skin, nose, and pharynx with anterior nares as the main reservoir. The primary mode of transmission of *S. aureus* is by direct contact, usually skin-to-skin contact with a colonized or an infected individual, although contact with contaminated objects and surfaces also plays a role. Various host factors, like disruption of the normal skin barrier, presence of underlying diseases such as diabetes, acquired immunodeficiency syndrome and defects in neutrophil function predispose to infection.

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It is known to cause illnesses ranging from minor skin infections to life-threatening diseases such as pneumonia, osteomyelitis, meningitis, Toxic Shock Syndrome, endocarditis and septicaemia [1]. Initially *S. aureus* was susceptible to almost every

antibiotic; over the time resistance to various antibiotics developed including penicillins, methicillin, semisynthetic penicillins, aminoglycosides & also to the novel drug, vancomycin. MRSA strains display intermediate resistance (VISA), heteroresistance (hVISA) and occasionally complete resistance to vancomycin (VRSA). Vancomycin creates pressure that favours the outgrowth of rare, vancomycin-resistant clones leading to hVISA clones, and eventually, with continued exposure, to a uniform population of VISA clones [2, 3].

The first report of *S. aureus* with hVISA and VISA was from Japan in 1996 and 1997 respectively and VRSA from US in 2002 [2, 3]. Fortunately, since it's emergence in 1997, VISA is still a rare event. However, the phenomenon of hVISA has been described more frequently in the literature, although the best method to detect hVISA strains and their clinical significance are still ill-defined.

Since, there is widespread empirical use of vancomycin against Gram positive infections including MRSA and many health care facilities have reported an upward trend of Minimum Inhibitory Concentration (MIC) for vancomycin, an attempt was made to study about rising reduced susceptibility to vancomycin in the form of VISA, hVISA by calculating the MIC for MRSA isolates.

MATERIAL AND METHODS: This study was conducted after taking ethical approval from the institution's ethical committee (No.BFUHS/2k21p-TH/6470). All the clinical specimens received in the Microbiology department were inoculated on culture plates [blood agar and MacConkey's agar; cystine lactose electrolyte deficient (CLED) agar for urine specimens] and incubated at 37 °C for 24-48 hours and direct microscopy was performed [4].

Based on the colony characteristics, Gram staining, the strains that were catalase positive and both slide and tube coagulase test positive were taken as *S. aureus*. The antibiotic susceptibility pattern of all *S. aureus* isolates to various antibiotics was determined by Kirby-Bauer's disc diffusion method according to CLSI guidelines [5].

Control Strains used in the Study: *S. aureus* ATCC 25923 and MRSA 43300 Methicillin resistance was detected using cefoxitin disc (30µg), which is a surrogate marker and a potent inducer of mecA gene. The strains of *S. aureus* with a zone diameter of ≤ 21mm were considered as MRSA.

Determination of MIC of Vancomycin:

Vancomycin Agar Screen with 6µg /ml of Vancomycin [5]: This test was used presumptively to screen for vancomycin resistance. Direct colony suspension of the test organism was made and matched with 0.5 McFarland turbidity standards. With the help of a micropipette, 10µl was delivered to the brain heart infusion (BHI) agar plate supplemented with 6µg/ml of vancomycin and incubated at 35°C in ambient air for 24 hours.

By using transmitted light plates were examined and greater than 1 colony was considered as reduced susceptibility to vancomycin. For quality control *Enterococcus faecalis* ATCC 29212 and *E. faecalis* ATCC 51299 strain was used.

Broth Micro-dilution [5]:

Preparation of Antibiotic Stock Solution: 0.1ml of serial doubling dilutions of the vancomycin stock solution were dispensed in a sterile, plastic microdilution tray.

Inoculum Preparation and Incubation: Direct colony suspension was made from the isolated colony and adjusted to match 0.5 McFarland turbidity standards ($1x10^8$ CFU/ml). Within 15 minutes it was diluted 1:20 to yield $5x10^6$ CFU/ml.

