Original Article

Performance of vancomycin and teicoplanin disk diffusion test in isogenic vancomycin non-susceptible *Staphylococcus aureus*

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Abstract

Introduction: Detection of heterogeneous vancomycin-intermediate *Staphylococcus aureus* (hVISA) is currently problematic. Although the population analysis profile with area under the curve (PAP-AUC) is the gold standard for detecting hVISA strains, this method is time consuming. This study aimed to induce vancomycin non-susceptible *Staphylococcus aureus* isolates in methicillin-resistant *S. aureus* (MRSA) and to determine the performance of the vancomycin and teicoplanin disk diffusion test for screening of induced and natural vancomycin non-susceptible isolates.

Methodology: Vancomycin resistance was induced *in vitro* in methicillin-resistant *S. aureus* by serial passage in media with increasing vancomycin concentrations. All test isolates were confirmed for their susceptibility to vancomycin by using a PAP-AUC method. The performance of the vancomycin and teicoplanin disk diffusion test for detecting both induced and natural hVISA/VISA isolates was analyzed using the MedCal program version 10.2.0.

Results: The induction test revealed that 42 of 78 MRSA isolates (53.8%) became hVISA and/or VISA. Using 10, 15, 20, 30 µg vancomycin disks and a 30 µg teicoplanin disk, the highest performance (88.9%) for hVISA/VISA detection (71.1%, sensitivity, 100% specificity, 100% positive predictive value, and 75% negative predictive value) was obtained when a 20 µg vancomycin disk was used at 1.0 McFarland inoculum for a 24-hour incubation.

Conclusions: The results indicated that using a 20 µg vancomycin disk and bacterial inoculum of 1.0 McFarland is simple to perform and provides a primary result for hVISA/VISA screening within 24 hours.

Key words: *Staphylococcus aureus*; vancomycin resistance; teicoplanin; induction; PAP-AUC.

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Introduction

Vancomycin and teicoplanin are glycopeptide drugs of choice for treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections and are also used for empirical therapy to cover infections by Gram-positive organisms [1]. Consequently, there has been an upward trend of vancomycin minimum inhibitory concentration (MIC) for MRSA isolates reported worldwide as vancomycin-resistant *S. aureus* (VRSA), or with reduced susceptibility to vancomycin such as low-level vancomycin-resistant *S. aureus* including vancomycin-intermediate *S. aureus* (VISA) and heterogeneous vancomycin-intermediate *S. aureus* (hVISA) [2]. Moreover, infections with MRSA and hVISA/VISA were significantly associated with vancomycin treatment failure and prolonged bacteremia [3].

Based on MICs recommended by the Clinical and Laboratory Standards Institute (CLSI), isolates with vancomycin MICs of ≥ 16 µg/mL and 4–8 µg/mL are considered as VRSA and VISA, respectively. In addition, isolates with vancomycin MICs within a susceptible range (≤ 2 µg/mL) but containing approximately 10^{-5} to 10^{-6} cells of resistant population are interpreted as hVISA [4,5].

An emergence of VISA strain Mu50 was first reported in 1996 from a Japanese patient with pneumonia who had no response to vancomycin therapy [6]. This strain was developed from the resistant subpopulation of hVISA precursor (Mu3 strain); both isolates showed indistinguishable pulsed-field gel electrophoresis patterns. Moreover, when the Mu3 was grown in conditions with increasing vancomycin concentrations, it could develop the VISA

strain. This indicates that exposure to vancomycin is a selective pressure in the evolutionary development of the resistant phenotype. Katayama *et al*. reported in an *in vivo* study that vancomycin exposure is a main risk for the evolution of hVISA to VISA [7]. However, some VISA strains do not easily convert to vancomycin-susceptible *S. aureus* (VSSA) strains, even after serial passage in an antimicrobial-free medium, although vancomycin MIC is gradually reduced by this treatment [8]. In Thailand, the first hVISA case was reported from Siriraj hospital in 2001 [9]. Furthermore, the VISA strain was first discovered at the Srinagarind hospital in northeast Thailand after the hVISA strain was presented at this location [10,11]. The prevalence of hVISA phenotype in this area was 2.2%.

