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Vancomycin MIC Distribution among Methicillin-Resistant *Staphylococcus Aureus*. Is Reduced Vancomycin Susceptibility Related To MIC Creep?

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Keywords: VISA; h-VISA; Vancomycin screening agar; BMD; Vancomycin MIC creep

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Abstract

AIM: To determine the distribution of vancomycin MIC and the frequency of *S. aureus* strains with reduced vancomycin susceptibility among Methicillin-Resistant *Staphylococcus aureus* (MRSA) isolates.

METHODS: MRSA isolates (n = 100) were tested for reduced susceptibility to vancomycin using MIC broth microdilution method (BMD), vancomycin screening agar with different vancomycin concentrations with and without casein, and Vitek 2 system.

RESULTS: BMD detected (22%) vancomycin-intermediate *S. aureus* (VISA) and (78%) vancomycin-susceptible *S. aureus* (VSSA) but couldn't detect nine (Heterogeneous VISA) (hVISA) isolates (9%) with MIC ≤ 2 µg/ml that grew on screening agar 4 µg/ml or 6 µg/ml. Adding casein to vancomycin screening agar increased detection rate of VISA by 4.5%. Screening agar with 6 µg/ml vancomycin overall detection rate for VISA was 95.45%. Probable 'pre-hVISA'isolates (17%) showed growth on vancomycin screening agar 2 µg/ml with casein. Vitek 2 system failed to detect any VISA isolates.

CONCLUSION: Vancomycin screening agar; 2 µg/ml and (4 and 6 µg/ml) were able to detect; probable "pre hVISA and (hVISA and VISA) isolates respectively based on their BMD MIC values. Decreased vancomycin susceptibility in MRSA isolates might be related to MIC creep. Analysis of vancomycin MIC values over longer periods is recommended to further study this phenomenon and its impact on vancomycin treatment failure.

Introduction

Staphylococcus aureus is a virulent microorganism responsible for many serious infections among the general population. The emergence of vancomycin resistance in *S. aureus* has been anticipated since vancomycin-resistant enterococci (VRE) has been recognised. Hiramatsu et al. in 1997 described the first documented case of infection caused by *S. aureus* with reduced susceptibility to vancomycin [1].

Reduced susceptibility could be either due to strains that showed intermediate resistance to vancomycin with MIC 4-8 µg/mL [vancomycin intermediate *S. aureus* (VISA)] or heteroresistant strains (Heterogeneous VISA) (h-VISA) that are defined as strains with minimal inhibitory concentrations (MICs) within the susceptible range (MIC ≤ 2 µg/ml), but containing subpopulations of cells in the vancomycin-intermediate range (VISA, MIC 4-8 µg/ml) These strains have been described for both MRSA and methicillin-susceptible *S. aureus* (MSSA) respectively [2].

There has been special interest in vancomycin MIC creep phenomenon that was associated with greater rates of complications, and vancomycin therapeutic failures with vancomycin MICs within the susceptible range (MICs of 1-2 mg/L) [3], [4], [5]. Unfortunately, there has been uncertainty regarding optimal laboratory detection of *S. aureus* with reduced susceptibility to vancomycin [6].

Center for Disease Control and Prevention (CDC) in 2012 recommended that screening of VISA should be done by MIC method plus vancomycin screening agar method with 6ug of vancomycin per ml, but this is not reliable to detect all VISA; some strains for which the vancomycin MICs are 4 ug/ml will fail to grow [7], [8].

Screening for h-VISA by the population analysis profile-area under the curve (PAP-AUC) method has been the most reliable and reproducible approach but is labour-intensive, costly, and unsuitable for routine use in clinical laboratories [9], [10].

Moreover, standardized reference methods for susceptibility testing, such as CLSI broth microdilution, agar dilution, and standard Etest methods, can detect VISA but fail to detect h-VISA due to several factors as small inoculum size, the relatively poor support of growth on Mueller-Hinton agar plates, the slow growth of h-VISA strains and its unique pleomorphic features, such as small-colony variant [9], [11].