When 0.01ml of this suspension was inoculated into 0.1ml broth, the final concentration turned out to be $5x10^5$ CFU/ml. One well served as positive growth control (broth plus inoculum) and the other as negative control (containing broth only). The microtitre tray was incubated at 35^0C for 16 to 20 hours. To prevent drying it was sealed with tight fitting plastic cover.

Turbidity or a button of > 2mm in the control well indicated adequate growth. The lowest concentration of the antibiotic that completely inhibited the growth of the organism as detected by unaided eye was taken as MIC.

E-Test (Epsilometer Test) [4, 5]: The MIC was determined using Vancomycin, Teicoplanin (Hi media India Pvt. Ltd) E-strips and interpreted as provided in CLSI guidelines.

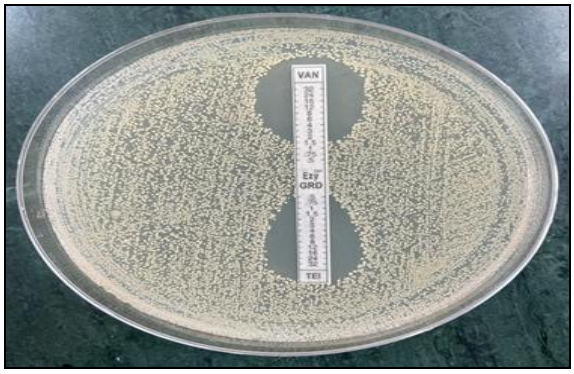

FIG. 1: SHOWS EPSILOMETER TEST USING HI-MEDIA VANCOMYCIN-TEICOPLANIN GRADED E-STRIP

Simplified Population Analysis for detection of Hvisa [2]: This is the most common screening method described by Hiramatsu *et al.*, for the characterization of the prototype hVISA and VISA strains. In this 10µl of a 10^8 CFU/ml bacterial suspension was inoculated onto BHI agar containing 4µg of vancomycin per ml (BHIA-V4). Growth at 24 h was considered "potential VISA," while growth at 48 hours as "potential hVISA." Strains were confirmed VISA when vancomycin MICs for them was 8µg/ml.

Plates positive for growth on BHIA-V4 were confirmed for hVISA by subculturing them onto Muller hinton agar (MHA) plates for sub clones and the isolated colonies were randomly selected and MIC was determined for them by micro-broth dilution method. They were confirmed as hVISA when MIC was > 8µg/ml and remained resistant for more than 9 days on media without antibiotic.

RESULTS: Of the total 11726 specimens received, 230 *S. aureus* were isolated, detection of methicillin resistance by cefoxitin disc (30µg) method showed 85.22% *S. aureus* were MRSA. Of all the MRSA isolated 150 consecutive MRSA isolates were considered for this study.

More no. of MRSA were isolated from patients with age-group 21- 40 years (33.33%) followed by 29.33% from 41-60 years and 64.67% isolates were from males and 35.33% from females. The male to female ratio was 1.83. Maximum MRSA were isolated from pus (79.33%) followed by blood (19.33%) and 0.67% each from biopsy tissue and tracheal/endotracheal aspirate.

