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Detection of Methicillin Resistant *Staphylococcus aureus* with Reduced Susceptibility to Vancomycin

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ABSTRACT

Background: As there is no molecular-based assays available for the detection of hVISA and VISA. However, increasing amounts of data support a number of methods for the screening and confirmation of heterogeneous vancomycin intermediate S. aureus (hVISA) and vancomycin intermediate S. aureus (VISA) infection. The vancomycin MIC result alone is unable to accurately distinguish hVISA from VSSA isolates, and the use of MIC testing alone will fail to detect hVISA strains that are relatively common among isolates of *Staphylococcus aureus (S. aureus*) with broth MICs of 2 g per ml.

Objective: The aim of the present work was to detect the efficacy of phenotypic and automated methods for detection of MRSA with reduced susceptibility to vancomycin. It aimed also, to determine the best MIC concentration in vancomycin screening agar for detection of VISA among MRSA isolates.

Methods: One hundred MRSA isolates were obtained from 100 patients from different departments of Ain Shams University Hospitals during the period from October 2015 to the end of April 2016. They were isolated from different clinical specimens; sputum, wound swabs, blood, pus, urine, and body fluid that were referred to central microbiology laboratory for routine culture and sensitivity. Detection of *S. aureus* with reduced susceptibility to vancomycin was done by vancomycin screening agar with different concentrations 2,4,6 ug/ml with and without casein, MIC broth microdilution method for vancomycin according to ***CLSI 2015***, and Vitek 2 automated system for determination of vancomycin MIC.

Results: Out of 100 MRSA isolates, vancomycin screening agar 2ug/ml with casein showed highest detection rate for VISA isolates (48 %) among other screening agars. Vancomycin screening agar 6 ug/ml without casein gave the lowest detection rate (29%). So, adding casein to vancomycin screening agar did not increase detection of VISA in any of vancomycin screening agar except for that with 2ug/ml vancomycin. Vancomycin screening agar 2ug/ml with casein gave the best sensitivity among all vancomycin screening agar tested. VITEK 2 system failed to detect any isolates with reduced susceptibility to vancomycin. They were sensitive to linezolid (100%) followed by tigecyclin (99%) then Quinupristin-dalfopristin (91%). However, most of the isolates were resistant to tetracycline (85%) followed by gentamicin (80%) then ciprofloxacin (63%).

Conclusion: BHI agar with 2ug/ml vancomycin and 16 g/l casein is a reliable, easy to perform, and inexpensive method to screen large number of *S. aureus* isolates for detection of reduced susceptibility to vancomycin on a daily basis. Applying quadruplicate technique in vancomycin screening agar may increase the yield for detection of VISA isolates. Although vancomycin screening agar 6 ug/ml is recommended by CLSI as a screening method for detection of VISA, yet it did not perform well and underestimated VISA isolates. VITEK 2 system is not an appropriate method for detection of *S. aureus* with reduced susceptibility to vancomycin (VISA). MRSA isolates with reduced susceptibility to vancomycin can be treated effectively with Linezolid.

Keywords: VISA, h VISA (heterogeneous vancomycin intermediate S. aureus), vancomycin screening agar, Minimal Inhibitory Concentration.

INTRODUCTION

S. aureus is a major cause of hospital acquired infections, causing high morbidity and mortality throughout the world. The proportion of methicillin resistant *Staphylococcus aureus (*MRSA) has risen worldwide during the last decades. The recommended treatment for multiresistant MRSA are glycopeptides, particularly vancomycin[1]**.** In January 2006, the Clinical and Laboratory Standards Institute (CLSI) updated MIC breakpoints for vancomycin susceptibility testing for *S. aureus* such that an MIC less than 2 ug/L is considered to represent susceptibility to vancomycin, 4-8 ug/L intermediate susceptibility and greater than 16 ug/L resistant to vancomycin Additionally, in 2009, the CLSI altered the guidelines for *Staphylococci* such that disk diffusion was no

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longer an acceptable means for testing vancomycin susceptibility in these organisms[2].

