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Original Research Article

A study of hVISA among Methicillin Resistant *Staphylococcus aureus* isolates in a tertiary care hospital in Central India

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Abstract

Introduction: Since the first stain of *S. aureus* with reduced susceptibility to vancomycin was reported from Japan, there has been an increase in the number of cases with both VISA and VRSA (Vancomycin-Intermediate and Vancomycin-Resistant *S. aureus* respectively). hVISA (heterogenous Vancomycin-Intermediate *S. aureus*) strains are phenotypically susceptible to vancomycin by routine laboratory methods but contain vancomycin-intermediate subpopulations on culture. Heterogeneous VISA strains are responsible for clinical failures to vancomycin treatment of otherwise apparently susceptible *S. aureus* strains.

Materials and methods: A total of 287 *Staphylococcus aureus* clinical isolates were included in study. In-house vancomycin screen agar plate was prepared by addition of 6 mg/l vancomycin (Hi- media laboratories Pvt, Ltd. Mumbai) to brain heart infusion (BHI) agar. MIC (Minimum Inhibitory Concentration) of vancomycin was performed by E strip method. 15µg vancomycin disc was prepared in-house using pure drug procured from Hi media with code no CMS217 for detection of hVISA.

Results: Out of total 287 *Staphylococcus aureus* clinical isolates, 46.68% were found to be MRSA (Methicillin resistant *S. aureus*). A total of 8 isolates were detected by Vancomycin screen agar for screening hVISA. Detection of hVISA by 15µg vancomycin disc by disc diffusion method was performed. In this, maximum number of isolates i.e. 10 isolates were detected using MHA (Mueller Hinton Agar) 2.0 (Mac Farland), MHA plus 2% NaCl (MHAS) using both 0.5 and 2.0 (Mac Farland). Vancomycin MIC (Minimum Inhibitory Concentration) among MRSA isolates by E test showed 9 isolates to be hVISA (heterogenous Vancomycin-Intermediate *S. aureus.*

Keywords: *S. aureus*, Heterogeneous VISA, Vancomycin, Minimum Inhibitory Concentration.

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1. Introduction

Vancomycin is regarded as the first-line drug for treatment of MRSA. It was introduced in 1858 and it was thought no resistance to this antibiotic can be encountered as the resistance was very difficult to induce. [1]

In 1997, the first stain of *S. aureus* with reduced susceptibility to vancomycin was reported from Japan. Since then, there has been an increase in the number of cases with both VISA and VRSA (Vancomycin-Intermediate and Vancomycin-Resistant *S. aureus* respectively). There is a fear in the medical community as *S. aureus* causes life-threatening infections in hospitalized and non-hospitalized patients especially with VRSA and VISA.[2]

Although it is accepted that hVISA (heterogenous Vancomycin-Intermediate *S. aureus*) strains are phenotypically susceptible to vancomycin by routine laboratory methods but contain vancomycin-intermediate subpopulations on culture, there is no precise definition for hVISA. These subpopulations are typically present at frequencies of 10^{-6} to 10^{-4}. [3]

Population analysis profile (PAP) testing remains the gold standard for detection of these subpopulations One example is the modified PAP method from United Kingdom by Wootton *et al*, in which the area under the curve of a PAP test (PAP/AUC) determined in a comparison of a test organism to hVISA reference strain Mu3 (ATCC 700698) is calculated. Using this method,

vancomycin-susceptible *S. aureus* (VSSA) is defined with a PAP/AUC ratio < 0.9 and hVISA (heterogenous Vancomycin - Intermediate *S. aureus*) with a PAP/AUC ratio ≥ 0.9. [4] But it is a tedious procedure taking a long time.

The clinical significance of heterogeneous VISA is not clear. It is unknown whether levels of resistance are responsible for treatment failures or if these strains are as virulent as vancomycin-susceptible strains of *S. aureus*. It has been suggested that heterogeneous VISA strains are responsible for clinical failures to vancomycin treatment of otherwise apparently susceptible *S. aureus* strains. [4]

1.1 Aim

To study heterogenous Vancomycin-Intermediate *Staphylococcus aureus* isolates obtained from various clinical specimens by vancomycin screen agar method, 15µgm vancomycin disc and E strip.

2. Material and methods

The present study is a hospital based cross sectional study, carried out for a period between January 2014 and June 2017 in the Microbiology Diagnostic Laboratory of a tertiary care hospital. The study was approved by the Institutional Ethical Committee.

A total of 287 *Staphylococcus aureus* clinical isolates were included in the study.

Sample size was calculated by the statistician using standard guidelines, as per the public service of Creative Research Systems survey software.