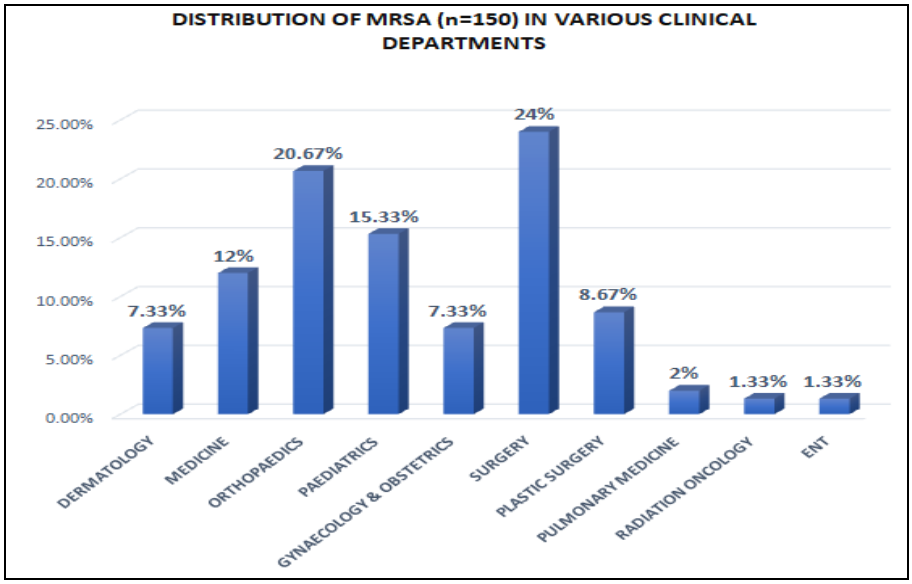

FIG. 1: SHOWS THE DEPARTMENT WISE DISTRIBUTION OF MRSA ISOLATES

All the MRSA isolates (100%) were resistant to ampicillin, followed by resistance to ciprofloxacin (88%) and erythromycin (79.33%). Rifampicin and linezolid showed 100% susceptibility in MRSA isolates **Table 1.**

TABLE 1: ANTIMICROBIAL SUSCEPTIBILITY PROFILE OF VARIOUS ANTIBIOTICS OF MRSA ISOLATES (n=150)

Antibiotic	No. of isolates sensitive (percentage)	No. of isolates resistant (percentage)
Ampicillin	0(0%)	150(100%)
Ciprofloxacin	18(12%)	132(88%)
Erythromycin	31(20.67%)	119(79.33%)

Cotrimoxazole	53(35.33%)	97(64.67%)
Clindamycin*	57(47.9%)	62(52.1%)
Amikacin	73(48.67%)	77(51.3%)
Linezolid	150(100%)	0(0%)
Rifampicin	150(100%)	0(0%)

*Clindamycin was used only in pus samples.

130(86.67%) MRSA isolates had vancomycin MIC of ≤0.5µg/ml and 20(13.33%) isolates had vancomycin MIC=1µg/ml by broth microdilution method. None of the MRSA isolate showed MIC >1µg/ml by this method **Table 2.** 101(67.33%) MRSA isolates showed MIC = 0.5µg/ml, 25(16.67%) isolates showed MIC = 1µg/ml, 22(14.67%) isolates showed MIC = 1.5µg/ml while 2(1.33%) isolates showed MIC = 2µg/ml by Epsilometer test. None of the isolate showed MIC>2 µg/ml **Table 2**. All the MRSA isolates were susceptible to vancomycin using vancomycin screen agar. Vancomycin screen agar using BHI agar supplemented with 4µg of vancomycin did not show any growth at 24 hours and 48 hours. Therefore, no potential VISA and hVISA were detected.

TABLE 2: MINIMUM INHIBITORY CONCENTRATION (MIC) OF VANCOMYCIN

MIC value	No. of MRSA isolates by broth microdilution method (in µg/ml)	No. of MRSA isolates by epsilometer test (in µg/ml)
MIC =0.5µg/ml	130(86.67%)	101(67.33%)
MIC=0.75µg/ml	NA	0(0%)
MIC=1µg/ml	20(13.33%)	25(16.67%)
MIC=1.5 µg/ml	NA	22(14.67%)
MIC=2 µg/ml	NA	02(1.33%)
Total	150	150

DISCUSSION: *S. aureus* is the most common cause of morbidity and mortality in hospital settings worldwide. In clinical practice multidrug resistance in Staphylococci is rapidly increasing especially in MRSA strains. MRSA is known to be more virulent than sensitive ones and it is a cause of concern as these strains are mostly nosocomially transmitted. These strains are resistant to most of the antimicrobial agents like penicillins, β-lactam/ β-lactamase inhibitor combinations, cephems except cephems with anti-MRSA activity like parenteral ceftaroline, ceftobiprole. Increased use of glycopeptides like vancomycin, teicoplanin has led to a rapidly evolving concern of reduced susceptibility to these agents. Decreased susceptibility to vancomycin may be expressed homogeneously (VISA) or the strains may be hetero-resistant (hVISA). However the incidence of decreased vancomycin susceptibility in *S. aureus* is low in some countries such as the United States (<1%) while it is rapidly increasing in others) [6-8].

