DETECTION OF HETEROGENEOUS, INTERMEDIATE-VANCOMYCIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (HVISA) USING LOW-CONCENTRATION VANCOMYCIN DISKS

Aroonlug Lulitanond[1,2], Aroonwadee Chanawong[1,2], Pipat Sribenjalux[1,2], Wanlop Kaewkes[3], Malai Vorachit[4], Piriyaporn Chongtrakool[4], Danaisak Leumsai[1] and Pensri Monpou[1]

[1]Faculty of Associated Medical Sciences, [2]Center for Research and Development in Medical Diagnostic Laboratories, [3]Faculty of Medicine, Khon Kaen University, Khon Kaen; [4]Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand

Abstract. Heterogeneous, intermediate-vancomycin-resistant *Staphylococcus aureus* (hVISA) represents a threat of an incurable infection since the first report in 1997. The method used to detect hVISA isolates is a population analysis profile (PAP); however, it is impractical for routine laboratory analysis. We therefore tested a simple, reliable and inexpensive method for the detection of hVISA. Eighteen isolates of hVISA and 22 of vancomycin-sensitive *S. aureus* (VSSA) were included. The organisms were tested by the disk diffusion method, using 15-μg vancomycin disks on four different media: Mueller-Hinton agar (MHA), MHA plus 2% NaCl (MHAS), Brain Heart Infusion agar (BHA), and BHA plus 2% NaCl (BHAS). In addition, two different inoculum sizes, bacterial suspensions adjusted to 0.5 and 2.0 McFarland, were tested. The inhibition zone was read independently by three medical technologists after incubation at 37ºC for 24 and 48 hours. The use of MHAS with an inoculum size of 2.0 McFarland and 48-hour incubation period yielded the highest sensitivity (94.4%), specificity (81.8%), positive predictive value (80.9%), and negative predictive value (94.7%). The disk diffusion test with 15-μg vancomycin disk is simple and may be used as a screening method for the detection of hVISA.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first observed approximately 40 years ago and remains among the top three clinically important pathogens (Van Belkum and Verbrugh, 2001; Deresinski, 2005). Vancomycin, a glycopeptide antibiotic, has long been reserved for the treatment of infections with this organism. However, intermediately since the late 1990s, vancomycin-resistant *S. aureus* (VISA) has emerged in many countries (Centers for Disease Control and Prevention, 1997; Hiramatsu *et al*, 1997; Bierbaum *et al*, 1999; Rotun *et al*, 1999; Dos Santos Soares *et al*, 2000; Ferraz *et al*, 2000; Song *et al*, 2004). According to the Clinical and Laboratory Standards Institute (CLSI), isolates with a minimum inhibitory vancomycin concentration (MIC) of ≤ 4μg/ml are considered susceptible, whereas isolates with an MIC > 16 μg/ml are considered vancomycin-resistant.

Some clinical isolates have been reported with vancomycin MICs of 8 μg/ml and are considered to have an intermediate resistance. These isolates are of clinical importance because treatment failure has been reported (Hiramatsu *et al*, 1997a; Rotun *et al*, 1999; Smith *et al*, 1999). Most isolates displayed a heterogeneous phenotype that has a subpopulation of at least 10^{-6} resistance to vancomycin, although the overall MIC is ≤ 4 μg/

Correspondence: Aroonlug Lulitanond, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand.
Tel 66 (0) 4320-2086; Fax 66 (0) 4320-2086
E-mail: arolul@kku.ac.th

ml (Rotun *et al*, 1999). The isolates of heterogeneous, vancomycin intermediate-resistant *S. aureus* (hVISA) cannot be detected by routine disk diffusion and MIC determination (Tenover *et al*, 1998, 2001). The reference procedure for the detection of hVISA is a population analysis profile (PAP) (Hiramatsu *et al*, 1997b); however, it is time-consuming, laborious, and therefore inappropriate for routine use.

