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Original Article

Microbiology Section

Determination of Vancomycin Minimum Inhibitory Concentration in Methicillin-resistant *Staphylococcus aureus* by Various Phenotypic Methods: A Cross-sectional Study

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ABSTRACT

Introduction: Methicillin-resistant *Staphylococcus aureus* (MRSA) has become a global threat, causing nosocomial and community-acquired infections, thus significantly contributing to morbidity and mortality. These organisms often exhibit Multidrug Resistance (MDR) to various groups of antimicrobials. The emergence of reduced susceptibility to vancomycin and resistance further exacerbates the problem, leaving clinicians with few therapeutic options to treat serious, life-threatening infections.

Aim: To determine the vancomycin susceptibility of MRSA using various phenotypic methods.

Materials and Methods: The present cross-sectional study was conducted in the Department of Microbiology, SHKM Government Medical College, Nalhar, Haryana, India, from February 2019 to January 2020. All MRSA isolates were included, totaling 66 MRSA isolates. MRSA isolates were screened using the cefoxitin disc (30 µg) according to the Clinical and Laboratory Standards Institute (CLSI) recommended disc diffusion method. The Minimum Inhibitory Concentration (MIC) of vancomycin was determined using phenotypic methods such as broth dilution, agar dilution and Epsilometer test (E-test). Demographic details such as age, sex, site of the sample and diagnosis were recorded. Statistical analysis was performed using the Pearson's product-moment correlation. The significance level was set at a probability of <0.05 with a confidence interval of 95%.

Results: A total of 66 (30.3%) isolates of *Staphylococcus aureus (S. aureus)* were identified as MRSA using standard laboratory tests and were studied for vancomycin MIC using phenotypic methods: Broth dilution, agar dilution and E-test. Vancomycin-intermediate *S. aureus* (VISA) was observed in 54.5%, 42.4% and 51.5% of MRSA by E-test, agar dilution and broth dilution, respectively. The presence of Vancomycin-resistant *S. aureus* (VRSA) was not detected in the region. When compared with broth dilution, E-test results demonstrated higher accuracy (97%) than agar dilution (90.9%).

Conclusion: The present study highlights the emergence of VISA in more than 40% of the MRSA in the region. The higher MIC of vancomycin emphasises the need for strict adherence to antibiotic prescription guidelines to prevent further emergence of vancomycin resistance.

Keywords: Antibiotic resistance, Infection control, Vancomycin-intermediate *Staphylococcus aureus*, Vancomycin-resistant *Staphylococcus aureus*

INTRODUCTION

Vancomycin was the first glycopeptide antibiotic to be discovered for Gram-positive cocci in the 1950s. However, due to its toxicity profile and the availability of beta-lactamases, it was rarely used. It was only after the large-scale emergence and spread of MRSA strains and extensive beta-lactamase resistance that this agent gained prominence. Merely 30 years later, the first clinical isolate with reduced susceptibility to vancomycin emerged [1].

Vancomycin resistance was first described in *Staphylococcus epidermidis* [2]. The first vancomycin-resistant clinical isolates of Enterococcus species were reported in Europe in 1988, which subsequently spread to most developing countries. After the emergence of vancomycin-resistant enterococci, significant concern existed regarding outbreaks of VRSA due to the acquisition of the vanA gene from enterococci [3]. The first isolate of *Staphylococcus aureus* with reduced susceptibility to vancomycin, also known as VISA, was reported from Japan in 1997 [4]. A fully resistant strain of VRSA emerged in 2002 and was first reported from Michigan, United States of America (USA). In the same year, the second VRSA strain was isolated in Pennsylvania, USA. Since then, a total of 52 VRSA strains have been reported, including 14 isolated in the USA, 16 in India, 11 in Iran, nine in Pakistan, one in Brazil and one in Portugal [5].