The vancomycin non-susceptible strains (hVISA/VISA) have thickened cell walls and have slower growth rates than the susceptible strains [12]. The use of standard concentration of bacterial suspension (0.5 McFarland) according to the CLSI broth and agar dilution methods could not detect the resistant subpopulation of the cells presenting in the hVISA strains. A current standard method for hVISA detection is a population analysis profile with area under the curve (PAP-AUC). However, this method is too complicated, expensive, and time consuming [6]. Although the disk diffusion method using standard 30 µg vancomycin disk is simple to perform, it cannot distinguish the hVISA/VISA strains from VSSA strains. Several agar screening plates supplemented with growth-enhancing substances such as casein or horse serum combined with vancomycin and testing with heavy inoculums (such as 2.0 McFarland turbidity) have been proposed for the detection of hVISA/VISA isolates [13,14]. The use of media supplemented with casein or horse serum supports the growth of vancomycin non-susceptible isolates and enhances the expression of vancomycin resistance. A previous report of hVISA screening methods using a low vancomycin concentration and the disk diffusion method on Mueller-Hinton agar (MHA) containing 2% NaCl could detect approximately 80% of hVISA [15]. Therefore, we experimentally induced vancomycin non-susceptible *S. aureus* strains from MRSA isolates. Using these isolates and natural vancomycin-resistant isolates, we evaluated the effectiveness of the disk diffusion test on media supplemented with various enriched substances such as casein, horse serum, or NaCl to screen for vancomycin non-susceptible isolates using various inoculums and vancomycin disk concentrations: 30, 20, 15, and 10 µg disks and a 30 µg teicoplanin disk.

Methodology

Bacterial isolates

A total of 78 MRSA isolates, including 58 VSSA and 20 hVISA isolates, were collected from patients of a hospital in northeast of Thailand between 2010 and 2011. These isolates were subjected to the induction of vancomycin resistance and used for the disk diffusion tests. Another set of MRSA isolates composed of 29 natural hVISA, 5 natural VISA, and 130 VSSA isolates were also included to evaluate the disk diffusion tests with various concentrations of vancomycin. All isolates were identified by biochemical tests such as coagulase, phenol red mannitol, and DNAse tests [16] and stored in skimmed milk (Difco Laboratories, Detroit, USA) with 20% glycerol at -20°C until the time of study.

This study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee of Khon Kaen University (project number HE552272).

Reference strains

Reference strains of *S. aureus* ATCC29213 (VSSA), ATCC25923 (VSSA), ATCC700699 (Mu50, VISA), and ATCC700698 (Mu3, hVISA) were used as control strains for vancomycin-susceptible, vancomycin-intermediate, and heterogeneous vancomycin-intermediate *S. aureus*, respectively.

Induction of vancomycin non-susceptible isolates

The MRSA isolates were induced to be vancomycin non-susceptible by the method of Pfeltz *et al*. with some modifications [17]. Briefly, the isolates were grown in trypticase soy broth (TSB) (Oxoid, Basingstoke, UK) plus 2 µg/mL of vancomycin (Sigma Chemical, St. Louis, USA) and with shaken (250 rpm) for 1–7 days at 37°C until the turbid growth was seen. The cultures were then spread on trypticase soy agar (TSA) (Oxoid, Basingstoke, UK) containing the same vancomycin concentration as in the broth cultures. After incubation, the mutant colony was further cultured in TSB supplemented with a higher concentration of vancomycin and incubated at the same condition for 1–7 days. The cycles of broth and agar cultures were repeated with increasing concentrations of vancomycin until the vancomycin concentration reached to 16 µg/mL in the media.

Modified population analysis profile/area under the curve (PAP-AUC ratio)

A PAP method used in the present study was modified from the method of Wootton *et al*. [18]. Briefly, an overnight broth culture of each MRSA strain was adjusted to 0.5 McFarland turbidity and serial dilution from undiluted to 10^{-6} was performed. A 100 µL aliquot of each suspension was spread on brain-heart infusion agar (BHIA) (Oxoid) containing vancomycin concentrations of 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 µg/mL. Colonies were counted after 48 hours of incubation at 37°C. Log_{10} of the colony numbers (log_{10} CFU/mL) were plotted versus the vancomycin concentrations using Graph Pad Prism 5.0.1 (GraphPad Software Inc., San Diego, USA). The area under the curve (AUC) of each isolate was calculated and interpreted as VSSA, hVISA, and VISA strains according to the ratio of the AUC of the test strain and that of the reference hVISA strain (Mu3). The ratios of < 0.90, 0.90–1.30, and > 1.30 were interpreted as VSSA, hVISA, and VISA, respectively. The PAP-AUC ratios of the reference strains ATCC29213 and ATCC700698 (Mu3) were determined simultaneously.

