The Professional Medical Journal
www.theprofesional.com

DOI: 10.29309/TPMJ/2019.26.09.1328

ORIGINAL PROF-0-1328

COMPARISON OF E TEST GRD AND VANCOMYCIN SCREEN AGAR (BHI-V3 AND BHI-V4) IN DETECTION OF HETERORESISTANT VANCOMYCIN INTERMEDIATE STAPHYLOCOCCUS AUREUS (hVISA).

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Article received on:
20/11/2018
Accepted for publication:
15/03/2019
Received after proof reading:
28/08/2019

ABSTRACT… Objectives: MRSA isolates with vancomycin MIC of 1-2 µg/ml have been linked with treatment failure and heteroresistant VISA phenotype. This study was aimed at comparing two screening methods i.e. GRD Etest and Vancomycin Screen agar in detection of heteroresistance. **Study Design:** Comparative Study. **Setting:** Pathology Department of Post Graduate Medical Institute, Lahore. **Period:** May 2014 to May 2015. **Material & Methods:** The present study was carried out on 41 Methicillin Resistant Staphylococcus aureus strains isolated from different clinical specimens collected from Lahore General Hospital, Lahore. After screening for methicillin resistance, vancomycin MIC was determined by standard E test. Isolates with a vancomycin MIC of 1-2 µg/ml were screened for heteroresistance by Glycopeptide Resistance Detection (GRD) E-test and Vancomycin screen agar. Data was entered and analyzed by using SPSS version 20.0. **Results:** When compared with E test GRD, Vancomycin screen agar (V3) showed 100% sensitivity with a 95% CI 39.76% to 100% and the specificity was 65 % with a 95 % CI 47.46% to 79.79%. Its PPV was 23% and NPV was 100% with an overall diagnostic accuracy of 68%. When compared with E test GRD, Vancomycin screen agar (V4) showed a sensitivity of 75% with a 95% CI 19.41% to 99.37% and a specificity of 86.47% with a 95% CI 71.91 to 95.59%. Its PPV was 37.5% and NPV predictive value was 96.96% with an overall diagnostic accuracy of 85.36%. **Conclusion:** In developing countries like Pakistan, where E tests are costly and difficult to use in routine laboratories, a screening test, which does not miss heteroresistant VISA may be of clinical use.

Key words: E Test GRD, Heteroresistance, MRSA, Staphylococcus aureus, Vancomycin, Vancomycin Screen Agar.

Article Citation: Cheema KH, Javed I, Mushtaq S, Anwar MS. Comparison of E test GRD and vancomycin screen agar (BHI-V3 and BHI-V4) in detection of heteroresistant vancomycin intermediate staphylococcus aureus (hVISA). Professional Med J 2019; 26(9):1419-1426. **DOI:** 10.29309/TPMJ/2019.26.09.1328

INTRODUCTION

Methicillin resistant Staphylococcus aureus (MRSA) is considered as one of the most important multi-resistant bacterial pathogen and a significant cause of hospital-acquired infections globally.[1] Vancomcyin has served as a keystone antibiotic for the treatment of severe MRSA infections for the last twenty years.[2]

Reduced Vancomycin susceptibility was first reported in Japan in 1996 and was designated as Vancomycin intermediate susceptible Staphylococcus aureus (VISA). The first Vancomycin resistant Staphylococcus aureus (VRSA) was reported in America, followed by thirteen confirmed cases of VRSA worldwide.[3,4,5]

Another population of Staphylococcus aureus, known as heteroresistant VISA emerged in 1996, which is defined as the presence of subpopulation of VISA within the population of MRSA at the rate of one organism per 10^5 to 10^6 organisms.[3,7]

The estimated prevalence of hVISA ranges from 1.3% to 27%.[7] hVISA infection is associated with prolonged fever and bacteremia thus lengthening hospital stay and an inability of vancomycin to cure the infection.[8]

Major factors leading to emergence of VISA and hVISA phenotypes are cell wall changes, which include reduced turnover of the cell wall, decreased autolysis, and in certain instances, triggered cell wall synthesis. All of these events

lead to decreased access of vancomycin to its functioning site that is confined at the septum of division.[9]

Infection caused by hVISA pose a clinical challenge, as these bacteria are usually not detected in the laboratory because these are considered vancomycin susceptible by routine MIC tests.[7]

Standardized susceptibility methods, like broth micro dilution, standard E test methods and agar dilution are unable to detect heteroresistant VISA probably due to small inoculums used or the poor growth support on Mueller-Hinton agar plates, or maybe both.[10]