Several methods have been proposed for the detection of *S. aureus* with reduced susceptibility to vancomycin, such as a commercially-prepared brain heart infusion agar plate with 6 μg/ml of vancomycin (Tenover *et al*, 2004), but this has lot-to-lot variation when prepared in-house (Tenover *et al*, 1998). Similarly, Walsh *et al* (2001) showed that this method gave 22% sensitivity and 97% specificity, compared to 20% sensitivity and 99% specificity using Mueller Hinton agar with 5 μg vancomycin/ml. From the same report, the Etest using McFarland 2.0 inoculum on brain heart infusion agar yielded a sensitivity and specificity of 88%. We tested the potential for routine laboratory detection of hVISA by the disk diffusion test using 15 μg vancomycin disks under various conditions.

MATERIALS AND METHODS

Bacterial isolates

Forty clinical isolates of methicillin-resistant *S. aureus* (MRSA) from patients in Srinagarind and Ramathibodi Hospitals were included. Using PAP (Hiramatsu *et al*, 1997b), 18 isolates were proved hVISA, and 22 were vancomycin-susceptible. All isolates were susceptible to 30 μg vancomycin-disk by routine disk diffusion test.

The reference strains of *S. aureus* Mu50 (VISA) and *S. aureus* Mu3 (hVISA) were kindly provided by Professor K Hiramatsu at Juntendo University, Japan and *S. aureus* ATCC 29213 (VSSA) was from Professor M Vorachit at Ramathibodi Hospital, Bangkok.

All test isolates were identified by conventional biochemical tests and stored in skim milk with 10% glycerol at -70ºC. Culture media (including brain heart Infusion agar (BHA) and Mueller Hinton agar (MHA) (BBL, USA)) were prepared according to the instructions of the manufacturer. Vancomycin powder and Vancomycin Etest strips were purchased from Sigma (Switzerland) and AB Biodisk (Sweden), respectively.

Disk diffusion test

All isolates were tested in triplicate by a disk diffusion test using 15 μg vancomycin disks on four different media: MHA, MHA plus 2% NaCl (MHAS), BHA, and BHA plus 2% NaCl (BHAS). Both 0.5 and 2.0 McFarland inoculum sizes were used. The isolates were grown overnight on blood agar plates. Several colonies were suspended in normal saline solution and adjusted to 0.5 and 2.0 McFarland. The suspension of each strain was then inoculated onto the four different agar plates with a sterile cotton swab. The plates were left at room temperature for approximately 10 minutes. A sterile paper disk was placed and an aliquot of 15 μl of 1,000 μg/ml vancomycin solution was applied to each paper disk. After incubation at 37ºC for 24 and 48 hours, the diameter of each inhibition zone was measured independently by three medical technologists.

Etest

The Etest MICs of vancomycin for the 18 hVISA and 12 VSSA were determined using the same types of media, inocula, and incubation periods as for the disk diffusion test. The MICs were read after incubation at 37ºC for 24 and 48 hours, by the three medical technologists.

RESULTS

The modes of the inhibition zone sizes to the 15 μg vancomycin disk for all isolates are shown in Table 1. Based on a cut-off point of ≥15 mm for an inhibition zone (susceptible),

Table1
Inhibition zone sizes of the 40 isolates with a 15-μg vancomycin disk.