The structure of vancomycin is based on a heptapeptide domain in which five amino acid residues are common to all glycopeptides. The mode of action of vancomycin is bactericidal, achieved by inhibiting bacterial cell wall synthesis [6]. Vancomycin is a useful drug against MRSA. The isolates of *S. aureus* with reduced susceptibility to vancomycin are classified into three groups by the CLSI. These are Vancomycin-susceptible *S. aureus* (VSSA) with MIC ≤2 µg/mL, VISA with an MIC of 4-8 µg/mL, and VRSA with an MIC ≥16 µg/mL [7]. Since many VISA and VRSA strains cannot be detected by the disc diffusion method, MIC determination using broth/agar dilution or the E-test is recommended to confirm vancomycin resistance. MIC is defined as the lowest concentration of antibiotic able to inhibit bacterial growth. Phenotypic methods for determining MIC range from broth micro/macro dilution to agar dilution and E-test [8].

Vancomycin is the last-resort drug available for treating MRSA infections. Therefore, it is crucial to detect and report resistance or reduced susceptibility to this drug, as early as, possible. Few studies have addressed vancomycin susceptibility among *S. aureus*

in India, and none have been conducted in Haryana [9-13]. This creates a knowledge gap regarding this aspect.

The present study emphasises the need for frequent surveillance programs to promptly identify VISA and VRSA in hospitals and communities. There is a constant need for strict handwashing practices, implementation of stringent infection control measures, education of healthcare workers and other personnel involved in patient management, and the rational use of antibiotics, especially vancomycin. The message must be widely and clearly disseminated that the emergence of drug resistance in Gram-positive cocci must be contained, as we are running out of treatment options. A well-coordinated team approach involving physicians, microbiologists, and intensive care specialists is crucial for combating these infections. The findings of the current study will contribute to existing data on vancomycin susceptibility among Gram-positive cocci and will aid in formulating an antibiotic policy for the institute, helping to curb the emergence of vancomycin-resistant strains. The aim of the present study was to determine the vancomycin susceptibility of MRSA using various phenotypic methods, namely broth dilution, agar dilution and E-test. The objectives were to detect and report the presence of vancomycin resistance or reduced susceptibility among *S. aureus* in Haryana, India.

MATERIALS AND METHODS

The present cross-sectional study was conducted in the Department of Microbiology, SHKM Government Medical College, Nalhar, Haryana, India, from February 2019 to January 2020. Ethical approval was obtained from the Institutional Ethics Committee of SHKM GMC hospital, with approval letter number EC/0A. 33/2018 dated 29.10.2018, and informed consent forms were collected.

Inclusion criteria: Gram-positive isolates that were MRSA species were included in the study.

Exclusion criteria: The Methicillin-sensitive Staphylococcus species and repeat samples isolates were excluded from the study.

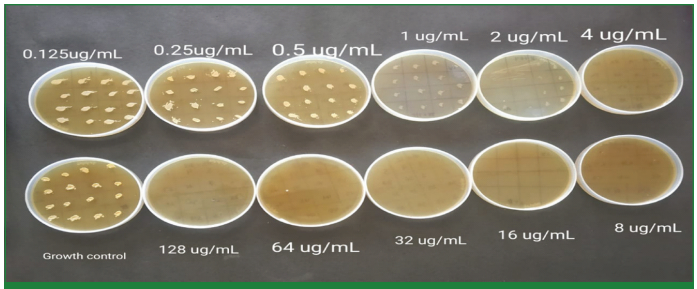
Study Procedure

The study included 66 clinical samples such as pus swabs, urine, blood, Cerebrospinal Fluid (CSF), sputum and Endotracheal Tube (ET) aspirates, which were processed in the laboratory. Demographic details such as age, sex, site of the sample and diagnosis were recorded. Risk factors such as details of prior hospitalisation and antibiotic usage within 12 months, immunosuppression, history of surgical procedures, co-morbid illnesses and the presence of any invasive device were documented. Invasive devices were defined as any foreign objects that entered the body and had an external segment (e.g., urinary catheter, central vascular access, suprapubic urinary catheter, tracheostomy tube, ET) [14].