Satola et al. showed that the use of BHI screen agar with 4 µg/ml vancomycin with increase incubation time to 48 hours and the addition of casein could increase the sensitivity and specificity for detection of VISA and may be useful for clinical detection of h-VISA [12].

The study aimed to determine the distribution of vancomycin MIC, and the frequency of *S. aureus* strains with reduced vancomycin susceptibility among Methicillin-Resistant *Staphylococcus aureus* (MRSA) isolates.

Materials and Methods

The study included 100 MRSA isolates analysed by Kirby Bauer disc diffusion method [13] recovered from different specimens referred to Central Microbiology Laboratory of Ain Shams University Hospitals for routine culture and sensitivity. Isolates were collected throughout four months from January till April 2017 and were preserved on tryptone soy broth with 15% glycerol at -80°C until use.

Detection of MRSA with reduced susceptibility to vancomycin was performed using vancomycin screening agar with different vancomycin concentrations 2, 4, and 6 µg/ml with and without casein and compared to MIC broth microdilution method for vancomycin (BMD) [13]. Finally, MIC susceptibility testing for MRSA isolates was performed by Vitek 2 automated system (Biomerieux, France).

Brain heart infusion (BHI) agar without casein (Oxoid, UK) was prepared according to the manufacturer's instructions and BHI agar with casein was similarly prepared but with the addition of eight gram pancreatic digest of casein (Sigma Aldrich, USA) to every 500 ml of media [12]. A stock solution of vancomycin was prepared by dissolving 500 mg of vancomycin powder in 10 ml of sterile distilled water (final concentration was 50 mg/ml). At the time of media preparation, further dilution of 1:10 was done twice to produce a working solution of 0.5 mg/ml vancomycin. For the final preparation of vancomycin screening agar with and without casein; six ml, four ml and two ml of 500 ml prepared media were removed under complete aseptic precautions and replaced by 6 ml, 4 ml and 2 ml of working solution of vancomycin to prepare *Vancomycin screening agar* with 6 µg/ml, 4 µg/ml and 2 µg/ml respectively.

All isolates were subcultured by taking a small piece of a frozen organism with a sterile loop and plated twice onto blood agar plates. The used vial was returned immediately to the deep freezer to be used if needed as repeated thawing and re-freezing can reduce the viability of the organism. The cultivated plates were incubated aerobically at 35°C for 24 hours. Two to three colonies were picked up by the sterile loop and adjusted to 0.5 McFarland standards in 5 ml sterile tubes. Quadruplicate technique was performed (i.e. four droplets, 10ul each, from 0.5 McFarland MRSA suspension, was dropped by a pipette onto the 2, 4, and 6 vancomycin screening agars) and the plates were incubation for a full 48 hours at 35°C to enhance the sensitivity of detection of MRSA with vancomycin reduced susceptibility [12]. Plates were examined at 24 and 48 hours. Vancomycin-Resistant *Enterococcus Faecalis* ATCC 51299 and *Staphylococcus aureus* ATCC 25923 (NAMRU-3) were used as positive and negative controls respectively.

No growth in any of the four droplets was denoted as sensitive to vancomycin. Growth in any of the four droplets was considered as MRSA with vancomycin reduced susceptibility.

The broth microdilution method was used for determination of the MICs of vancomycin [14]. Vancomycin suspension used was prepared by dissolving 500 mg of vancomycin powder in 10 ml of sterile distilled water (50 mg/ml), then further dilution 1:10 was done twice (0.5 mg/ml). From the prepared dilution, 640 µl was added to 10 ml of D.W to reach a final concentration of 32 µg/ml vancomycin.