Staphylococcus aureus isolates obtained from various clinical specimens like blood, pus, wound swabs, pleural / ascitic / synovial fluid, aspirates, sputum, ear swabs, urine received in Microbiology Diagnostic Laboratory for the microbiological investigations were selected for the study.

The quality control and rejection criteria for the inappropriate specimen were followed as per the standard guidelines. [5,6]

Specimens were processed within 2 hours of collection by the standard microbiological technique.[6,7]

Specimens showing pus cells with Gram positive cocci in clusters in primary smear were given special attention.

Sheep blood agar and Mac Conkey's medium were used for inoculation of all specimens. The plates were then incubated at 35 ± 2^0 Celsius for 18 - 24 hours in aerobic atmosphere.[5]

Gram positive cocci uniform in size, appearing characteristically in groups mostly, but also seen singly and in pairs were further identified by the scheme described for the identification of the gram positive cocci arranged in clusters using following tests like Catalase test, Modified Oxidase test, Furazolidone susceptibility test, Coagulase test(slide and tube coagulase), Mannitol sugar fermentation test[5].

2.1 Cefoxitin disk diffusion testing [8,9]

All the *S. aureus* isolates were subjected to cefoxitin disk diffusion test using a 30 µg disk. A 0.5 McFarland standard suspension of the isolate was prepared and lawn culture done on Mueller–Hinton Agar plates with 4% NaCl.

Plates were incubated at 37^0 C for 18 hour and zone diameters were measured.

2.2 Interpretive Criteria (in mm) for Cefoxitin Disk Diffusion Test

	Susceptible	Resistant
S. aureus	≥ 22	≤ 21

2.3 Vancomycin screen agar plate method

In-house vancomycin screen agar plate was prepared by addition of 6 mg/l vancomycin (Hi- media laboratories Pvt, Ltd. Mumbai) to brain heart infusion (BHI) agar (Hi media laboratories Pvt, Ltd. Mumbai). Inoculum suspension was prepared by transferring colonies from overnight growth on nutrient agar plate to sterile saline to produce a suspension that matches the turbidity of a 0.5 McFarland standard. A 10µl inoculum of a 0.5 McFarland suspension was spotted on the agar using a micropipette (final concentration=10^6 CFU/ml) and was incubated for 24 h at 35 °C in ambient air. Any visible growth indicated the vancomycin resistance. In addition, *S. aureus* ATCC 29213 was used as control strain.[10,11]

MIC (Minimum Inhibitory Concentration) of vancomycin was performed by E strip method.

2.4 Vancomycin disc preparation in-house.

15µg vancomycin disc was prepared in-house using pure drug procured from Hi media with code no CMS217 for detection of hVISA. Whatman number 1 filter paper was used to prepare the discs. The discs were sterilized in hot air oven and 5 µl prepared vancomycin inoculum was dispersed on every disc. The discs were dried and stored in refrigerator for further use. [12]

2.5 E – Test method to determine MIC (Minimum Inhibitory Concentration) of Vancomycin [8]

All isolates of MRSA were tested for minimum inhibitory concentration to vancomycin by E- test strips (Hi-media laboratories Pvt. Ltd. Mumbai).

0.5 McFarland standard suspension of the isolate was prepared and lawn culture done on Mueller–Hinton Agar plates. After ensuring that the agar surface was completely dry, using the applicator, E - test strip was held & its bottom edge was placed against the inoculated agar surface, holding it at an angle to the surface with the MIC scale facing upwards. It was then released onto inoculated agar surface and was made sure that the strip is in complete contact with the agar surface. Plates were incubated at 37° C for 24 hours.

2.6 Reading and Interpretation:

MIC was taken at the point where inhibition zone intersects / ellipse intersects the MIC scale on the test strip.

3. Results

A total of 287 *Staphylococcus aureus* clinical isolates were included in the study.

Table 1: Detection of Methicillin resistant *S. aureus* (MRSA) by cefoxitin (30µg) disc using Kirby Bauer method (n=287)

Cefoxitin (30µg) disk diffusion	Number
MRSA (Methicillin resistant *S. aureus*)	134(46.68%)
MSSA (Methicillin sensitive *S. aureus*)	153(53.31%)
Total (n)	287(100%)

Table 2: Vancomycin screen agar for screening MRSA for detecting hVISA

MRSA isolates inoculated	Number of isolates showing visible growth
n=134	8

A total of 8 isolates were detected by Vancomycin screen agar for screening hVISA.