According to CLSI, broth microdilution (BM) is considered the gold standard to determine vancomycin MIC. However, because it is time consuming, a considerable number of clinical laboratories do not use it as routine methodology. Other techniques have been widely used, with variable sensitivity and specificity, such as E-test and automated systems.[3]

The definition and optimal laboratory detection of hetergenous vancomycin intermediate *S. aureus* (hVISA) remain uncertain. Essentially, hVISA isolate is *a S. aureus* isolate with a vancomycin MIC within the susceptible range when tested by routine methods, but where a proportion of the population of cells are in the vancomycin-intermediate range .[4]

A variety of alternative methods for detection of the heteroresistant phenotype have been evaluated with varying success e.g. standard E-test, E-test GRD, E-test macromethod, BHI screen agar plates .[5]

AIM OF THE WORK

The aim of the present work was to detect the efficacy of phenotypic and automated methods for detection of MRSA with reduced susceptibility to vancomycin. It aimed also, to determine the best MIC concentration in vancomycin screening agar for detection of VISA among MRSA isolates

PATIENTS AND METHODS

One hundred MRSA isolates recovered from clinical specimens referred to central microbiology laboratories of Ain Shams University Hospitals for routine culture and susceptibility were collected during the period from October 2015 to April 2016. Ethical committee for sharing in the study was taken from the patients .The isolates were stored in a Tryptic soya broth media at-70 ^{o}C until use and were subcultured twice on blood agar plates and incubated aerobically at 37^{o}c for 24 hours to be ready for use .

All isolates were inoculated on:

Vancomycin screening agar containing 2, 4, 6 ug/ml vancomycin with and without casein for detection of *S. aureus* with reduced susceptibility to vancomycin. , as the addition of supplements that enhance growth of hVISA such as casein could potentially improve their detection by screen agar methods. Also, it is reported that using the quadruplicate technique (i.e., use of four 10ul droplets of 0.5 McFarland *S. aureus* suspension) and the incubation of the plates for full 48 hours may enhance the sensitivity of detection of VISA.[5] so we did so, Broth microdilution method for determination of Vancomycin MICs as reference method and Vitek 2 automated system for determination of vancomycin MIC (Biomerieux, France).

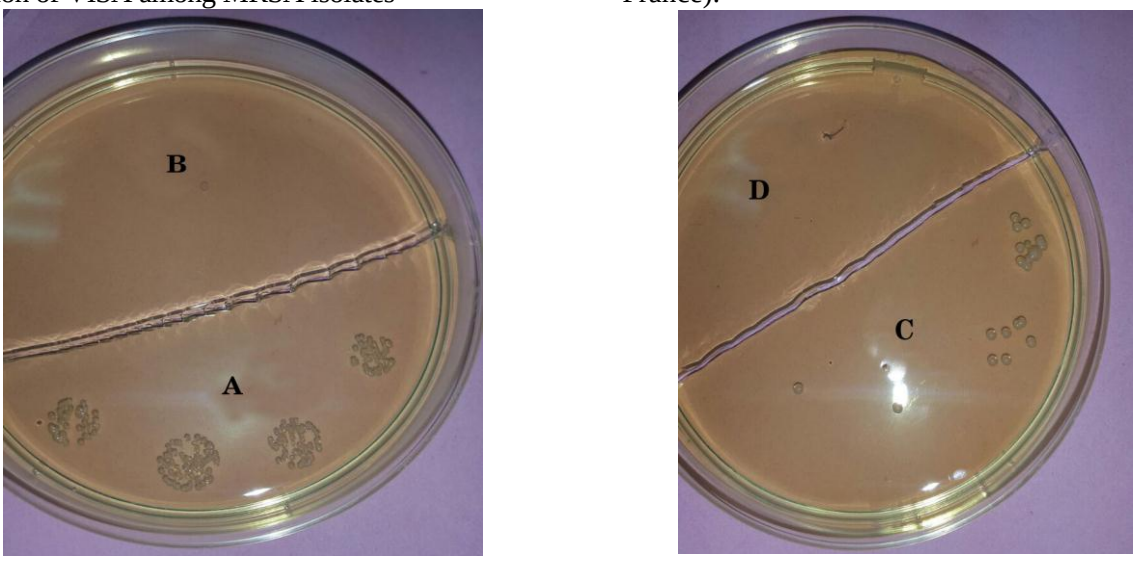

Figure (1): Growth of VISA on vancomycin screening agars

A ,C : show growth in four droplets on vancomycin screening agar 2 ug/ml with casein consider as VISA.
B,D: No growth consider as VSSA.

Statistical analysis

Statistical analysis was carried out using SPSS statistics software version 20. According to the type of data, data was presented and analyzed:

A. Descriptive statistics:

Categorical variables were described using frequencies and percentages.

B. Analytical statistics

To assess the performance of a diagnostic test, ***sensitivity***; defined as the probability that the test is positive in patients with the disease and ***specificity***; defined as the probability that the test is negative in patients without the disease were determined.