Media[a] and inoculum size	Time (hr)	Organisms	Number of isolates with various inhibition zone diameters (mm)														Total
			7	8	9	10	11	12	13	14	15	16	17	18	19	20	
MHA	24	hVISA									2	4	3	7	1	1	18
0.5 McF		VSSA										4	5	7	5	1	22
	48	hVISA							1	2	3	4	2	4	2		18
		VSSA										4	7	7	3	1	22
MHAS	24	hVISA							2	2	5	5	2	2			18
0.5 McF		VSSA										5	9	7	1		22
	48	hVISA						2	1	5	5	3	1	1			18
		VSSA									1	4	14	3			22
MHA	24	hVISA							2	1	10	2	3				18
2.0 McF		VSSA									4	12	4	2			22
	48	hVISA					1		3	2	9	2	1				18
		VSSA									6	10	6				22
MHAS	24	hVISA					1	2	2	6	4	1	2				18
2.0 McF		VSSA								1	7	9	4	1			22
	48	hVISA					4		6	7		1					18
		VSSA								4	6	8	4				22
BHA	24	hVISA				3	3		1	4	3	4					18
0.5 McF		VSSA							3	2	13	1	2	1			22
	48	hVISA			2	1	4	1	1	2	4	3					18
		VSSA							2	6	11	2	1				22
BHAS	24	hVISA			3		2	4	3	3	3						18
0.5 McF		VSSA								7	8	6	1				22
	48	hVISA		4	1		1	5	2	4	1						18
		VSSA							2	8	8	4					22
BHA	24	hVISA		1	2	2	1	1	7	4							18
2.0 McF		VSSA							7	10	3	2					22
	48	hVISA		1	2	2	3	2	5	3							18
		VSSA						2	5	12	1	2					22
BHAS	24	hVISA		3	1	1	2	6	2	2	1						18
2.0 McF		VSSA						4	8	6	4						22
	48	hVISA	1	3	1	1	4	2	5		1						18
		VSSA					1	7	5	9							22

[a]MHA: Mueller Hinton agar, MHAS: Mueller Hinton agar + 2% NaCl, BHA: Brain heart infusion agar, BHAS: Brain heart infusion agar + 2% NaCl, McF: McFarland

according to CLSI guidelines that use a 30 μg vancomycin disk, the inhibition zone for each of the 40 isolates was ≥15 mm after 24-hour incubation when tested on MHA with a 15 μg vancomycin disk and 0.5 McFarland inoculum.

When the incubation time was increased to 48 hours, three of the 18 hVISAs were detected (inhibition zone ≤14 mm). When tested on MHAS, four and eight isolates of hVISA were detected after 24 and 48 hours, respectively. Slightly better results were obtained when testing with 2.0 McFarland inoculum, as three and six isolates of hVISA were detected on MHA after 24 and 48 hours, respectively.

MHAS permitted detection of 11 and 17 of hVISA after 24 and 48 hours but resulted in one and four isolates of VSSA being false positives, respectively.

The organisms grew better on BHA and BHAS than on MHA and MHAS. When tested with 0.5 McFarland on BHA, 11 hVISA isolates were detected after 24- and 48-hour incubation periods with five and eight false positive VSSA isolates, respectively. Fifteen and 17 isolates of the 18 hVISA were detected after 24- and 48-hour incubation when tested with 0.5-McFarland on BHAS. Seventeen hVISA isolates were detected, when tested with 2.0-McFarland inoculum albeit the number of false positives also increased to between 17 and 22 isolates.

The sensitivity, specificity, positive (PPV) and negative predictive value (NPV) for hVISA detection by the disk diffusion test under various conditions are summarized in Table 2. In addition, the cut-off points were varied to evaluate where the best results were obtained. It was shown that the use of MHAS with an inoculum of 2.0 McFarland and 48-hour incubation gave the best result (*ie*, 94.4% sensitivity, 81.8% specificity, 80.9% PPV, and 94.7% NPV), based on a cut-off point of ≥15 mm for the inhibition zone diameter as susceptible.

Using a cut-off point at vancomycin MIC of ≤4 μg/ml (susceptible), as recommended by the CLSI, the MIC determined by Etest on MHA after 24-hour incubation with 0.5 McFarland inoculum permitted detection of two hVISA. The 28 remaining isolates had an MIC for vancomycin of ≤4 μg/ ml: six were suspected of being hVISA when read after a 48-hour incubation. Better results were obtained when tested on MHAS. The number of suspected hVISA also increased when tested with 2.0 McFarland; that is, 4 and 10 suspected isolates after 24- and 48-hour incubation on MHA *vs* 10 and 11 suspected isolates after 24- and 48-hour incubation on MHAS, respectively.