Serial two-fold dilution of the prepared vancomycin concentration was carried in a 96 well plate. Fifty microliters of double-strength Muller Hinton Broth (MHB), 50 µl of the antibiotic dilutions, and (5 µl of the organism suspension adjusted to 0.5 McFarland standards and then diluted 1:20) were mixed and incubated at 35°C for 24 hours. MICs; ≤ 2 µg/ml is considered as sensitive, 4-8 µg/ml as VISA and ≥ 16 µg/ml as vancomycin resistant *S. aureus* (VRSA) (Figure 1).

Susceptibility testing on Vitek 2 system was performed with AST PG 76 cards according to the manufacturer's instructions, and susceptibility breakpoints of *Staphylococcus aureus* were interpreted per CLSI 2015 [13].

Results

Broth microdilution (BMD) method (Figure 1) revealed that, out of 100 MRSA isolates, 22/100 (22%) were VISA (14/22 VISA with MIC = 8 µg/ml and 8/22 VISA with MIC = 4 µg/ml) and 78/100 (78%) were VSSA (VSSA MIC ≤ 2 µg/ml).

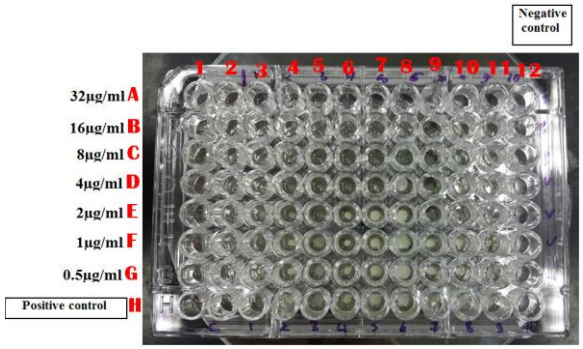

Figure 1: Broth microdilution plate for detection of vancomycin susceptibility in MRSA isolates; A12 to G12 are negative growth control wells. H1 to H11 wells are positive growth control wells. MIC of vancomycin for isolate; 4 (well E4) is 2 µg/ml, 5 (well E5) is 2 µg/ml (VSSA), 6 (well D6) is 4 µg/ml and for isolate 8 (well C8) is 8 µg/ml (VISA)

In vancomycin screening agar method, h-VISA was reported if one or two colonies on at least one droplet showed growth on screening agar with 4 µg/ml or 6 µg/ml [12]. Among MRSA isolates that showed MIC ≤ 2 µg/ml by BMD; 9 isolates (9%) grew on screening agar 4 µg/ml or 6 µg/ml and were designated as h-VISA (Figure 2).

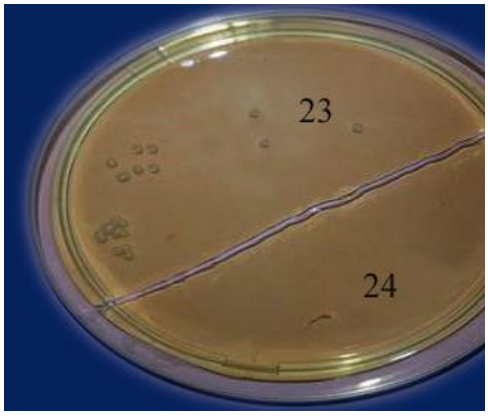

Figure 2: Vancomycin screening agar 6 µg/ml with casein; isolate [23] shows the growth of hVISA. Isolate [24] shows no growth (VSSA)

Seventeen isolates with susceptible MIC by BMD (17%) showed growth on vancomycin screening agar 2 µg/ml, six out of them with MIC of 2 µg/ml by BMD. These isolates were considered as probable 'pre-hVISA', which represent small subpopulations of cells capable of growth in the presence of 2-4 mg/L vancomycin [14] (Table 1 and Figure 3).