The study was approved by the Ethics Board of Ain Shams University.

RESULTS

Out of 100 MRSA isolates, 30/100 (30%) were VISA (21/30 VISA MIC =8 ug/ml), (9/30 VISA MIC = 4 ug/ml) and 70/100 (70%) were VSSA (VSSA MIC ≤ 2ug/ml) by broth microdilution test.

Vancomycin screening agar 2ug/ml with casein detected 30/30 of isolates that were VISA by BMD with 100% sensitivity and 74.3% specificity. Vancomycin screening agar 2ug/ml without casein detected 20/30 0f isolates that were VISA by BMD with 66.7% sensitivity and 85.7% specificity. The agar without casein failed to detect 10 isolates out of 30 (33.3%) that were VISA positive by BMD. Vancomycin screening agar 4ug/ml with casein detected 20/30 0f isolates that were VISA by BMD with 66.7% sensitivity and 84.3% specificity. Vancomycin screening agar 4ug/ml without casein detected 20/30 0f isolates that were VISA by BMD with 66.7% sensitivity and 85.7% specificity. Both agar 4ug/ml with and without casein failed to detect 10 isolates out of 30 (33.3%) that were VISA positive by BMD. Vancomycin screening agar 6ug/ml with casein detected 20/30 0f isolates that were VISA by BMD with 66.7% sensitivity, 85.7% specificity and failed to detect 10 isolates out of 30 (33.3%) that were VISA positive by BMD. Vancomycin screening agar 6ug/ml without casein detected 19/30 0f isolates that were VISA by BMD with 63.3% sensitivity, 85.7% specificity and failed to detect 11 isolates out of 30 (36.7%) that were VISA positive by BMD.

Table (1): Comparison between vancomycin screening agar 2ug/ml with casein and BMD.

	Broth microdilution (BM)	
Vancomycin screening agar 2ug/ml concentration with casein	**Positive**	**Negative**
Positive	30 (100%)	18 (25.7%)
Negative	0 (0%)	52 (74.3%)
Total	30 (100%)	70 (100%)

Table (2): Comparison between vancomycin screening agar 2ug/ml without casein and BMD.

	Broth microdilution (BM)	
Vancomycin screening agar 2ug/ml concentration without casein	**Positive**	**Negative**
Positive	20 (66.7%)	10 (14.3%)
Negative	10 (33.3%)	60 (85.7%)
Total	30 (100%)	70 (100%)

Table (3): Comparison between vancomycin screening agar 4ug/ml with casein and BMD.

	Broth microdilution (BM)	
Vancomycin screening agar 4ug/ml concentration with casein	**Positive**	**Negative**
Positive	20 (66.7%)	11 (15.7%)
Negative	10 (33.3%)	59 (84.3%)
Total	30 (100%)	70 (100%)

Table (4): Comparison between vancomycin screening agar 4ug/ml without casein and BMD.

	Broth microdilution (BM)	
Vancomycin screening agar 4ug/ml concentration without casein	**Positive**	**Negative**
Positive	20 (66.7%)	10 (14.3%)

Negative	10 (33.3%)	60 (85.7%)
Total	30 (100%)	70 (100%)

Table (5): Comparison between vancomycin screening agar 6 ug/ml with casein and BMD.

	Broth microdilution (BM)	
Vancomycin screening agar 6ug/ml concentration with casein	**Positive**	**Negative**
Positive	20 (66.7%)	10 (14.3%)
Negative	10 (33.3%)	60 (85.7%)
Total	30 (100%)	70 (100%)

Table (6): Comparison between vancomycin screening agar 6ug/ml without casein and BMD.

	Broth microdilution (BM)	
Vancomycin screening agar 6ug/ml concentration without casein	**Positive**	**Negative**
Positive	19 (63.3%)	10(14.3%)
Negative	11 (36.7%)	60 (85.7%)
Total	30 (100%)	70 (100%)

Table (7): Diagnostic performance of vancomycin screening agar with different concentrations 2,4,6 ug/ml of vancomycin, with and without casein.