Table 3: Detection of hVISA by 15µg vancomycin disc by disc diffusion method

Media/inoculum size	Incubation (hr)	MRSA (n=134)
MHA (Mueller Hinton Agar) 0.5 McF (Mac Farland)	24	9(6.71%)
	48	9(6.71%)
MHA(Mueller Hinton Agar) 2.0 McF (Mac Farland)	24	10(7.46%)
	48	10(7.46%)
MHAS (Mueller Hinton Agar plus 2% NaCl) 0.5 McF (Mac Farland)	24	10(7.46%)
	48	10(7.46%)
MHAS (Mueller Hinton Agar plus 2% NaCl) 2.0 McF(Mac Farland)	24	10(7.46%)
	48	10(7.46%)

The above table shows number of hVISA by 15µg vancomycin disc by disc diffusion method. In this, maximum number of isolates i.e. 10 isolates were detected using MHA (Mueller Hinton Agar) 2.0 (Mac Farland), MHA plus 2% NaCl (MHAS) using both 0.5 and 2.0 (Mac Farland)

Table 4: Vancomycin MIC (Minimum Inhibitory Concentration) among MRSA isolates by E test (n=134)

S. No	Vancomycin MIC	MRSA (%)
1	0.5	10(7.46%)
2	1	40(29.85%)
3	2	75(55.97%)
4	4	9(6.71%)
5	8	Nil
6	≥16	Nil

4. Discussion

The first hVISA strain, Mu3, was isolated in Japan in 1996 from a 64-year-old man with MRSA pneumonia that did not respond to vancomycin. Although the vancomycin MIC for this isolate was 4 µg/ml, the isolate contained subpopulations that were able to grow in media containing 5 to 9 µg of vancomycin/ml, thus demonstrating heterogeneous resistance. [2]

4.1 Mechanisms of resistance to vancomycin in Heterogeneous VISA (hVISA)

Currently, the mechanism of intermediate resistance in *S. aureus* is unknown. However, none of the VISA strains have been shown to have any of the *van* determinants (*van A*, *van B*, *van C1*, *van C2*, or *van C3*) that are present in VRE; thus, interspecies transfer of resistant genes is not responsible for intermediate resistance to vancomycin in *S. aureus*. VISA strains have been observed to have lower growth rates and thicker cell walls than fully susceptible strains. [13]

Hanaki *et al* [14] found that hVISA produced three- to fivefold-greater quantities of penicillin-binding proteins 2 and 2′ and three- to eightfold increased quantities of cell wall precursors than vancomycin-susceptible strains did.

Cui *et al* [15] noted that cell wall thickening correlated with increased vancomycin MICs and was a common phenotype observed in VISA strains. Increased cell wall thickness appears to play a role in resistance by sequestering vancomycin molecules in the cell wall peptidoglycan, thus reducing the susceptibility of *S. aureus* to vancomycin.

4.2 Definition of Heterogeneous VISA (hVISA)

Heterogeneous VISA (hVISA) appears to be the stage that precedes the development of intermediate-level resistance in *S. aureus* or VISA. These are strains of *S. aureus* containing subpopulations of vancomycin-intermediate daughter cells; the MICs for the parent strains of these daughter cells fall within the susceptible range of 1 to 4 µg/ml. Vancomycin creates a selective pressure that favors the outgrowth of rare, vancomycin-resistant clones leading to hVISA clones, and eventually, with continued exposure, to a uniform population of VISA clones. However, the criteria for identifying hVISA strains have not been standardized, complicating any determination of their clinical significance and role in treatment failures.[16]

4.3 Clinical relevance of hVISA

The emergence of *S. aureus* with reduced susceptibility to vancomycin arising from MRSA is of utmost concern. It is particularly worrisome if there are heteroresistant strains that appear to be susceptible by conventional testing but can express resistance at a low frequency. These strains, which are currently difficult to detect in most clinical laboratories, may potentially give rise to strains homogeneously resistant to vancomycin *in vivo*. Furthermore, it has been recently been shown that such isolates adhere to artificial surfaces 5- to 20-fold more than does MRSA, providing a further problem for its management in nosocomial environments.[17]

The clinical significance of hVISA and VISA has been difficult to assess. It is unknown whether these strains are fully virulent or perhaps even more virulent than vancomycin-susceptible strains of *S. aureus* and whether levels of resistance are responsible for treatment failures. A

variety of complicating factors make it difficult to ascertain whether the reported deaths in patients with VISA infections are directly attributable to the organism.[18]

Moore *et al* found that hVISA was associated with treatment failure in a patient with endocarditis. Paired *S. aureus* isolates (the pre-treatment and relapse clinical isolates) from this patient were tested. Both strains had similar genotypes, and the vancomycin MICs for both strains were ≤2 µg/ml.[19]

Population analysis profile (PAP) testing remains the gold standard for detection of these subpopulations One example is the modified PAP method from United Kingdom by Wootton *et al*, in which the area under the curve of a PAP test (PAP/AUC) determined in a comparison of a test organism to hVISA reference strain Mu3 (ATCC 700698) is calculated. Using this method, vancomycin-susceptible *S. aureus* (VSSA) is defined with a PAP/AUC ratio < 0.9 and hVISA with a PAP/AUC ratio ≥ 0.9.[4] But it is a tedious procedure taking a long time.