Identification of organism: Various clinical samples (pus swab, urine, blood, CSF, sputum and ET aspirate) underwent standard laboratory procedures to isolate *S. aureus*. All clinical samples, except urine, were inoculated on blood agar and MacConkey agar plates, while the urine samples were inoculated on Cystine Lactose Electrolyte Deficient (CLED) agar plates. The plates were then incubated and identified following standard microbiological procedures. All *S. aureus* isolates underwent the cefoxitin disc diffusion test using a 30 µg cefoxitin disc in accordance with CLSI guidelines. Isolates showing a zone size of <21 mm were interpreted as MRSA and were further analysed for vancomycin MIC estimation following CLSI 2018 guidelines [7].

Determination of MIC: The MIC of vancomycin was determined by broth dilution, agar dilution method (AD) and by E-test.

a) Agar dilution method: A 0.5 McFarland standard suspension of MRSA was prepared. Mueller Hinton agar (MHA) containing vancomycin in concentrations of 0.25-256 mg/L was then prepared. Subsequently, the bacterial suspension was inoculated onto these plates [Table/Fig-1]. The plates were incubated at 35°C for 24 hours in an ambient air incubator [7].



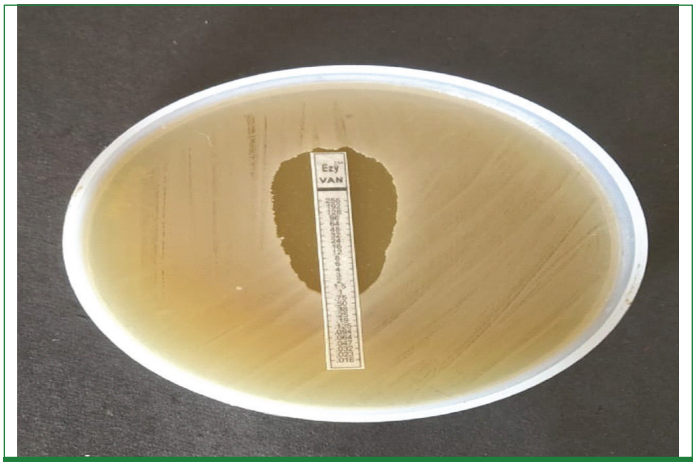
[Table/Fig-1]: Vancomycin MIC determination of MRSA isolates by agar dilution.

Interpretation: If there was no growth on the agar plate, the organism was considered sensitive to that concentration of vancomycin. If the organism formed a colony on the agar, then it was considered resistant to that concentration of vancomycin. The least concentration of antibiotic that inhibited the visible growth of the organism was taken as the MIC of that organism.

b) Epsilometer test (E-test) (vancomycin Ezy MIC™ strip) [7]:

In this test, a predefined, continuous and exponential gradient of antibiotic concentrations (0.016-256 µg/mL) was immobilised along a rectangular plastic strip.

A 0.5 McFarland standard suspension of each MRSA isolate was prepared. This was lawn cultured on the MHA agar. Then, an E-test strip was applied over the plate with the help of an applicator. The plate was incubated overnight at 37ºC, and a tear drop zone of inhibition was observed [Table/Fig-2].



[Table/Fig-2]: Vancomycin MIC determination of MRSA isolates by Epsilometer test.

The zone edge intersecting the graded strip at the given concentration of the antibiotic was interpreted as the MIC for that test organism in accordance with CLSI 2018 guidelines.

c) Broth dilution: A broth inoculum was prepared for each MRSA isolate by suspending an isolated colony in Brain Heart Infusion (BHI) broth. This broth was adjusted to achieve a turbidity equivalent to a 0.5 McFarland suspension. A vancomycin stock solution was prepared and further diluted to give a concentration range from 0.25-256 mg/L. One mL of the McFarland-adjusted inoculum was added to each tube containing 1 mL of vancomycin in the dilution series [Table/Fig-3]. The test tubes were incubated at 35°C for 24 hours in an ambient air incubator following CLSI 2018 guidelines [7].

[Table/Fig-3]: Vancomycin MIC determination of MRSA isolates by broth macrodilution.

Interpretation: The amount of growth in the tubes containing vancomycin was compared with growth-control tubes. If the organism formed turbidity in the tube, then it was considered resistant to that concentration of vancomycin. The least concentration of antibiotic that inhibited the visible growth of the organism was taken as the MIC of that organism.

Staphylococcus aureus ATCC 29213 was used as the vancomycin susceptible control strain.