The present study revealed that of all *S. aureus* isolates 85.22% isolates were MRSA. However, other studies have reported lower rates of MRSA isolation [9, 10]. This variation may be due to the differences in the hospital environment, inclusion criteria of the samples, and the infection control practices. Most of the (50, 33.33%) MRSA isolates were obtained from patients in the age group 21-40 years followed by (44, 29.3%) 41-60 years of age. More no. of MRSA isolates were obtained from males 97 (64.67%) as compared to females 53 (35.33%). Dhillon PS *et al.,* have also reported male predominance, while almost equal no. of isolates were obtained from either gender in a study done by Darboe *et al.,*[11, 12].

119 (79.33%) MRSA were isolated from pus followed by blood 29 (19.33%) and 1 (0.67%) were biopsy tissue and tracheal/endotracheal aspirate respectively. This is in accordance with the studies conducted by Rajaduraipandi *et al.,* and Sharma NK *et al.,* [13, 14].

In the present study 32.67% MRSA strains were isolated from surgical units (surgery and plastic surgery) and 20.67% from orthopaedics ward. This is in accordance with the study conducted by Kaur G *et al.,* where 22.4% MRSA isolates were from surgery department, 11.9% from orthopaedics followed by 11.1% from plastic surgery [10].

Antimicrobial susceptibility testing of MRSA isolates performed using Kirby Bauer's disc diffusion method revealed 100% resistance to ampicillin, followed by ciprofloxacin (88%), erythromycin (79.33%), cotrimoxazole (64.67%), clindamycin (52.1%) and amikacin (51.3%). Similar findings of higher resistance among MRSA isolates to various antibiotics have been reported by various authors [10, 15]. However, lower resistance rates were reported to amikacin (32%), ciprofloxacin (63%), erythromycin (40.5%), clindamycin (43.5%) by Kaur G *et al.,* [10].

No resistance was reported against linezolid among MRSA isolates in the present study. This corroborates the findings of Kaur G *et al.,* Xu Yanlei *et al.,* Singh S *et al.,* who have also reported 100% susceptibility to linezolid [10, 15, 16]. All the MRSA isolates were susceptible to rifampicin. Similar findings were reported by Singh S *et al.,* However studies conducted by Kaur G *et al.,* Naimi HJ *et al.,* reported 13% and 9.5% resistance to rifampicin [10, 17]. In the present study susceptibility to teicoplanin was determined using MIC by Epsilometer test. Hundred percent susceptibility to teicoplanin was reported among the MRSA isolates. Kaur G *et al.,* and Xu Yanlei *et al.,* Singh S *et al.,* have also reported 100% susceptibility to teicoplanin [10, 15, 16].

In the present study all MRSA isolates were found to be susceptible to vancomycin by all the 3 methods depicting 100 percent susceptibility to vancomycin. Comparison of MIC by broth microdilution method and E-test, showed that MIC's by E test were higher as compared to broth microdilution method. Sader *et al.,* have also reported higher MIC by E-test [18]. Higher MIC in E-test as compared to the broth microdilution may be considered more reliable as it can guide vancomycin treatment responses in a better way. However a major disadvantage is that it is expensive for a resource constrained laboratory. No MRSA isolate in the present study had MIC >1μg/ml by broth microdilution however, 22 isolates had MIC=1.5μg/ml and 2 isolates had MIC=2μg/ml in E-test. These 2 MRSA strains were isolated from elderly female patients aged 78 and 65 admitted in surgery and radiation oncology department respectively. Though previous study conducted in this institute reported no resistance to vancomycin, however no study was done to evaluate the MIC of vancomycin for MRSA isolates in our institute [11]. Also the observation of the current study that 2 strains had MIC on higher side of susceptible range is a matter of concern. MIC values of vancomycin have an impact on physician's decision for treatment of MRSA infections. There have been reports of reduced susceptibility to frank resistance to vancomycin among clinical isolates of MRSA from India and other parts of world. Kaur G *et al.,* reported 3 (1.12%) isolates as VISA on the basis of MIC by broth-dilution [10].