As there is no single genetic mechanism responsible for hVISA and the genetic determinants are still under evaluation, one has to rely on the phenotypic methods for its detection.[11]

Detection of hVISA, therefore, relies on the testing of a higher inoculum and techniques to enhance the growth of this sub-population, such as longer incubation period (up to 48 hours) or using additional nutritive media (e.g. BHI agar and Mueller-Hinton agar with 5 % blood).[9]

PAP-AUC has been considered a gold standard but it is time-consuming, laborious and not suitable for routine microbiology laboratory.[1]

Alternatively, various screening methods, including Macro E test method (MET), Glycopeptide Resistance Detection (GRD) E test and agar screening methods have been developed.[9]

The burden of heteroresistant Vancomycin Intermediate Staphylococcus aureus and the issue of reduced vancomycin sensitivity are still unknown in Pakistan. It is vital for the clinicians to update themselves with the changing trends of vancomycin sensitivity.

Therefore, our study was aimed at detection of heteroresistance among clinical isolates of MRSA and to compare GRD E test and vancomycin screen agar in detection of hVISA.

PATIENTS AND METHODS

Our study was a Comparative Study carried out at the Pathology Department of Post Graduate Medical Institute, Lahore from May 2014 to May 2015. Clinical isolates of Staphylococcus aureus that were Methicillin resistant on Cefoxitin disc diffusion method were included in the study. Sample size was calculated by formula for determination of sample size for proportion where the assumed proportion of heteroresistance in MRSA was 12 % with 1.96 for 95% confidence level and acceptable difference of 10%. Non-probability consecutive sampling was used.

In the present study, we isolated 41 Methicillin-resistant Staphylococcus aureus from various clinical specimens including, blood, wound swab, urine, pus, sputum and aspirates of patients admitted in Lahore General Hospital, Lahore.

All specimens were brought to microbiology laboratory, Department of Pathology, PGMI, Lahore for culture and sensitivity within two hours of collection. These specimens were inoculated on blood agar and MacConkey agar plates and incubated at 37 °C for 24 hours.

Identification of Staphylococcus aureus was done by observing colony morphology, Gram staining, catalase and coagulase test.

Antimicrobial sensitivity was carried out on all isolates according to modified Kirby-Bauer method and interpreted according to CLSI 2015 criteria.

Resistance to methicillin was detected by using Cefoxitin disc diffusion method. MRSA ATCC 43300 and MSSA ATCC 25923 control strains were used. Interpretation was done according to CLSI criteria 2015.

All MRSA isolates were subjected to E test for determination of Vancomycin MIC. Interpretation of the MICs was done according to CLSI 2015 guidelines.

All MRSA isolates having MIC ≤ 2 µg/ml as determined by Vancomycin E strips were subjected to further testing for heteroresistance by using Glycopeptide Resistance Detection (GRD) E test and Vancomycin Screen agar (BHI-V3 and BHI-V4).

Glycopeptide Resistance Detection (GRD) E test

E-test GRD is a double-sided predefined gradient (0.5-32 µg/ml) of vancomycin and teicoplanin for the detection of VISA or hVISA phenotypes. MIC test strip GRD consists of a screening method and can be tested with 0.5 McFarland and Mueller-Hinton blood agar plates.

GRD E test strips, manufactured by Liofilchem Diagnostics were used. The strips were stored at -20 °C.

A 0. 5 McFarland turbidity standard suspension prepared from well isolated colonies of the bacterial isolates was swabbed onto the surface of Mueller-Hinton blood agar plates.

GRD E strips were applied on the Mueller-Hinton blood agar plates and incubated at 35 °C for up to 48 hours.

The zone of the GRD E test strip was read at 24 hour and 48 hour of incubation. MIC showing complete inhibition of growth was recorded.

The isolate was considered a potential hVISA if the MIC was ≥ 8 µg/ml for either vancomycin or teicoplanin (Figure-1).

Reference control strains used with each batch testing include ATCC 29213 (VSSA), ATCC 700698 (hVISA; Mu3) and ATCC 700699 (VISA; Mu50).

Vancomycin Screen agar (BHI-V3 & BHI-V4)

These are screening media to which Vancomycin is added to Brain Heart Infusion (BHI) agar in order to achieve a final concentration of 3 µg/ml and 4 µg/ml. These are termed as BHI-V3 and BHI-V4 respectively.