There are various screening methods to detect hVISA like vancomycin screen agar in BHI (Brain Heart Infusion Agar) with 6 µgm vancomycin, E-test on BHI with 2.0 Mac Farland standard, agar dilution, microbroth dilution method, 15 µgm vancomycin disc, PCR, Microscan method.[20]

Out of these methods we evaluated vancomycin screen agar with 6 µgm, vancomycin 15µgm disc diffusion method and E test with 2.0 Mac Farland standard.

We found 7 isolates by E Test, 8 isolates by vancomycin screen agar, 9and 10 isolates by 15 µgm disc in 0.5 Mac Farland and 2.0 Mac Farland respectively. Chi square tests were performed with the computer software SPSS (version 15). A *P* value of <0.05 was considered to indicate statistical significance.

It means any one test is not reliable for detecting hVISA. A combination of two or more methods is necessary for its detection in screening. We found the maximum no of isolates in vancomycin with 15 µgm disc that is 10 isolates.

Tenover *et al* also compared several of above methods to detect hVISA. He found Broth microdilution tests held a full 24 h were best at detecting strains along with 6µgm vancomycin screening method for reduced glycopeptide susceptibility.[21]

Lulitanond *et al* used 15 µgm vancomycin disc for screening on MHA (Mueller Hinton Agar) plate with 0.5 Mac Farland and 2 Mac Farland for 24 hrs and 48 hrs. He found this method to be useful for detecting hVISA. [12] We also found similar results.

Since the recognition of hVISA and VISA, it has been suggested that hVISA strains are responsible for clinical failures to vancomycin treatment of otherwise apparently susceptible *S. aureus* strains.

Ariza *et al* [16] reported that 86% (12 of 14) of orthopaedic surgery patients with MRSA infections whose isolates tested positive for hVISA experienced treatment failure compared to 20% (1 of 5) of patients with MRSA-positive and hVISA-negative infections. For all of these MRSA strains, the vancomycin MICs were between 1 and 4 µg/ml. Lui *et al* found E test as best method for evaluating hVISA.[20]

Tenover, *et al* selected 12 isolates of staphylococci for which the vancomycin MICs were ≥4 µg/ml or for which the teicoplanin MICs were ≥8 µg/ml and 24 control strains for which the vancomycin MICs were ≤2 µg/ml or for which the teicoplanin MICs were ≤4 µg/ml to determine the ability of commercial susceptibility testing procedures and vancomycin agar screening methods to detect isolates with reduced glycopeptide susceptibility. [21]

By PCR analysis, none of the isolates with decreased glycopeptide susceptibility contained known vancomycin resistance genes. Broth microdilution tests held a full 24 h were best at detecting strains with reduced glycopeptide susceptibility. Disk diffusion did not differentiate the strains inhibited by 8 µg of vancomycin per ml from more susceptible isolates. Most of the isolates with reduced glycopeptide susceptibility were recognized by MicroScan conventional panels and E-test vancomycin strips. Vitek results were 4 µg/ml for all strains for which the vancomycin MICs were ≥4 µg/ml. Vancomycin MICs on Rapid MicroScan panels were not predictive, giving MICs of either ≤2 or ≥16 µg/ml for these isolates. Commercial brain heart infusion vancomycin agar screening plates containing 6 µg of vancomycin per ml consistently differentiated those strains inhibited by 8 µg/ml from more susceptible strains. [21]

K. Riederer *et al* also compared E test with agar screening method for hVISA. He found agar screening method to be a good alternative to Population analysis profile (PAP) testing.[22]

5. Conclusion

The lack of a reliable method of detecting hVISA currently limits our ability to understand the role of the heterogeneous resistance phenotype in clinical treatment failures, to predict them, and to prevent them. Various studies have varying results for screening hVISA.

Important questions regarding the prevalence and clinical significance of hVISA cannot be accurately addressed without an improved, standardized, practical, and validated means of testing for the organism. There are many areas of controversy surrounding the most appropriate way of identifying hVISA. E-test and 15 µgm vancomycin discs method seems promising as an alternative to agar plating methods but will need further study before it can be adopted for standard use.

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