A gold standard is a test that determines absolutely and without error whether the isolate is resistant or sensitive. Since an adequate gold standard is not available, we have taken broth dilution as the gold standard according to the CLSI [7].

STATISTICAL ANALYSIS

The software used for statistical analysis was the Statistical Package for the Social Sciences (SPSS) version 21.0 and Epi-Info version 3.0. The categorical variables were summarised as frequencies and percentages. The pearson's correlation coefficient and coefficient of determination were calculated. A p-value <0.05 was considered statistically significant, with a 95% confidence interval.

RESULTS

A total of 332 Gram-positive Cocci (GPC) were received in the Department of Microbiology. Out of these, 218 (65.6%) GPCs were identified as *S. aureus*. Of all, 66 (30.3%) of the *S. aureus* isolates were confirmed as MRSA using the cefoxitin disc diffusion method as per CLSI guidelines. The prevalence of MRSA was higher in males compared to females. The maximum MRSA isolates were obtained from the elderly (>50 years), followed by the middle-aged and younger population. Pus swab was the most common sample associated with MRSA, followed by urine, blood and the least number of isolates were from catheter tips. The Intensive Care Unit (ICU) and Surgery Departments were major contributors [Table/Fig-4].

Parameters		MRSA, n (%)
Age (years)	Elder (>50)	19 (28.8)
	Middle aged (31-50)	18 (27.3)
	Young (16-30)	13 (19.7)
	Paediatric (0-15)	16 (24.2)
Gender	Male	42 (63.6)
	Female	24 (36.4)
Samples	Pus swab	42 (63.6)
	Urine	12 (18.1)
	Blood	8 (12.1)
	Catheter tip	4 (6.1)
Department	ICU	22 (33.3)
	Surgery	13 (19.7)
	Orthopaedics	12 (18.1)
	Medicine	10 (15.1)
	Other departments	9 (13.6)

[Table/Fig-4]: Distribution of MRSA based on different parameters.

Based on the details collected via case history and also based on literature published in the past and the risk factors observed in ICU patients included the presence of intravenous catheters, patients on endotracheal intubation, patients on urinary catheterisation, the presence of co-morbid conditions such as diabetes and heart disease, previous hospitalisation and the use of broad-spectrum antibiotics such as third-generation cephalosporins continuously or intermittently. In medicine patients, co-morbid conditions, continuous or intermittent use of broad-spectrum antibiotics, and recurrent infections were the associated risk factors. Paediatric patients, malnutrition and prolonged hospitalisation were observed.

The antimicrobial susceptibility pattern of the MRSA isolates was studied using the Kirby Bauer disc diffusion method, as mentioned in [Table/Fig-5]. Inducible clindamycin resistance was reported in 27 (40.9%) isolates, and aminoglycoside resistance was observed in 49 (74.2%) isolates. MDR MRSA was defined as being resistant to ≥3 antibiotics belonging to different classes. Out of 66 isolates, 50 (75.7%) MRSA strains were observed to be MDR, as they were resistant to Cephalosporins, Aminoglycosides, Macrolides, Sulphonamides and Penicillins [Table/Fig-5].

Antimicrobials	MRSA, n (%)
Oxacillin	66 (100)
Cefoxitin	66 (100)
Penicillin	66 (100)
Clindamycin	52 (78.8)
Gentamycin (Aminoglycoside)	49 (74.2)
Erythromycin	49 (74.2)
Trimethoprim-sulfamethoxazole	49 (74.2)
Vancomycin	11 (16.6)
Teicoplanin	14 (21.2)
Linezolid	13 (19.7)

[Table/Fig-5]: Percentage of MRSA resistance against antimicrobial agents.

