Brief communication (Original)

An evaluation of 2.0 McFarland Etest method for detection of heterogeneous vancomycin-intermediate *Staphylococcus aureus*

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Background: *Staphylococcus aureus* with reduced susceptibility to vancomycin or heterogeneous vancomycin-intermediate *S. aureus* (hVISA) have become increasingly reported from various parts of the world. hVISA cannot be detected by routine test for minimal inhibitory concentration (MIC) for vancomycin. The gold standard method for detection, population analysis profiles (PAP) method, is complicated, time-consuming, expensive, and needs well-trained microbiologists.

Objective: Evaluate of 2.0 McFarland Etest method, in comparison with the PAP method, for detection of hVISA in clinical specimens.

Methods: All methicillin-resistant *S. aureus* strains from clinical specimens isolated from consecutive patients attended at King Chulalongkorn Memorial Hospital and Siriraj Hospital, Bangkok between 2006 and 2007 were studied. 1 hundred nineteen specimens were obtained. The PAP method detected six hVISA strains (5 from blood and from cultures) from four patients at King Chulalongkorn Memorial Hospital, accounting for a prevalence of 6.35%. The MIC determined by agar dilution method was in the range of 2-3 µg/mL.

Results: 2.0 McFarland Etest method detected no false positive and five false negatives (42%), and gave a sensitivity and a specificity of 16.7% and 100%, respectively. The one-point population analysis screening method detected two false positives and 1 false negative, and gave a sensitivity of 83.3% and a specificity and 98.2%.

Conclusion: The 2.0 McFarland Etest method had a very good specificity but a poor sensitivity for detecting hVISA. It may be used as an alternative method to confirm detection of hVISA.

Keywords: Etest, heterogeneous vancomycin-intermediate *S. aureus*, population analysis profiles, *Staphylococcus aureus*, susceptibility

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections are a major health problem worldwide. The increasing use of glycopeptides makes the organism more virulent and resistant [1]. *S. aureus* with reduced susceptibility to glycopeptides was first reported from Japan in 1996 in a patient with MRSA pneumonia [2, 3]. Since then, similar strains have been isolated in the United States [4],

Europe [5], Asia [6], and Thailand [7]. These isolates display vancomycin minimal inhibitory concentration (MIC) below the breakpoint, but contain some bacterial subpopulations ($>10^6$) growing in the presence of ≥ 4 µg/mL of vancomycin. These isolates are termed heterogeneous vancomycin-intermediate *S. aureus* (hVISA). With the revised Clinical and Laboratory Standards Institute (CLSI) breakpoints for vancomycin [8], most hVISA strains with the MICs in the range of 3-6 µg/mL may be reclassified as homogeneous VISA or VISA, making the differentiation between these strains less important than the accurate determination of hVISA strains with

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the MICs between 1-2 $\mu\text{g/mL}$, which would be identified as vancomycin-susceptible *S. aureus* (VSSA).

Recently, a SENTRY study showed that four of 253 MRSA strains were hVISA with the MICs between 1-2 $\mu\text{g/mL}$ [9]. A recent study of 152 MRSA reported that 90% had vancomycin MIC of $\geq 2 \mu\text{g/mL}$ as determined by Etest strips, while 9.8% exhibited vancomycin of 3 $\mu\text{g/mL}$ [10]. In addition, hVISA strains may represent a gateway to high-level vancomycin resistance as VISA and vancomycin-resistant *S. aureus* (VRSA) strains [11, 12]. All these evidences emphasize an urgent need for a suitable and reliable method to detect hVISA with vancomycin MICs of 1-2 $\mu\text{g/mL}$, especially isolated from patients who fail to respond to glycopeptide treatment.

The population analysis profiles (PAP) method is generally used for detecting hVISA, but it is complicated, time-consuming, expensive, and needs well-trained microbiologists [2]. Using a laboratory method with comparable sensitivity and specificity but more convenience, simpler, and cheaper would be an alternative to the PAP. A modification of the Etest system with a heavy inoculum [200 μL of bacterial suspensions with the density equivalent to a 2.0 McFarland turbidity (6×10^8 colony-forming units/mL)] may improve the determination of hVISA strains. In this study, we aimed to evaluate the Etest at 2.0 McFarland inocula, in comparison with the PAP method, for detection of hVISA in clinical specimens.