Minimum inhibitory concentration (MIC)

All isolates were subjected to vancomycin MIC determination by an agar dilution

method according to the CLSI [4].

Disk diffusion test

The disk diffusion method was preliminarily tested against all the isogenic induced isolates including reference strains using 1.0 and 2.0 McFarland inoculums with different concentrations of vancomycin disks (V10, V15, V20, and V30 µg/disk, using sterile 6 mm diameter blank disks supplemented with 20 µL of each stock vancomycin solution) and 30 µg teicoplanin disk (Oxoid) on three kinds of media: BHIA supplemented with 16 g/L of casein, 20% horse serum, and 2% NaCl. The best of these media was used for further testing with all isolates. The diameters of inhibition zones were measured by a vernier caliper (Insize Co., Ltd., Suzhou, China) after 24 and 48 hours of incubation at 37°C.

Statistical analysis

The percentages of sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated using the MedCal program version 10.2.0.0 (MedCalc Software, Ostende, Belgium). The receiver operating characteristic (ROC) curves were achieved by plotting the sensitivity (true positive rate) values versus the 100-specificity (false positive rate) values using the MedCal program, and the area under the ROC curve was used to estimate the accuracy of the test.

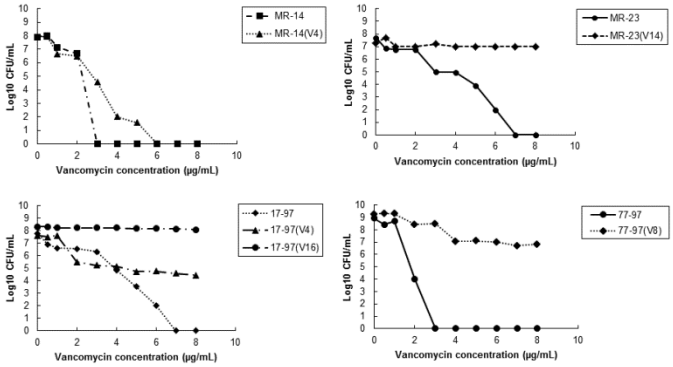
The inhibition zones of isogenic isolates were compared using the paired t-test. For non-isogenic isolates, the unpaired t-test was used. $P < 0.05$ was considered statistically significant.

Results

Induction of reduced vancomycin-susceptible isolates

Of the 78 MRSA isolates, 58 (74.4%) were VSSA and 20 (25.6%) were hVISA (vancomycin MICs of 1–2 µg/mL) and their PAP-AUC ratios were 0.12–0.88 and 0.90–1.15, respectively. After serial passage in the media containing vancomycin, 26 VSSA (33.3%) and 9 hVISA isolates (11.5%) turned to VISA, and 7 VSSA isolates (9%) became hVISA. Among 20 hVISA isolates, 10 isolates (12.8%) could reverse to be VSSA after serial passage in vancomycin-free media. The PAP-AUC ratios of laboratory-induced hVISA isolates were increased from 0.58–0.85 to 0.99–1.27 and those of the induced VISA isolates were changed from 0.12–1.14 to 1.35–3.91 (Figure 1). The vancomycin MICs for the hVISA and VISA isolates were 1–3 µg/mL and 4–16 µg/mL, respectively. About a half (45.2%) of these isolates grew within one day after vancomycin exposure, followed by growth after two days (30.9%), three days (7.8%), seven days (6.3%), four and five days (4% each), and six days (1.8%). Of the 78 isolates, 36 (46.2%) isolates with PAP-AUC ratios of 0.40–1.26 could not develop vancomycin resistance after seven-day shaking in TSB plus 2 µg/mL vancomycin.