Vancomycin screening agar concentrations	**Sensitivity**	**95% CI**	**Specificity**	**95% CI**	**PPV**	**NPV**
2ug/ml	66.7	(48.8,80.8)	85.7	(75.7,92.1)	66.6	85.7
2ug/ml (with casein)	100	(88.7,100)	74.3	(63,83.1)	62.5	100
4ug/ml	66.7	(48.8,80.8)	85.7	(75.7,92.1)	66.6	85.7
4ug/ml (with casein)	66.7	(48.8,80.8)	84.3	(74,91)	66.6	85.7
6ug/ml	63.3	(45.6,78.1)	85.7	(75.7,92.1)	65.5	84.5
6ug/ml (with casein)	66.7	(48.8,80.8)	85.7	(75.7,92.1)	66.6	85.7

DISCUSSION

The only CLSI vancomycin screen agar method in place for clinical isolates for the detection of vancomycin-resistant *S. aureus* (VRSA) and possibly VISA is BHI agar containing 6 mg/liter vancomycin (BHIA6V), a method originally established for detection of vancomycin resistance in enterococci [6] ,however this method have a very low sensitivity for the detection of h-VISA . [5]

The CDC recommends this as a supplemental test for VISA detection, with the caveat that strains with vancomycin MICs of 4 ug/ml will not be reliably identified and screen agar plates with a lower concentration of 3 g/ml vancomycin have a very high false positive rate. [7,6]

In our study, Broth microdilution method detected 30% positive VISA isolates, (21/30 VISA MIC =8 ug/ml), (9/30 VISA MIC = 4 ug/ml). Our results were nearly similar to the study by sewson in 2009[9] who used 129 isolates of S. aureus, detected 34.9% VISA isolates by Clinical and Laboratory Standards Institute broth microdilution.

In our study, vancomycin screening agar 2ug/ml with and without casein detected 48% and 30% respectively as VISA with sensitivity 100% and 66,7% as compared to broth microdilution method as the gold standard. Vancomycin screening agar 2ug/ml with casein gave the best sensitivity among all vancomycin screening agar tested. The 48 isolates that were detected as VISA by screening agar 2 ug/ml vancomycin with casein were 18 isolates were MIC=8 ug/ml and 12 isolates were MIC=4 ug/ml ,the false positive 18 isolates, 2 isolates were MIC =2 ug/ml, 10 isolates were MIC=1ug/ml and 6 isolates were MIC =0.5ug/ml). Although vancomycin screening agar 2ug/ml with casein detected all isolates that were VISA positive by BMD, however that without casein failed to detect 10/30 (33.3%) of them with specificity 74.3% and 85.7% respectively. This could be due to absence of casein which is reported that it increase sensitivity of detection of VISA. As the false positive result by this method were in the range of 0.5-2 ug/ml which fall in the sensitive range, they may be h VISA but we could not confirm that as we didn't use PAP-AUC.

In the present study, using vancomycin screening agar 4 ug/ml with and without casein detected 31%, 30 % as VISA with sensitivity 66.7%,specificity 84.3 %, 85.7% respectively. The false positive for both were in the range of 0.5-2 ug/ml which may be h VISA but that could not be confirmed. The 10 false negative isolates for both may be due to increase concentration of vancomycin 4 mg/liter.

Satola and his coworkers [5] collected 140 MRSA blood isolates with vancomycin MICs of 2 ug/ml by reference broth microdilution and screened for reduced susceptibility to vancomycin using PAP-AUC as the reference method, where they detected 15% h-VISA. They evaluated brain heart infusion (BHI) screen agar containing 16 g/liter casein and 4 mg/liter vancomycin for the detection of hVISA. Vancomycin screening agar 4ug/ml with casein was 90% sensitive and 95% specific with a 0.5 McFarland inoculum and 100% sensitive and 68% specific with a 2.0 McFarland inoculum.

A study by ***Riederer and his coworkers*** [10] compared the performance of two E-test screening methods macromethod [MAC] and glycopeptide resistance detection [GRD] plus brain heart infusion (BHI) agars supplemented with 3(BHI-V3) and 4(BHI-V4) mg/liter vancomycin in detecting hVISA and/or VISA phenotypes. Using 485 saved MRSA blood isolates with vancomycin MICs of 0.5 to 4 ug/ml available for testing. The modified PAP/AUC was measured for all isolates revealing seven VISA and 33 hVISA phenotypes. Growth on BHI-V3 was noted in all hVISA/VISA and 24 (5.4%) vancomycin susceptible MRSA isolates. Growth on BHI-V4 was noted in all VISA and four (12.1%) hVISA isolates. None of the vancomycin susceptible MRSA isolates grew on BHI-V4 agar. The sensitivity, specificity 100%, 94.6% for BHI-V3; and 100%, 99.2%, for BHI-V4 for detecting VISA. These observations differ from those of[2] who reported 100% sensitivity and 65% specificity for detecting VISA with BHI-V3. The reason for the difference is unclear but might be related to isolate selection as ***Burnham et al.*** selected their isolates based on MIC results and did not perform PAP/ AUC[2].