Table 1: Detection rate of MRSA with reduced susceptibility to vancomycin among 100 tested isolates

Vancomycin screening Agar	BMD MIC	
	VSSA (MIC ≤ 2 µg/ml)	VISA (MIC 4-8)
No growth	52 (52%)	0
2 µg/ml (probable pre-hVISA)	17 (17%)	0
4 µg/ml or 6 µg/ml	9 (9%) hVISA	22 (22%)
Total	78 (78%)	22 (22%)

All of the results of screening agar with and without casein were similar except for two isolates; one isolate showed growth on screening agar with 4 µg/ml, with casein but not in that without casein, and one more isolate grew on screening agar 2 µg/ml without casein only after 48 hours. So, adding casein to vancomycin screening agar increased detection rate of VISA by 4.5% (only one VISA out of 22). Screening agar with 6 µg/ml vancomycin (with and without casein showed similar results) detected 7 out of 8 VISA with BMD MIC equal to 4 µg/ml (87.5%) and 14 out of 14 with BMD MIC equal to 8 µg/ml (100%), with overall detection rate of VISA 95.45% (Table 2).

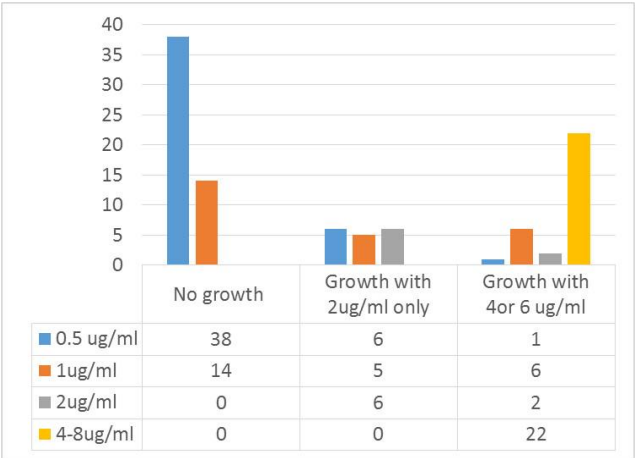

Figure 3: Results of Broth microdilution and vancomycin screening agar among 100 tested isolates

Increasing incubation time did not increase the detection rate for vancomycin with reduced susceptibility among screening agar with casein and only affected one isolate grown on 2 µg/ml screening agar without casein (Table 2).

Table 2 shows vancomycin MIC results using Broth microdilution and vancomycin screening agar. It is noted that Broth microdilution method was not able to detect nine (9) h-VISA isolates.

Table 2: Vancomycin MIC results using Broth microdilution and vancomycin screening agar for detection of MRSA with reduced susceptibility to vancomycin

	BMD MIC	Vancomycin screening agar MIC						
		No growth (< 2 µg/ml) with and without casein	> 2 µg/ml (< 4 µg/ml)		> 4 µg/ml-< 6 µg/ml		> 6 µg/ml	
			With casein*	Without casein	With* casein	Without* casein	With* casein	Without* casein
Sensitive	MIC ≤ 1	52	11	10 + 1●	0	0	7	7
	MIC 2	0	6	6*	0	0	2	2
Interme-diate	MIC 4	0	0	0	1	0	7	7
	MIC 8	0	0	0	0	0	14	14
Total		52	17	16 + 1●	1	0	30	30

*No difference between 24 and 48 hours; ●the Only one showed no growth at 24 hours but detected at 48h.

All of MRSA isolates (100%) were susceptible for both vancomycin and linezolid by VITEK 2 system. (Figure 4) shows the result of susceptibility testing for MRSA isolates on the Vitek 2 system.