Table 2
The percentages of sensitivity, specificity, PPV, and NPV of hVISA detection by the disk diffusion test using a 15-μg vancomycin disk under various conditions.

Media/inoculum size	Incubation (hr)	Cut-off point (mm)	Sensitivity	Specificity	PPV	NVP
MHA 0.5 McF	24	≥19	88.9	27.3	50.0	75.0
	48	≥19	88.9	18.1	47.1	66.7
2.0 McF	24	≥17	83.3	27.3	48.4	66.7
	48	≥17	94.4	27.3	51.5	85.7
MHAS 0.5 McF	24	≥17	77.8	77.3	73.7	80.9
	48	≥17	88.9	77.3	76.2	89.5
2.0 McF	24	≥15	61.1	95.5	91.7	75.0
	48	≥15	94.4	81.8	80.9	94.7
BHA 0.5 McF	24	≥16	77.8	18.1	43.8	50.0
	48	≥16	83.3	13.6	44.1	50.0
2.0 McF	24	≥14	77.8	68.2	66.7	78.9
	48	≥14	83.3	68.2	68.2	83.3
BHAS 0.5 McF	24	≥15	83.3	68.2	68.2	83.3
	48	≥15	94.4	54.5	62.9	92.3
2.0 McF	24	≥14	83.3	45.5	55.6	76.9
	48	≥14	94.4	40.9	56.7	90.0

PPV: positive predictive value, NPV: negative predictive value, McF: McFarland

Table3
Number of isolates with various MIC values for vancomycin by Etest.

Media[a]/ Inoculum size	Incubation (hr)	Organisms	Minimum inhibitory concentration (MIC μg/ml)										Total
			1	1.5	2	3	4	6	8	12	16	24	
MHA	24	hVISA		2	4	7	3	2					18
0.5 McF		VSSA	6	3	2	1							12
	48	hVISA		2	2	6	2	2	4				18
		VSSA	3	5	1	2		1					12
MHAS	24	hVISA		2	1	3	3	7	2				18
0.5 McF		VSSA		3	3	5	1						12
	48	hVISA		1	2	2	3	4	6				18
		VSSA		1	5	4	1	1					12
MHA	24	hVISA			4	5	5	2	2				18
2.0 McF		VSSA	4	4	3	1							12
	48	hVISA			4	2	2	4	4	2			18
		VSSA	1	2	7	1	1						12
MHAS	24	hVISA		1	2	2	3	6	4				18
2.0 McF		VSSA		3	3	4	1	1					12
	48	hVISA		1	2	2	2	4	5	2			18
		VSSA		1	5	3	2	1					12
BHA	24	hVISA				4	4	2	4	2	1	1	18
0.5 McF		VSSA		3	4	3	1	1					12
	48	hVISA				2	4	2	4	2	2	2	18
		VSSA		3	3	2	2	1	1				12
BHAS	24	hVISA			1	3	2	1	5	4	2		18
0.5 McF		VSSA			2	5	2	2	1				12
	48	hVISA				4	2	1	5	4	2		18
		VSSA			1	6	1	3	1				12
BHA	24	hVISA				3	4	2	4	2	2	1	18
2.0 McF		VSSA		2	5	1	2	2					12
	48	hVISA				3	4	1	4	1	2	3	18
		VSSA			4	3	1	2	2				12
BHAS	24	hVISA				3	2	2	4	5	2		18
2.0 McF		VSSA				4	4	2	1	1			12
	48	hVISA				3	2	2	4	5	2		18
		VSSA				4	3	2	2	1			12

[a] MHA: Mueller Hinton agar, MHAS: Mueller Hinton agar + 2% NaCl, BHA: Brain Heart Infusion agar, BHAS: Brain Heart Infusion agar + 2% NaCl, McF: McFarland

Most isolates showed a higher MIC value when tested on BHA and BHAS. Ten and 12 of 18 hVISA showed an MIC of >4 μg/ml on BHA after 24- and 48-hour incubation, respectively, with 0.5 McFarland inoculum. The use of BHAS gave a slightly better result with 12 of 18 hVISA isolates showing an MIC of >4 μg/ml (Table 3 and 4).