Figure 1. Population analysis profile of parent strains MR-14, MR-23, 17-97, 77-97 and their mutants after exposure to vancomycin 4 µg/mL (V4), 8 µg/mL (V8), 14 µg/mL (V14) and 16 µg/mL (V16)



Performance of vancomycin and teicoplanin disk diffusion test

The induced hVISA and VISA isolates grew better on BHIA supplemented with 16 g/L of casein than those containing 20% horse serum or 2% NaCl. However, the best performance of vancomycin and teicoplanin disk diffusion test to detect these isolates were obtained when testing on BHIA supplemented with 2% NaCl. The ROC curves of 10, 15, 20, and 30 µg vancomycin (V10, V15, V20, V30) disk diffusion tests using 1.0 McFarland inoculum for 24-hour incubation showed that the V20-curve was closest to the top-left corner than that representing other disk concentrations. Based on their AUC of 0.882, 0.885, 0.889, and 0.846, respectively, it was apparent that the tests were quite satisfactory for hVISA/VISA detection (Figure 2). The 2.0 McFarland inoculum for 24- and 48-hour incubations also gave a comparable AUC to that of 1.0 McFarland inoculum at 24-hour incubation. The 20 µg vancomycin disk diffusion test using 1.0 McFarland inoculum for 24-hour incubation gave the highest accuracy for the detection of the vancomycin non-susceptible isolates (88.9%) when using a cut-off at ≤ 12 mm (71.1% sensitivity, 100% specificity, 100% PPV, and 75% NPV) (Table 1).

Figure 2. ROC curves of 10, 15, 20, 30 µg vancomycin disks and 30 µg teicoplanin disk diffusion on BHIA supplemented with 2% NaCl T30: teicoplanin disk 30 µg; V30: vancomycin disk 30 µg; V20: vancomycin disk 20 µg; V15: vancomycin disk 15 µg; V10: vancomycin disk 10 µg

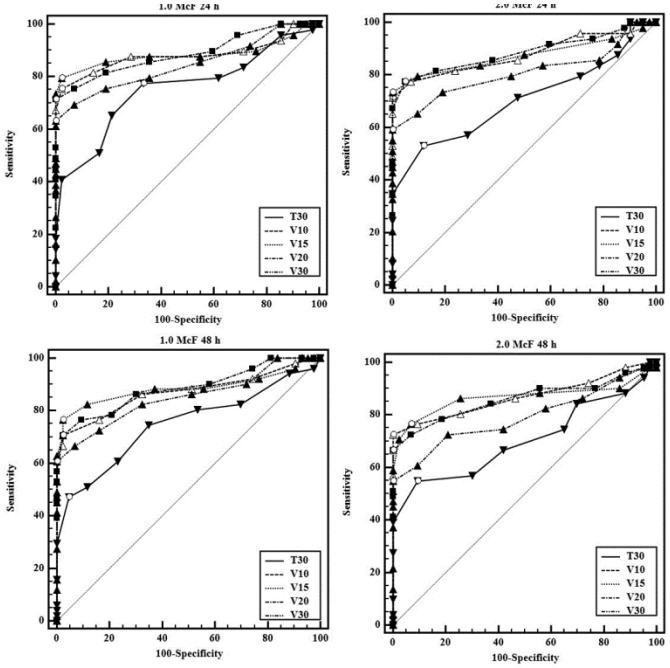


Table 1. Performance of detecting hVISA/VISA mutants by disk diffusion test on BHIA supplemented with 2% NaCl

Type of disk/ concentration (µg/disk)	Isogenic MRSA (n = 94; VSSA = 43, hVISA = 16, VISA = 35)							
	Inoculum sizes (McF)	Incubation (hours)	Cut-off point (mm)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
T30	1.0	24	≤ 18	77.6	66.7	73.1	71.8	75.5
		48	≤ 18	81.4	46.5	64.1	66.7	-
	2.0	24	≤ 15	53.1	88.1	83.9	61.7	70.4
		48	≤ 15	56.9	69.8	69	57.7	-
V30	1.0	24	≤ 14	63.3	100	100	70	84.6
		48	≤ 14	66.7	93	91.9	70.2	-
	2.0	24	≤ 13	59.2	100	100	67.7	80.9
		48	≤ 13	60.8	90.7	88.6	66.1	-
V20	1.0	24	≤ 12	71.4	100	100	75	88.9
		48	≤ 12	76.5	90.7	90.7	76.5	-
	2.0	24	≤ 12	77.6	95.2	95	78.4	88.7
		48	≤ 12	78.4	81.4	83.3	76.1	-
V15	1.0	24	≤ 12	79.6	97.6	97.5	80.4	88.5
		48	≤ 12	82.4	88.4	89.4	80.9	-
	2.0	24	≤ 11	73.5	100	100	76.4	87.5
		48	≤ 11	76.5	93	92.9	76.9	-
V10	1.0	24	≤ 10	75.5	97.6	97.4	77.4	88.2
		48	≤ 10	76.5	83.7	84.8	75	-
	2.0	24	≤ 9	71.4	100	100	75	87.9
		48	≤ 9	76.5	90.7	90.7	76.5	-