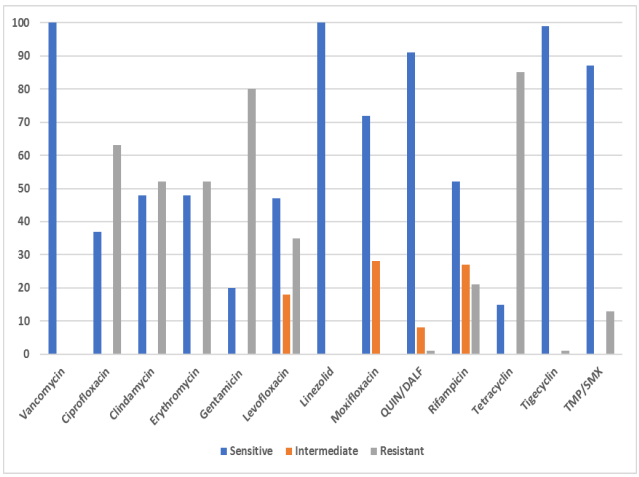

Figure 4: Result of susceptibility testing for 100 MRSA isolates using AST GP 76 cards on Vitek 2 system [14]

Discussion

Overuse of vancomycin has led to the development of a selective pressure over time with the result of the emergence of *S. aureus* with reduced vancomycin susceptibility. The emergence of MRSA with reduced susceptibility to vancomycin is worrisome as the available drugs for MRSA treatment are limited [6].

We report 22% VISA isolates by broth microdilution method. Vaudaux et al. reported that Broth microdilution assay led to under detection of the vancomycin-intermediate *S. aureus* VISA) phenotype, yielding only three VISA isolates, for which vancomycin MICs were 4 µg/ml compared to 8 and 19 VISA isolates detected by microdilution and agar testing, respectively [14].

In the present study, among the MRSA isolates that showed MIC less than or equal to 2 µg/ml by BMD; (9%) of h-VISA isolates showed growth on screening agar 4 µg/ml or 6 µg/ml. Whereas, (17%) of isolates with susceptible MIC by BMD showed growth on vancomycin screening agar 2 µg/ml (probable 'pre-hVISA') [15]. The pre-hVISA strains may be correlated with the 'MIC creep' phenomenon observed in hospitals where anti-MRSA chemotherapy is frequently implemented [15].

Lodise et al. observed that patients with MRSA bloodstream infections with elevated vancomycin MICs but within the susceptible range (≥ 1.5 mg/mL) had higher probabilities of recurrent bacteremia and longer hospital stays [16]. Sakoulas et al. reported that the likelihood of treatment success is significantly lower in patients with MRSA isolate with a vancomycin MIC of 1-2 mg/mL compared with patients infected by isolates with a vancomycin MIC ≤ 1.5 mg/Ml [17]. Edwards et al. suggested lowering vancomycin breakpoints further, to avoid clinical failure and the increased risk of mortality [4].

Satola et al., tested 140 MRSA blood isolates with vancomycin MICs 2 µg/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 h-VISA, revealing 90% and 95% sensitivity and specificity with a 0.5 McFarland inoculum and 100% and 68% sensitivity and specificity with a 2.0 McFarland inoculum respectively [12].

In the present work, adding casein to vancomycin screening agar increased detection rate of VISA by 4.5% (only one VISA out of 22). The base medium of the screening agar might be as important as the vancomycin concentration. Enhancement of detection of h-VISA by screen agar methods could be obtained by the addition of supplements to the agar. Willey et al. reported that the addition of pancreatic digest of casein to BHI agar and 4 g/ml vancomycin improved the detection of VISA on screen agars, as 97.7% of VISA strains in their study were successfully detected with high specificity within 24 h [18]. Other supplement suggested differentiating between h-VISA and VSSA was the addition of 20% horse serum to BHI [19].

Riederer et al., tested 485 MRSA blood isolates with vancomycin MICs 0.5 to 4 µg/ml using BHI-V3, BHI-V4 and other methods. The modified PAP/AUC was measured for all isolates revealing seven VISA and 33 h-VISA phenotypes. The sensitivity and specificity for detecting VISA were 100% and 94.6% for BHI-V3, 100% and 99.2%, for BHI-V4 respectively [20]. These observations differ from those of Burnham et al., who reported 100%

sensitivity and 65% specificity for detecting VISA with BHI-V3 [21]. The reason for the difference is unclear but might be related to isolates selection as Burnham et al., selected their isolates based on MIC results and did not perform PAP/AUC [21].