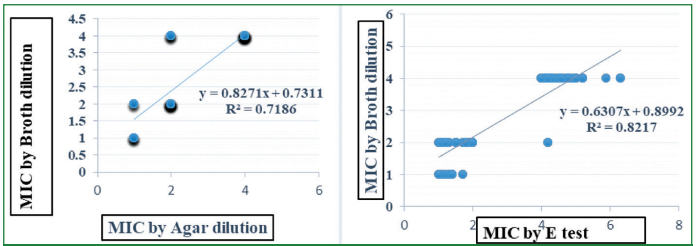
The MRSA isolates showing MIC ≤2 µg/mL were termed VSSA, and isolates which had MIC 4-8 µg/mL were termed VISA, as per the current CLSI guidelines. Thirty-two (48.5%) isolates were found to be VSSA and 34 (51.5%) isolates were VISA by broth dilution. When tested using agar dilution, 38 (57.6%) isolates were found to be VSSA, and 28 (42.4%) isolates were found to be VISA, with no VRSA. Thirty (45.5%) isolates were found to be VSSA, and 36 (54.5%) isolates were VISA by E-test [Table/Fig-6].

Variables	MIC by broth dilution n (%)	MIC by agar dilution n (%)	MIC by E-test n (%)
4-8 µg/mL VISA	34 (51.5)	28 (42.4)	36 (54.5)
≤2 µg/mL VSSA	32 (48.5)	38 (57.6)	30 (45.5)
Total	66 (100)	66 (100)	66 (100)

[Table/Fig-6]: Comparison of vancomycin MIC of MRSA isolates by E-test and agar dilution with broth dilution (n=66).

No VRSA was observed by broth dilution, as well. The incidence of VISA was 54.5%, 42.4% and 51.5% of MRSA by E-test, agar dilution and broth dilution, respectively. The authors could not demonstrate the presence of VRSA. In comparison to the broth dilution results, the E-test results were more reliable (97% accuracy) than agar dilution (90.9% accuracy).

The pearson's product-moment correlation coefficient for E-test and agar dilution with respect to the broth dilution was found to be statistically highly significant (p-value ≤0.0001) (r-value=0.91 and r-value=0.85, respectively), thus indicating a strong positive correlation. When the MIC of all MRSA isolates by agar dilution (R^2=0.7186) and E-test (R^2=0.8217) was compared with broth dilution, a large positive linear association was observed [Table/Fig-7].



[Table/Fig-7]: Comparison of vancomycin MIC by agar dilution and E-test with broth dilution (n=66).

When the MIC of broth dilution was compared with E-test, the sensitivity, specificity, positive predictive value and negative predictive value were 100%, 93.8%, 94.4%, and 100%, respectively, whereas in the case of agar dilution, it was 82.3%, 100%, 100%, and 84.2%, respectively.

DISCUSSION

Staphylococcus aureus is emerging as one of the most common agents of nosocomial infections in hospitals and also causes opportunistic infections in immune-compromised individuals. It is well-known that they can cause a variety of serious life-threatening infections such as endocarditis, bloodstream infections, wound infections and urinary tract infections. Their ability to survive under adverse environmental conditions, along with intrinsic and acquired resistance to a variety of antibiotics, makes them difficult pathogens to treat, resulting in significant morbidity and mortality. Hence, it is essential to detect them early and institute adequate therapy based on the antimicrobial susceptibility pattern. With the emergence of vancomycin resistance, the situation has worsened, leaving very few antibiotic options for the treatment of these MDR organisms [15].

During the study period, out of the 332 GPC, 218 isolates were *S. aureus*. Out of the 218 *S. aureus* isolates, 66 (30.3%) were confirmed as MRSA. Similar to the present study, Rajaduraipandi K et al., and Dar JA et al., also showed MRSA prevalence of 37.9% and 35.1%, respectively, in their studies [16,17].

While studying MRSA isolates, it was observed that the prevalence was higher in males, 42 (63.6%) than in females, 24 (36.4%). Out of the total study isolates, the majority of MRSA were isolated from the >50 years age group, 19 (28.8%). In another study conducted by Sharma S and Mall A, the maximum number of MRSA isolates was among the elder age group (>55 years) at 52%, but more in females as compared to males, which was contrary to the present study [18]. The risk factors were also observed. Callejo-Torre F et al., had reported in a multicenter cohort study of 69,894 patients that the risk factors on ICU admission included male gender, urgent surgery, trauma critical patient, immunosuppression, admission from other ICUs, hospital ward, or long-term facility and Skin and Soft Tissue Infections (SSTI) [19].