McF: McFarland; T30: 30 µg teicoplanin disk; V30: 30 µg vancomycin disk; V20: 20 µg vancomycin disk; V15: 15 µg vancomycin disk; V10: 10 µg vancomycin disk; PPV: positive predictive value; NPV: negative predictive value

Table 2. Comparison of inhibition zones between isogenic hVISA/VISA and VSSA

Disk (µg)	Inoculum McFarland	VSSA (n = 32) (inhibition zone, mm)		hVISA/VISA (n = 32) (inhibition zone, mm)		P
		range	X±SD	range	X±SD	
T30	1	16–24	19.7 ± 1.8	13–23	16.7 ± 2.5	< 0.0001
	2	15–24	18.6 ± 2.3	12–22	16.4 ± 3.0	0.0025
V30	1	15–25	19.0 ± 2.0	8–19	13.1 ± 3.1	< 0.0001
	2	14–24	18.0 ± 2.2	7–20	12.9 ± 3.5	< 0.0001
V20	1	14–23	17.2 ± 2.1	6–17	9.8 ± 3.2	< 0.0001
	2	13–22	16.1 ± 2.2	6–17	9.6 ± 3.1	< 0.0001
V15	1	13–20	16.8 ± 1.7	6–17	8.8 ± 2.9	0.0006
	2	12–20	14.8 ± 1.6	6–16	8.7 ± 2.9	0.2922
V10	1	11–19	14.1 ± 1.9	6–15	7.7 ± 2.2	0.0004
	2	10–18	13.1 ± 1.8	6–15	7.5 ± 2.2	0.414

V30: 30 µg vancomycin disk; V20: 20 µg vancomycin disk; V15: 15 µg vancomycin disk; V10: 10 µg vancomycin disk

Table 3. Performance of natural hVISA/VISA detection by disk diffusion test on BHIA supplemented with 2% NaCl

Type of disk/ concentration (µg/disk)	Natural MRSA (n = 164; VSSA = 130, hVISA = 29, VISA = 5)							
	Inoculum sizes (McF)	Incubation (hours)	Cut-off point (mm)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
T30	1.0	24	≤ 21	88.2	30	24.8	90.7	51.5
		48	≤ 21	97.1	10.8	22.1	93.3	-
	2.0	24	≤ 17	79.4	40.8	26	88.3	56.2
		48	≤ 17	52.9	57.7	24.7	82.4	-
V30	1.0	24	≤ 21	94.1	17.7	23	92	53.7
		48	≤ 21	97	10.8	22.1	93.3	-
	2.0	24	≤ 16	82.4	28.5	23.1	86	51.7
		48	≤ 16	58.8	42.3	21.1	79.7	-
V20	1.0	24	≤ 17	67.7	47.7	25.3	84.9	55.9
		48	≤ 17	82.4	24.6	22.2	84.2	-
	2.0	24	≤ 15	52.9	56.9	24.3	82.2	55.7
		48	≤ 15	70.6	38.5	23.1	83.3	-
V15	1.0	24	≤ 13	20.6	93.1	43.8	81.8	50.8
		48	≤ 13	26.5	84.6	31	81.5	-
	2.0	24	≤ 14	52.9	55.4	23.7	81.1	53.9
		48	≤ 14	70.6	32.3	21.4	80.8	-
V10	1.0	24	≤ 11	85.3	6.2	19.2	61.5	50.7
		48	≤ 11	82.4	14.6	20.1	76	-
	2.0	24	≤ 12	67.7	40.8	23	82.8	51.7
		48	≤ 12	35.3	60.8	19	78.2	-

McF: McFarland; T30: 30 µg teicoplanin disk; V30: 30 µg vancomycin disk; V20: 20 µg vancomycin disk; V15: 15 µg vancomycin disk; V10: 10 µg vancomycin disk; PPV: positive predictive value; NPV: negative predictive value