In the present study, screening agar with 6 µg/ml vancomycin detected 7 out of 8 VISA with BMD MIC equal to 4 µg/ml (87.5%) with overall detection rate of VISA 95.45%. CDC 2015 stated that growth of more than one colony on screening agar with 6 µg/ml vancomycin is considered a positive result for VISA [13]. All *S. aureus* isolates for which the vancomycin MIC ≥ 8 µg/ml grow on these plates and some isolates for which the vancomycin MIC = 4 µg/ml will also grow [22].

As a vancomycin MIC of 4 to 8 µg/ml is considered an 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 adequate for this purpose, as those strains having a vancomycin MIC greater than 2 but less than 6 µg/ml could not be detected by this method [23].

Swenson et al. reported that BHI-V6 agar failed to detect 33% (12 of 36) of VISA isolates with MIC 4 µg/ml [24]. Similarly, Walsh et al. reported low sensitivity (22%) for the agar screening method using brain heart infusion agar (6 mg of vancomycin per litre), and 97% specificity [23].

In the present study, the VITEK 2 system failed to detect any isolates with reduced susceptibility to vancomycin. Swenson et al. reported that the Vitek 2 system tended to categorise VISA isolates as susceptible [24]. This was justified by Edwards et al., 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 [4]. Also, Mason et al. pointed out that the prevalence of vancomycin MIC creeps may be underestimated because of the cryopreservation effect [25].

On the other hand, the study performed by Burnham et al., showed that Vitek2 using card GP67 had the worst sensitivity (7.7%), detecting only one out of the 13 VISA isolates compared to Microscan system which had the highest sensitivity (92%), followed by Etest (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 MIC 2 µg/ml is generated and/or the concomitant use of a screening medium such as BHI-V3 to ensure detection of VISA isolates [21]. Also, Kruzel et al. stated that it became evident that the automated susceptibility testing methods are inappropriate for the detection of VISA [26].

All of our MRSA isolates were susceptible to vancomycin using VITEK 2 system. They were also sensitive to linezolid (100%) followed by tigecycline (99%) then Quinupristin-dalfopristin (91%). A study by Cook et al. described the successful treatment of a ventriculoperitoneal shunt infection caused by h-VISA with linezolid due to its tolerability and excellent blood-brain barrier penetration [27]. High-dose of Quinupristin-dalfopristin (Synercid) significantly reduced the number of bacteria detected in the VISA hematogenous infection in murine models [28].

Since the first reports of hVISA/VISA, their prevalence differed among geographic regions: the incidence of h-VISA was 6.81% in Asia and 5.60% in Europe/America, and that of VISA was 3.42% and 2.75%, respectively. Several factors may be responsible for such condition; i) high public hygiene standards and meticulous antimicrobial treatments in most European and American countries [29], [30], [31], ii) the control of nosocomial infections is more successful in European and American countries [32, 33], iii) Asia is the most populous region of the world, susceptible to microbial transmission, and iv) more MRSA infections occur in Asian countries [34].

In the present work, Vancomycin screening agar; 2 µg/ml and (4 and 6 µg/ml) were able to detect; probable “pre hVISA and (hVISA and VISA) isolates respectively based on their broth microdilution MIC values. We believe that decreased vancomycin susceptibility in MRSA isolates might be related to MIC creep, but we could not indicate this phenomenon since an earlier data from our lab was not available for comparison. Similar factors as that found in Asia could be responsible for the occurrence of MRSA with reduced susceptibility to vancomycin in our country. Further studies on a large scale are needed to determine the prevalence of VISA and h-VISA and also to study the phenomenon of vancomycin MIC creep.

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