The samples that yielded the maximum MRSA were pus swabs, accounting for 42 (63.7%) of the cases, followed by urine, which accounted for 12 (18.1%) cases. This trend is similar to another study that reported the majority of samples being pus swabs (79.39%) [20]. In a separate study conducted in Iran, the majority of isolates were from blood (39.5%), followed by urine (23.7%) [21]. The prevalence of pus swabs can be explained by the fact that exposure of wounds to skin commensals is more likely with *S. aureus*, and in hospital settings, it is more likely to encounter MRSA.

In the present study, 75.7% of MRSA isolates were found to be MDR. Majumder D et al., from Assam reported that 23.2% of the MRSA isolated from clinical specimens were MDR [22]. Similarly, Anupurba S et al., from Uttar Pradesh reported 32% of MDR MRSA [23].

Vancomycin susceptibility of 66 MRSA isolates was measured using phenotypic methods, namely Kirby Bauer disc diffusion, broth dilution, agar dilution method and E-test. The incidence of VISA was found to be 54.5%, 42.4% and 51.5% by E-test, agar dilution, and broth dilution, respectively. VISA isolates could not be sent to a reference laboratory for molecular confirmation due to the Coronavirus Disease 2019 (COVID-19) pandemic. Furthermore, no VRSA was observed using the broth dilution method. In line with the broth dilution method, the E-test results were found to be more reliable than the agar dilution method. The MIC results of the broth dilution method were found to be more in agreement with the E-test as compared to the agar dilution method. The present study has clearly indicated that each susceptibility test has inherent advantages and limitations. According to CLSI guidelines, the broth dilution method was considered the gold standard [7].

This trend is alarming, as these isolates represent VISA with higher MIC values and could potentially become VRSA under further antibiotic pressure. Various national and international studies documenting VISA and VRSA, along with the strategies employed for detection, are tabulated in [Table/Fig-8] [24-29]. The present study is consistent with the majority of these studies.

Author of study	Year of study	No. of isolates studied	Isolates obtained with increased MIC of vancomycin (VISA or VRSA) n (%)	Method used
International studies				
Hasan R et al., [24] (Bangladesh)	2016	29	VISA-16/29 (55.17) VRSA-8/29 (28)	Broth microdilution method
Park JW et al., [25] (South Korea)	2019	66	VISA-14/66 (21.2)	Agar dilution, broth microdilution and E-test
Asadpour L and Ghazanfari N [26] (Iran)	2019	110	VISA- 8/110 (7.27) VRSA-3/110 (2.73)	Polymerase chain reaction
Indian studies				
Kaur K et al., [27]	2019	83	VISA-19 (11.7) VRSA-4 (2.46)	Agar dilution method
Mendem SK et al., [28]	2016	212	VRSA- 23/212 (10.84)	Agar dilution method
Solanki R and Javadekar TB [29]	2012	294	VRSA-1/294 (0.34)	E-test

[Table/Fig-8]: Summary of findings from other in-vitro studies reporting reduced susceptibility to vancomycin among *S. aureus* [24-29].

The findings of the present study will aid in formulating antibiotic policies. The current study emphasises the need for conducting frequent surveillance programs to promptly identify VISA and VRSA in hospitals and the community. There is also a constant need for strict handwashing practices, implementation of stringent infection control measures, education of healthcare workers and other personnel involved in patient management, and rational use of antibiotics, especially the last resort drugs like vancomycin.

Limitation(s)

Due to the lack of a molecular facility in the set-up, the authors were unable to perform PCR for the molecular characterisation of these VISA isolates. Additionally, the presence of heteroresistance for vancomycin could not be studied due to resource constraints.

CONCLUSION(S)

The results of the E-test were comparable to the gold standard broth dilution method rather than the agar dilution method and were found to be useful for determining the vancomycin MIC. Since performing the E-test is significantly less demanding on resources and requires less time compared to the broth dilution technique, it appears to be an acceptable alternative to broth dilution, particularly in laboratories that do not have adequate resources.

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- Plagiarism X-checker: May 31, 2023
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- iThenticate Software: Feb 06, 2024 (17%)

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- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. Yes

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