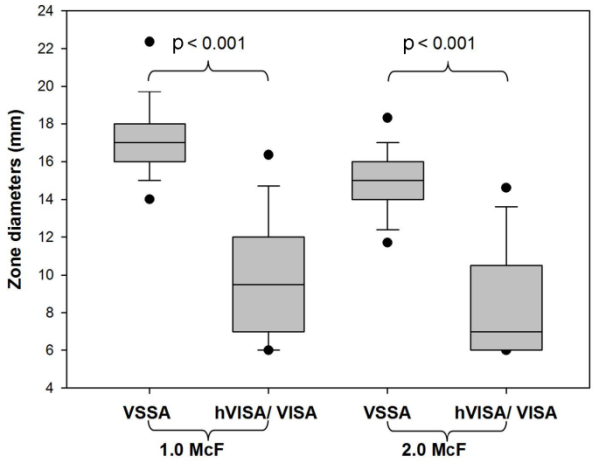


Figure 3. Comparison of zone diameters of 20 µg vancomycin disk diffusion test with 1.0 and 2.0 McFarland inoculum of VSSA and non-susceptible hVISA/VISA after 24-hr incubation ●, the 5th and 95th percentiles of the zone diameters of representative isolates

The disk diffusion performance slightly decreased with a longer incubation period (48 hours) (88.5% accuracy, 70.6% sensitivity, 95.7% specificity, 97.3% PPV, and 73.7% NPV). When 2.0 McFarland inoculum was used, the results of 24- and 48-hour incubations were similar to those of 1.0 McFarland (88.7 vs. 86% accuracy, 77.6% vs. 66.7% sensitivity, 95.2 vs. 100% specificity, 95% vs. 100% PPV, and 78.4% vs. 71.1 % NPV at the cut-off range of 10–12, mm respectively). The paired t-test between inhibition zones of the isogenic VSSA, hVISA, and VISA isolates showed significant difference ($p < 0.001$) when the V30 and V20 disks were tested (Table 2, Figure 3). The ranges of the accuracy, sensitivity, specificity, PPV, and NPV in detection of vancomycin non-susceptible isolates using other concentrations of vancomycin disks were 70.3%–88.5%, 47.1%–79.6%, 66.7%–100%, 73.1%–100%, and 60.3%–80.4%, respectively, when using the cut-off point range of 8–18 mm. Detection of natural hVISA/VISA isolates by vancomycin disk diffusion test gave rather low and diverse sensitivity, ranging from 20.6% to 94.1%, specificity from 6.2% to 93.8%, PPV from 19.2% to 43.8%, and NPV from 61.5% to 92%. The AUC values from the ROC curves of these natural isolates were 0.507–0.559 at the cut-off point range of 11–21 mm (Table 3).

The AUC values from ROC curves (0.703–0.755) were quite low when tested with 30 µg teicoplanin disk. This implied that the test was inferior to the vancomycin disk for hVISA/VISA detection. Using the 30 µg teicoplanin disk, 1.0 McFarland inoculum, and 24-hour incubation yielded the best performance (88.2% sensitivity, 30% specificity, 24.8% PPV, 90.7% NPV, and AUC of 0.515) for detecting natural hVISA/VISA isolates at a cut-off point ≤ 21 mm.

Discussion

The emergence of vancomycin treatment failure among MRSA infection cases is a serious problem. The major cause for the occurrence of hVISA/VISA phenotypes is the overuse of vancomycin and other glycopeptide antibiotics such as teicoplanin for the treatment of MRSA and Gram-positive bacterial infections [19]. In this study, the natural parental strains of VSSA with vancomycin MIC of 1–2 µg/mL were changeable to hVISA and VISA phenotypes by serial passage in the media with stepwise increase of vancomycin concentrations. Most of the increased vancomycin-resistant isolates occurred within one to two days after exposure to the increasing concentration of the drug. This may be due to the vancomycin-resistant sub-population concealed among the test organisms or because the parent VSSA strains tended to be hVISA and VISA easily in the environment with increasing drug pressure. This confirmed the presumption of what happened *in vivo* among patients after prolonged treatment with vancomycin [6]. On the other hand, the parental hVISA isolates turned into VSSA (lower MICs and PAP-AUC ratio < 0.90) after 15–30 serial passages in the vancomycin-free medium. This finding demonstrated that the vancomycin non-susceptible property of some isolates was reversible.