Materials and methods

Reference bacterial strains used were Mu50 (VISA) and Mu3 (hVISA) [2]. One hundred three clinical isolates of MRSA were consecutively obtained from patients hospitalized at King Chulalongkorn Memorial Hospital between January 2006 and March 2007. In addition, 16 additional clinical isolates of MRSA were collected from patients at Siriraj Hospital between May and November 2006. All MRSA isolates were tested for antimicrobial susceptibility by the disk-diffusion method according to the CLSI guidelines [8], and stored in skim milk glycerol at -70°C . All methods described in this study, apart from PAP method, were carried out in duplicate. Vancomycin standard powder was purchased from Sigma (Steinheim, Germany).

One-point population analysis

The one-point population analysis method was carried out as previously described by Hiramatsu

et al. [2]. Specific bacterial inocula were plated onto brain heart infusion (BHI) agar containing 4 $\mu\text{g/mL}$ of vancomycin. Growth of bacteria at 24 hours was considered a VISA strain. Growth occurring between 24 and 48 hours was considered a hVISA strain.

PAP

The PAP method was performed as previously described by Hiramatsu et al. [2]. 100 μL of a bacterial suspension [made by adjusting an overnight culture in BHI broth to optical density 578 nm of 0.3 (about 10^8 colony-forming units/mL) with fresh BHI broth]. Ten-fold dilutions of this suspension were plated onto BHI agar plates containing various concentrations of vancomycin. After incubation at 37°C for 48 hours, the number of colonies grown on each plate was counted. The number of resistant cells contained in 100 μL of the starting cell suspension was calculated and plotted on a semilogarithmic graph.

Etest

Etest MICs were determined using two inoculum densities of 0.5 and 2.0 McFarland [13]. Strains were grown overnight on blood agar plates. A randomly selected single colony was inoculated into fresh Mueller-Hinton broth, and grown overnight. The turbidity of the broth was adjusted to 0.5 and 2.0 McFarland standards using fresh broth. Then, 200 μL of this suspension was pipetted onto a 90-mm BHI agar (BBL, Becton Dickinson, Cockeysville, USA) plate and streaked out evenly with a swab. After being dried for approximately 10 minutes, Etest strips (AB BIO Disk) for vancomycin (0.06-256 $\mu\text{g/mL}$) were applied. All plates were incubated at 35°C for 24 hours.

Agar dilution

The method was carried out as recommended by the CLSI guidelines [14]. Colonies were taken from overnight blood agar plates, and sterile saline was inoculated to make a 0.5 McFarland suspension inoculum. Mueller-Hinton agar (Oxoid) was inoculated with the suspension inoculum. The plates were incubated at 35°C and read after 24-hour incubation.

Analysis

The performance of 2.0 McFarland Etest and one-point population analysis methods in detecting hVISA was determined, in comparison with the PAP method. Each method was evaluated for sensitivity and specificity in discriminating hVISA from MRSA

strains. The sensitivity was calculated based on the number of hVISA that were correctly identified, while the specificity was calculated based on the number of correct negative results. The positive predictive value (PPV) refers to the probability that a positive result is correct, while the negative predictive value (NPV) refers to the probability that a negative result is correct.

Results

One hundred nineteen clinical specimens were obtained during the study period. The PAP method detected 6 hVISA strains (five and one from blood and pus cultures, respectively) from four patients at King Chulalongkorn Memorial Hospital, accounting for a prevalence of 6.35% among MRSA isolates.

The performance of 2.0 McFarland Etest and one-point population analysis methods is shown in

Table 1. 2.0 McFarland Etest method detected no false positive (0%) and five false negatives (42%), hence showing a sensitivity of 16.7%, a specificity of 100%, a PPV of 100%, and an NPV of 95.8%. The one-point population analysis screening method detected two false positives and one false negative, hence giving a sensitivity of 83.3%, a specificity of 98.2%, a PPV of 71.4%, and an NPV of 99.1%.

The vancomycin MIC of five hVISA strains determined by agar dilution method was 2 µg/mL, and one strain isolated from the blood had the MIC of 3 µg/mL (**Table 2**). In addition, all these six hVISA strains had the MICs determined by 0.5 McFarland Etest method between 1.5 and 3 µg/mL, which were in the susceptible range based on the CLSI breakpoint criteria for vancomycin. The 2.0 McFarland Etest method detected two of these six strains with the MIC of 3 and 6 µg/mL.