When using Mueller Hinton agar at the 0.5 and 2.0 McFarland inoculum sizes, most (82.8-100%) of the inhibition zones read by the three readers after 48 hours were the same size (±1 mm) (data not shown). Similarly, at least two of the three inhibition zones, done in triplicate, were the same size (±1 mm) for each reader (90.6-100%; data not shown).

Table 4
The percentages of sensitivity, specificity, PPV, and NPV of hVISA detection by the Etest vancomycin strip under various conditions.

Media/ inoculum size		Incubation (hr)	Sensitivity	Specificity	PPV	NPV
MHA	0.5 McF	24	11.1	100.0	100.0	42.9
		48	33.3	91.7	85.7	47.8
	2.0 McF	24	22.2	100.0	100.0	46.2
		48	55.6	100.0	100.0	60.0
MHAS	0.5 McF	24	50.0	100.0	100.0	57.1
		48	55.6	91.7	90.9	57.9
	2.0 McF	24	55.6	91.7	90.9	57.9
		48	61.1	91.7	91.7	61.1
BHA	0.5 McF	24	55.6	91.7	90.9	57.9
		48	66.7	83.3	85.7	62.5
	2.0 McF	24	61.1	83.3	84.6	58.8
		48	61.1	66.7	73.3	53.3
BHAS	0.5 McF	24	66.7	75.0	80.0	60.0
		48	66.7	66.7	75.0	57.1
	2.0 McF	24	72.2	66.7	76.5	61.5
		48	72.2	58.3	72.2	58.3

Cut-off point at an MIC of ≤ 4 μg/ml as susceptible.

DISCUSSION

The emergence of *S. aureus* with reduced susceptibility to vancomycin arising from MRSA is insidious. These isolates are hetero-resistant and appear susceptible by routine testing, but express resistance at a low concentration. A grave crisis shall arise should they gain homogeneous resistance *in vivo*. Clinical laboratories therefore need a simple and economical method of detecting these isolates that is both sensitive and specific.

From this study, we found that the addition of 2% NaCl to MHA increased the sensitivity of hVISA detection. The use of a 15 μg vancomycin-disk on MHAS with an inoculum size of 2.0 McFarland after 24-hour incubation yielded 61.1% sensitivity and 95.5% specificity. After 48-hour incubation, the sensitivity increased to 94.4%, whereas the specificity decreased to 81.8%. BHA is more enriched than MHA, thereby allowing the organisms to grow better and yielding higher sensitivity for the detection of hVISA. Nevertheless, the number of isolates giving false positives also increased. The use of BHAS with a McFarland inoculum size of 0.5 after 24-hr incubation gave 83.3% sensitivity, but only 68.2% specificity.

The result of the Etest on MHA and MHAS yielded a low sensitivity (*ie*, between 11.1 and 61.1%), but 91.7% to 100% specificity. When tested on BHA and BHAS, the sensitivity increased to between 55.6% and 72.2%, with between 58.3% and 91.7% specificity. The best condition for Etest accuracy from this study was a 2.0 McFarland inoculum size on BHAS after a 24-hour incubation, which yielded 72.2% sensitivity and 66.7% specificity. From our study, the Etest gave a lower sensitivity level than reported by Walsh *et al* (2001), who reported a sensitivity and specificity of 88% when testing with McFarland 2.0 inoculum on BHA.

Our study demonstrated that the disk diffusion test, using a 15 μg vancomycin disk on MHA with 2% NaCl, permitted the detection

of hVISA isolates that could not be detected by ordinary concentration commonly used disk (30 μg); however, it is recommended that all hVISA positive results by this method should be confirmed by PAP.

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