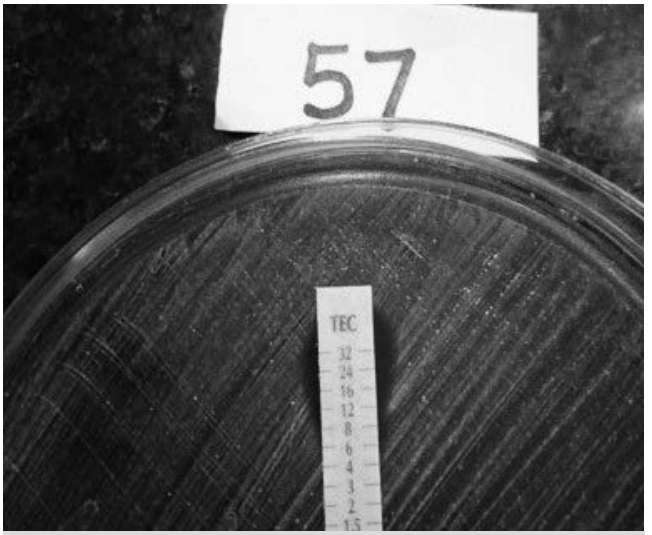

Figure-1. E test GRD showing a positive test (hVISA) MIC= 8µg/ml

A 10 µl of 0.5 McFarland standard suspension of the organism was spot inoculated onto 3 µg/ml Vancomycin BHI agar plate (V3) and 4 µg/ml Vancomycin BHI agar plate (V4) and incubated at 35°C for up to 48 hours.

The isolate was considered possible hVISA if a countable number of colonies (1-30) were apparent within 48 hours on either V3 or V4 screen agar.

In case of no growth, the isolate was considered susceptible to Vancomycin.

Reference control strains used with each batch testing include ATCC 29213 (VSSA), ATCC 700698 (hVISA; Mu3) and ATCC 700699 (VISA; Mu50).

Data was entered and analyzed in SPSS 20.0. A 2×2 table was generated to validate Vancomycin screen agar taking GRD E test as standard. Sensitivity, specificity, negative (NPV) & positive predictive values (PPV) and diagnostic accuracy (DA) was calculated. Chi–square test was applied to rule out role of chance while testing validity of Vancomycin screen agar method. P value < 0.05 was taken as significant.

RESULTS

The present study was conducted on a total of

41 consecutive clinical isolates of MRSA from patients admitted in Lahore General Hospital, Lahore. All isolates were recorded as Gram-positive, catalase and coagulase test positive and were resistant to cefoxitin. The frequency distribution of Vancomycin MIC for 41% isolates of MRSA is shown in Figure 2. Twenty-nine (71%) isolates had a MIC of 2 µg/ml, whereas for 12 (29%) isolates, the MIC was 1 µg/ml.

Forty-one percent of MRSA isolates showed on growth on V3 screen agar, whereas, 20 % of MRSA isolates showed growth on V4 screen agar (Figure-3 and Figure-4).

It is apparent that V3 screen agar is more useful for detection of possible hVISA among MRSA isolates as compared to V4 screen agar as shown in Table-I (P-value < 0.001).

To determine sensitivity and specificity of V3 screen agar and V4 screen agar for detection of possible hVISA, all MRSA isolates were tested by E test GRD.

The results of E test GRD are shown in Table-II. An isolate showing MIC value of ≥ 8 µg/ml was considered a possible hVISA. Four (9.75%) MRSA isolates were hVISA positive, whereas 37 (90.2%) had a MIC less than 8 µg/ml and therefore were taken as hVISA negative.

The comparison of V3 and V4 screen agar with E test GRD is shown in Table-III and Table-IV.

When compared with E test GRD, Vancomycin screen agar (V3) showed 100% sensitivity with a 95% CI 39.76% to 100% and the specificity was 65 % with a 95 % CI 47.46% to 79.79%. The positive predictive value of the test was 23% and the negative predictive value was 100%. The overall diagnostic accuracy of V3 screen agar was 68%.

When compared with E test GRD, Vancomycin screen agar (V4) showed a sensitivity of 75% with a 95% CI 19.41% to 99.37% and a specificity of 86.47% with a 95% CI 71.91 to 95.59%. The positive predictive value of the test was 37.5% and the negative predictive value was 96.96%. The overall diagnostic accuracy of V4 screen agar was 85.36%.