Table 1. Sensitivity and specificity of one-point population analysis, 2.0 McFarland Etest method, in comparison with the population analysis profile method, in detecting heterogeneous vancomycin-intermediate *Staphylococcus aureus*.

Method	False positive (number)	False negative (number)	Sensitivity (percent)	Specificity (percent)	PPV (percent)	NPV (percent)
2.0 McFarland Etest	0	5	16.7	100	100	95.8
One-point population analysis	2	1	83.3	98.2	71.4	99.1

PPV: positive predictive value, NVP: negative predictive value

Table 2. One-point population analysis, population analysis profile (PAP) and minimal inhibitory concentration (MIC) determined by agar dilution and 2.0 McFarland and Etest methods of all 6 heterogeneous vancomycin-intermediate *Staphylococcus aureus*.

Isolate	Specimen	One-point population analysis	PAP	MIC by agar dilution method (µg/mL)	MIC by 0.5 McFarland Etest (µg/mL)	MIC by 2.0 McFarland Etest (µg/mL)
1.	Blood	Positive	Positive	2	2	3
2.	Blood	Negative	Positive	2	1.5	2
3.	Blood	Positive	Positive	2	2	2
4.	Blood	Positive	Positive	2	2	2
5.	Pus	Positive	Positive	2	2	2
6.	Blood	Positive	Positive	3	3	6

Discussion

The detection of hVISA by routine susceptibility testing is unreliable, and this strain is most probably being underreported [11, 12]. Standardized reference methods for susceptibility testing (agar dilution method recommended by the CLSI) also performed suboptimally in detecting hVISA, even with the recently revised MIC breakpoint criteria for VISA with the MIC of 4-8 µg/mL. Most of hVISA strains have the MICs between 1 and 2 µg/mL, and hence would be identified as VSSA in clinical laboratory. Our results clearly confirmed this statement. All hVISA strains tested had MICs determined by agar dilution method in the susceptible range between 1.5 and 3 µg/mL.

Even though the PAP method represents the gold standard for detecting hVISA strains, a use of this method in the clinical laboratory will be inconvenient, time-consuming, expensive, and needs well-trained microbiologists. A simpler and reliable clinical laboratory test is urgently needed. Different strategies of susceptibility testing have been used to detect hVISA strain including the use of different culture media, higher bacterial inocula, longer incubation periods before obtaining of susceptibility results, and lower vancomycin concentrations. Previous studies evaluated several methods for detection of both VISA and hVISA, in comparison with the PAP method [13, 15, 16]. To the best of our knowledge, there has been only one small study by Lulitanond and colleagues to evaluate the disk diffusion method with low-concentration vancomycin disk for detecting hVISA strains [17]. In this prospective study with a larger sample size, we use two strategies including a high bacterial inoculum and the simple vancomycin Etest strips for detection of hVISA. Our results showed that the 2.0 McFarland Etest method had a 100% specificity and PPV, but a very low sensitivity, if the MIC breakpoints were between 4 and 8 µg/mL. This is in contrast with the results by Walsh and colleagues, which showed that the sensitivity and specificity of the 2.0 McFarland Etest method was 96% and 97%, respectively. However, they included both VISA and hVISA strains in their study. It is our opinion that after the recent revision of the CLSI MIC breakpoints for VISA to 4-8 µg/mL, most of hVISA strains in their study may be reclassified as VISA, and hence may be not difficult for differentiation from VSSA strains in a clinical laboratory. It seems that the 2.0 McFarland Etest method has the disadvantage of lower sensitivity

and produces higher false negative results in comparison with the one-point population analysis method. It should not be used as a screening test for detection hVISA. A convenient and reliable method for screening of hVISA is still needed for clinical laboratory.

In conclusion, this is the first prospective study to evaluate the 2.0 McFarland Etest method for detection of hVISA without including homogeneous VISA. The 2.0 McFarland Etest method has a very good specificity but a poor sensitivity for detection of hVISA. Due to its high convenience and lower cost in comparison with the PAP method, it may be used as an alternative method to confirm the detection of hVISA.

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We have no conflict of interest to report.

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