The MRSA isolates were categorized into VSSA, hVISA, and VISA groups by their PAP-AUC ratios. Almost 40% of the VSSA isolates in this study presented a borderline PAP-AUC ratio of 0.80–0.89. It has been described that 44% of isolates with a given PAP-AUC ratio between 0.80–0.89 were falsely classified as hVISA by three screening methods: Etest macromethod, Etest GRD, and BHI with casein screen agar [14]. These isolates may have been at the preliminary step of hVISA phenotype and displayed overlapping characteristics of VSSA and vancomycin non-susceptible isolates.

Although the addition of 16g/L of casein or 20% horse serum into the BHI screen agar has been recommended to improve the detection of hVISA or VISA isolates [14], the present study revealed that BHI with 2% NaCl supported hVISA and gave a better detection rate than those supplemented with casein or horse serum. This finding was similar to our previous study, which showed that a low-concentration vancomycin disk diffusion test using BHIA supplemented with 2% NaCl yielded 83.3% sensitivity and 45.5% specificity [13]. These disk diffusion tests gave comparable sensitivity and specificity to agar screen methods such as BHI screen agar plate containing 4 µg/mL of vancomycin and 16 g/L of casein, which had 43% and 76% of sensitivity, 100% and 67% of specificity using 0.5 and 1.0 McFarland inoculums, respectively, and 24-hour incubation. The test performance had significantly improved when it was incubated for 48 hours [14]. However, in the present study, no such significant improvement was observed after 48-hour incubation. In another study, screen agar plate of Mueller-Hinton supplemented with 5 µg/mL of vancomycin using a standard bacterial concentration (0.5 McFarland) gave poor sensitivity (1%–20%) and specificity (59%–99%) [20,21]. Although the use of a 20 µg vancomycin disk in the present study gave higher sensitivity for hVISA detection, it provided quite low specificity, especially

for the natural hVISA/VISA isolates. This may be due to the phenotypic differences between the natural- and induced-resistance isolates in their vancomycin MICs and the recent vancomycin exposure. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry is a new technology that reduces time for identification and drug susceptibility testing [22]. The most updated method of isothermal microcalorimetry provides excellent NPV for detection of *S. aureus* with reduced vancomycin susceptibility within eight hours [23]. However, these methods are expensive and unaffordable for routine testing in the diagnostic laboratory of rural hospitals in developing countries. Therefore, further study is necessary to identify other characteristics of hVISA to differentiate them from VSSA isolates.

The hVISA and VSSA phenotypes could not be differentiated correctly by their vancomycin MICs, and the reduced vancomycin-susceptible isolates like hVISA or VISA are still a public health concern. The development of these phenotypes is associated with various factors such as cell wall thickening, decrease of autolytic activity, slow growth rate, and decreased *agr* activity [24]. Their muropeptide cross-linking and adherence ability are also reduced compared to those of VSSA strains [8,25-27]. Another mechanism that was discovered in isogenic series of clinical MRSA isolates was one that produced an abnormal cell wall chemical composition (peptidoglycan), relieving cross-linking and poorly separated cells when it was grown in drug-free medium [28]. Determination of whole-genome sequences of VISA and their parental hVISA strains revealed that the VISA strains had mutations in 20 genes, and 15 of the 20 genes had novel mutations associated with phenotypic conversion of hVISA to VISA. The most frequently affected gene was *cmk* – encoding cytidylate kinase. Reduction of cytidylase kinase activity in *cmk* mutants encouraged the hVISA-to-VISA conversion by thickening the cell wall and reducing the cell growth rate [29].

Conclusions

The disk diffusion method using a 20 µg vancomycin disk, 1.0 McFarland inoculum on BHIA supplemented with 2% NaCl, and cut off point at ≤ 12 mm could detect 88% of hVISA/VISA phenotypes. It is simple to perform and yields a primary result within 24 hours. The test may be suitable for screening of low-level vancomycin-resistant strains from patients with chronic MRSA infection or failure of glycopeptide therapy. However, the isolate with a positive screening test should be confirmed by the PAP-AUC method because of low specificity. Other characteristics of hVISA should be further investigated to develop a simple screening method with more accuracy and higher performance for the detection of hVISA isolates.

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