In our study, on performing vancomycin screening agar 6 ug/ml with and without casein, it detected 30%, 29% isolates as VISA with sensitivity 66.7%, 63.3% respectively and specificity 85.7% for both. The false positive for both were in the range of 0.5-2 ug/ml which may be h VISA but that could not be confirmed. The false negative isolates for both may be due to increase concentration of vancomycin 6 mg/liter. As a vancomycin MIC of 4 to 8 ug/ml is now considered to represent intermediate susceptibility, the use of an agar medium such as BHI-V6 as a means to screen for vancomycin intermediate strains of *S. aureus* (VISA) is not adequated for this purpose, as those strains having a vancomycin MIC greater than 2 but less than 6 ug/ml could not detect by this method[2] **.**

Swenson and co worker [9] reported that BHI-V6 agar failed to detect 33% (12 of 36) of VISA isolates with an MIC of 4 mg/liter. Similarly, ***Walsh and his coworkers (2001)*** reported low sensitivity (22%) for the agar screening method using brain heart infusion agar (6 ug of vancomycin per ml), and a specificity of 97%.

In the present study, VITEK 2 system failed to detect any isolates with reduced susceptibolity to vancomycin. ***Swenson et al.*** [9] tested 43 *S. aureus (*20 isolates with MICs 2 g/L, 22 with MICs 4 g/L, and one isolate with MIC 8 g/L) by three automated systems. One of them was the Vitek 2 system which tended to categorize VISA isolates as susceptible (five isolates).This could be explained by ***Edwards et al.*** [11] *who* demonstrated that MICs from automated systems and the E-test were significantly lower after cryopreservation, if compared with those from the E-test analysis, at the time of isolation, Also, ***Mason et al.*** [12] pointed out that the prevalence of vancomycin MIC creeps may be underestimated because of the cryopreservation effect.

On the other hand, the study performed by[2] showed that Vitek2 using card GP67 had the worst sensitivity (7.7%), detecting only 1 of the 13 VISA isolates compared to Microscan which had the highest sensitivity (92%), failing to detect only one VISA strain, followed by E-test (85% sensitive) and then Sensititre (54% sensitive). Thereby, they suggested that laboratories using the GP67 AST card for vancomycin susceptibility testing of *S. aureus* should consider additional testing to rule out VISA when an MIC of 2 mg/liter is generated and/or the concomitant use of a screening medium such as BHI-V3 to ensure detection of VISA isolates. Also ***Kruzel et al.*** [13] stated that after the emergence of hVISA and VISA, it became clear that the automated susceptibility testing methods are inadequate for the detection of VISA.

All of our MRSA isolates were susceptibile to vancomycin using VITEK 2 system. They were sensitive to linezolid (100%) followed by tigecyclin (99%) then Quinupristin-

dalfopristin (91%). However, most of the isolates were resistant to tetracyclin (85%) followed by gentamicin (80%) then ciprofloxacin (63%). A study by ***Cook et al.*** described the successful treatment of a ventriculoperitoneal shunt infection caused by a h-VISA with linezolid due to its tolerability and excellent blood-brain barrier penetration. High-dose of Quinupristin-dalfopristin (synercid) significantly reduced the number of bacteria detected in the VISA hematogenous infection in murine models[14]. Combination therapy of synercid and vancomycin was effective in treatment of case with oxacillin-resistant *Staphylococcus aureus* bacteraemia that was not responding to vancomycin alone.

CONCLUSION

BHI agar with 2ug/ml vancomycin and 16 g/l casein is a reliable, easy to perform, and inexpensive method to screen large number of *S. aureus* isolates for detection of reduced susceptibility to vancomycin on a daily basis. Applying quadruplicate technique in vancomycin screening agar may increase the yield for detection of VISA isolates. Although vancomycin screening agar 6 ug/ml is recommended by CLSI as a screening method for detection of VISA, yet it did not perfom well and undersetimated VISA isolates. VITEK 2 system is not an appropriate method for detection of *S. aureus* with reduced susceptibility to vancomycin (VISA). MRSA isolates with reduced susceptibility to vancomycin can be treated effectively with Linezolid.

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