However no VRSA was reported. In a study conducted by Kaur K *et al.,* 19 (11.7%) MRSA isolates were VISA with MIC 4-8 μg/ml and 4 (2.46%) were VRSA with MIC>16 μg/ml, however one isolate was with MIC>32 μg/ml [9]. Moses VK *et al.,* reported 46.08% MRSA isolates as VISA[19]. Song *et al.,* have also reported VISA [20]. In our study no isolate of MRSA was found to be vancomycin resistant (MIC≥16 μg/ml or showed vancomycin intermediate resistance (MIC 4- 8 μg/ml); and the minimum inhibitory concentrations of vancomycin to the strains of MRSA ranged from ≤ 0.5μg/ml to 2 μg/ml. 100 percent susceptibility to vancomycin is in accordance with other studies from India and abroad [13, 21].

In the present study, for detection of hetero-resistance, vancomycin screen agar method was also used with vancomycin concentration at 4, 6 μg/ml on different plates. Hiramatsu *et al.,* used vancomycin in a concentration of 4μg/mLwhile CDC/ CLSI has recommended vancomycin concentration of 6 μg/mL in their respective vancomycin agar screen method for the detection of hVISA and VISA [22]. None of the isolate showed any growth on BHI agar screen by Hiramatsu method at 4μg/mL vancomycin at 48 hours of incubation. Also no growth was obtained by the CDC method/ CLSI recommended method that contained 6μg/ml of vancomycin at 24-48 hours of incubation. Currently there are no standardised methods for identifying hVISA. Population analysis profile (PAP) has been proposed as the most precise method of determining heteroresistance. All the studies that aimed at detection of hVISA have employed modified PAP

method as a confirmatory test, though it is labour intensive and requires technical expertise and manual dexterity. Hiramatsu *et al.*, simplified the above method in the characterisation of the prototype hVISA and VISA strains, Mu3 and Mu50 known as simplified population analysis [22].

However, in the present study none of the MRSA isolate was screening test positive for hVISA, hence simplied population analysis could not be performed. There are limited reports regarding hVISA from India [20, 23-25].

The clinical significance of hVISA is still debated. Such strains might be responsible for vancomycin treatment failures specially the infections with high bacterial load such as bone and joint infections. Moreover hVISA might be a pre-stage of VISA. Currently antibacterial agents such as linezolid, daptomycin, tigecycline and ceftobiprole are considered alternatives for the treatment of hVISA infected patients. Though no hVISA/VISA/VRSA was found in our institute, the high prevalence of MRSA and glycopeptide use, make the widespread dissemination of these organisms a realistic possibility; once it emerges. So there is a need for continuous monitoring of MIC levels of vancomycin in MRSA and screening for hVISA in routine.

CONCLUSION: The following conclusions could be drawn from the present study which was conducted on 150 consecutive MRSA strains isolated from the various clinical specimens received and processed in the department of microbiology. There is high prevalence of MRSA in our hospital and all the MRSA isolates showed high level of resistance to multiple classes of antibiotics except linezolid, rifampicin, and glycopeptides, thereby limiting the therapeutic options. This indicates that there is an urgent need for implementation of more effective infection control practices (like, screening of MRSA carriers, isolation or cohorting of patients and colonized healthcare workers, environmental decontamination, etc.). Also, the existing infection control practices need to be re-evaluated, along with the need for a comprehensive drug resistance surveillance and containment system to optimize the usage of antimicrobial agents. Hundred percent susceptibility to vancomycin was noticed among all MRSA strains using three different methods: vancomycin screen agar using 6 μg/ml, broth micro-dilution and Epsilometer test (using Hi-media Vancomycin, Teicoplanin E strips). E test is a good option for evaluating MIC, but it is costly for a resource constrained laboratory.

No MRSA isolate was found to be heteroresistant to vancomycin (hVISA) or showing intermediate susceptibility to vancomycin (VISA). This is a good sign but MIC of few MRSA isolates towards the higher side of susceptible range warrants the routine monitoring of MIC in our institute along with regular screening for heteroresistance. This would serve as a guide to the clinicians for empirical and definitive treatment.

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Ethical Approval: The study was performed after taking approval from the instituition's Ethical committee.

CONFLICTS OF INTEREST: None

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