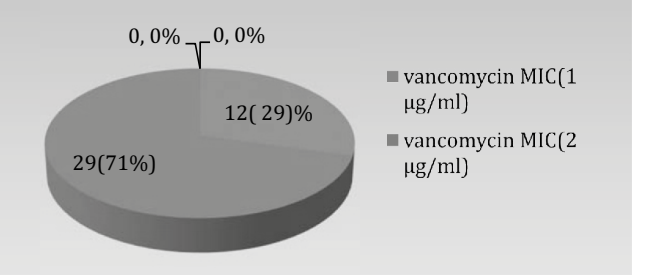

Figure-2. Distribution of MRSA isolates according to MIC for Vancomycin (n=41)

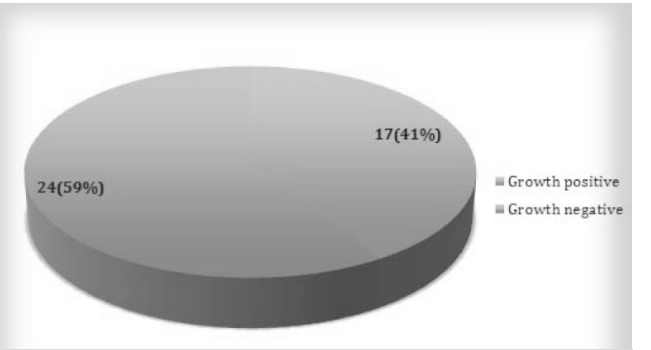

Figure-3. Frequency of hVISA in MRSA isolates by (V3) vancomycin screen agar (n=41)

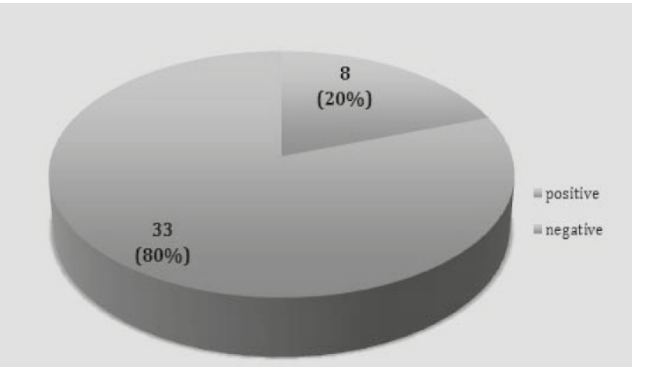

Figure-4. Frequency of hVISA in MRSA isolates by (V4) vancomycin screen agar (n=41)

Vancomycin Screen Agar	hVISA Positive	Percentage (%)
V3	17	41.4*
V4	08	19.5

Table-I. Comparison of V3 screen agar and V4 screen agar in determination of hVISA among MRSA isolates (n=41)
*** P –value significantly higher as compared to V4 (P-value <0.001)**

hVISA by Etest GRD	No. of Isolates	Percentage (%)
Positive*	04	9.75
Negative**	37	90.2

Table-II. Frequency of hVISA among MRSA isolates as determined by E test GRD (n=41)

***MIC ≥ 8 µg/ml**
**** MIC ≤ 8 µg/ml**

		hVISA Positive by Etest GRD		Total
		Positive	Negative	
hVISA positive by growth on V3 screen agar	Positive	04	13	17
	Negative	0	24	24
Total		04	37	24

Table-III. Comparison of V3 screen agar and E test GRD for detection of hVISA among MRSA isolates (n=41)

Remarks:

- Sensitivity: 100% (95% CI 39.76% to 100%)
- Specificity: 65% (95% CI 47.46% to 79.79%)
- Positive predictive value: 23% (95% CI 6.81% to 49.90%)
- Negative predictive value: 100% (95% CI 85.75% to 100%)
- Diagnostic accuracy: 68%

		hVISA positive by Etest GRD		Total
		Positive	Negative	
hVISA positive by growth on V4 screen agar	Positive	03	05	08
	Negative	01	32	33
Total		04	37	41

Table-IV. Comparison of V4 screen agar and E test GRD for detection of hVISA among MRSA isolates (n=41)

Remarks:

- Sensitivity: 75% (95% CI 19.41% to 99.37%)
- Specificity: 86.48% (95% CI 71.91% to 95.59%)
- Positive predictive value: 37.5% (CI 8.52% to 75.51%)
- Negative predictive value: 96.96% (CI 84.67% to 99.93%)
- Diagnostic accuracy: 85.36%

DISCUSSION

Multiple phenotypes with decreased sensitivity to glycopeptides are on the rise due to increased vancomycin usage in critically ill patients with MRSA infections.[12]

It is possible that vancomycin treatment failures against putatively vancomycin-susceptible Staphylococcus aureus are due to undiagnosed VISA or hVISA.[13]

In the present study, we used both BHI-V3 and BHI-V4 screen agar for detection of hVISA and compared it with E test GRD.

Table-I shows that vancomycin screen agar with 3 µg/ml (V3) was more useful in detection of hVISA as compared to V4 screen agar (P-value <0.001). In order to determine the accuracy of hVISA detection by V3 and V4 screen agar, we used E test GRD as the gold standard. Though Population Analysis Profile-Area under the Curve is considered the ideal test for hVISA detection, it is, however, laborious, costly and difficult to perform in our setup. Since, E test GRD has comparable sensitivity and specificity with PAP-AUC, we used this test as the gold standard in our study. The initial studies carried out on detection of hVISA by E test GRD showed it to be an important and practical tool that could be used in the laboratory. Yousuf et al compared E test GRD with the reference method and found the sensitivity and specificity to be 84% and 95% respectively.[14] Studies that followed, confirmed the high specificity of E test GRD ranging from 85.8% to 97% but lower sensitivity levels ranging from 57% to 82%.[15,16]

Presently, most of the studies recommend using two different methods for more accurate detection of VISA/hVISA strains (CDC).

When compared with E test GRD, V3 screen agar (BHI-V3) showed 100% sensitivity. Despite having an excellent sensitivity, there were 13

vancomycin-susceptible isolates that grew on this medium, corresponding to 65 % specificity.

Schick et al conducted a study in which they compared V3 screen agar and Macro E test (MET) for hVISA detection. V3 screen agar showed good sensitivity but with a high false positivity rate in their study.[17]

V4 screen agar showed a sensitivity of 75% and a specificity of 86.5% (Table-IV). Eight isolates grew on BHI-V4 screen agar. Out of these 8 isolates, 3 were confirmed as hVISA by E test GRD. However, there was one isolate, which showed growth on V3 screen agar and was positive by E test GRD, but did not grow on V4 screen agar.

Reiderer et al conducted a study in 2011 in Michigan, in which they compared the performance of Macro E test method (MET), E test GRD and vancomycin screen agars with 3 µg/ml (BHI-V3) and 4 µg/ml (BHI-V4) of vancomycin in detecting hVISA phenotypes.[18] The sensitivity and specificity of V3 screen agar in their study was comparable to ours i.e. 100% and 94% respectively, whereas the sensitivity and specificity of V4 screen agar was 28% and 100% respectively. This finding was in contrast to ours, in which the specificity of V4 screen agar was 86%.

Rizk and Zaki reported a similar finding in 2007 in Egypt, in which they found the sensitivity and specificity of V4 screen agar to be 28.6% and 100% respectively.[19]

However, another study conducted by Satola et al in 2011 compared BHI-V4 with MET and E test GRD and found the sensitivity and specificity to be 91% and 94% respectively.[19]

In conclusion, four hVISA (9.75%) strains have been detected among MRSA isolates in our study with a vancomycin MIC 2 µg/ml. Two hVISA strains were isolated from blood, whereas two were isolated from pus samples. This finding is of great concern as these isolates have a vancomycin MIC in the susceptible range (≤ 2 µg/ml) and can result in therapeutic failures in such patients.

In developing countries like Pakistan, where E tests are costly and difficult to use in routine laboratories, a screening test, which does not miss hVISA can be used in the detection of hVISA . Thus, the screen agar is an efficient and cost-effective approach to test MRSA isolates in the routine laboratory. However, it needs confirmation to rule out any false positives.

More studies at different institutions are required in order to define the burden of hVISA in our country and its clinical implications. As there is no single genetic mechanism for detection of hVISA, we have to rely on the phenotypic detection methods. The main limitation of our study was that we were unable to use PAP-AUC as the reference method because of its high cost in our setup.

CONCLUSION

The phenomenon of heteroresistant VISA is still unknown in Pakistan. This study is the first attempt at detection of hVISA among MRSA. We recommend BHI-V3 agar to be used as a screening method for detection of hVISA. If there is no growth on this agar, it is certain that the isolate is susceptible to vancomycin and can be reported as such. We strongly feel that this is a cost-effective algorithm for hVISA detection and can be incorporated in routine laboratory workflow.

ACKNOWLEDGEMENTS

This research was supported by the Postgraduate Medical Institute, Lahore.

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2	Iffat Javed	Study design & concept, Critical review.	
3	Suhaila Mushtaq	Analysis & Interpretation.	
4	M. Saeed Anwar	Analysis & interpretation, Critical review.	