Vancomycin Susceptibility Trends and Prevalence of Heterogeneous Vancomycin-Intermediate *Staphylococcus aureus* in Clinical Methicillin-Resistant *S. aureus* Isolates[∇][†]

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Due to the rise in methicillin-resistant *Staphylococcus aureus* (MRSA) infections and widespread use of vanco-mycin, MRSA isolates with reduced susceptibility to vancomycin are emerging (i.e., MIC creep). However, the prevalence of heterogeneous vancomycin-intermediate *S. aureus* (hVISA) is unknown due to the difficulty in detect-ing this phenotype. Recently, Etest glycopeptide resistance detection (GRD) strips have been developed to detect hVISA. This study assessed vancomycin susceptibility in MRSA isolates and determined the prevalence of hVISA by Etest GRD and population analysis profile-area under the curve ratio (PAP-AUC). The genetic backgrounds of 167 MRSA isolates collected from 2000 to 2008 were identified by pulsed-field gel electrophoresis. Vancomycin MICs were determined using Etest and two broth microdilution assays, MicroScan and Sensititre. Etest GRD was performed on all isolates, and those exhibiting a hVISA phenotype were further tested by PAP-AUC. The vanco-mycin MIC modes remained consistent at 1 μg/ml, as assessed by Sensititre and MicroScan. Etest reported a significant increase (mode MIC = 1.5 μg/ml) in the MIC between 2000 and 2008 ($P < 0.01$); however, this increase did not reflect a ≥2-fold change. In addition, the slight MIC increase did not increase linearly from 2000 to 2008, suggesting biological fluctuation, and is inconsistent with the concept of MIC creep. Etest GRD identified six hVISA isolates, two of which were confirmed to be hVISA by PAP-AUC. In conclusion, reduced vancomycin susceptibility was not detected in our hospital over a 9-year period using three different MIC methodologies, and the hVISA incidence was 1.2%, as determined by Etest GRD and PAP-AUC.

Vancomycin remains the antibiotic of choice to treat many methicillin-resistant *Staphylococcus aureus* (MRSA) infections. However, due to the dramatic rise in MRSA infections and widespread use of vancomycin, which is known to have mar-ginal tissue penetration and slow bactericidal activity, MRSA strains with reduced susceptibility to vancomycin are emerging (10, 30, 44, 46). Although most of these strains have a vanco-mycin MIC within the susceptible range, according to the Clin-ical and Laboratory Standards Institute (CLSI), some reports have shown a generalized increase in vancomycin MIC over time (also known as MIC creep) (38, 41–43, 52).

Associated with this issue is the presence of heterogeneous vancomycin-intermediate *S. aureus* (hVISA). These organisms are described as being susceptible to vancomycin but contain a subpopulation that possesses a thicker cell wall and expresses resistance to vancomycin (5, 6, 49). With these characteristics, hVISA is considered the precursor of vancomycin-intermedi-ate *S. aureus* (51). Infections caused by hVISA are a growing

concern in hospitals, resulting in prolonged bacteremia, endo-carditis, and osteomyelitis and ultimately leading to vancomy-cin treatment failure (4, 9, 16, 37). Contributing to this prob-lem of hVISA is the fact that current diagnostic methods often fail to detect this resistance phenotype, and additional screen-ing is required to detect hVISA (15, 24). The "gold standard" for identifying hVISA is the population analysis profile-area under the curve ratio (PAP-AUC), but this method is time-consuming, labor-intensive, and costly for clinical laboratories. Additional methods have been used to detect hVISA, but these are not standardized and have low predictability in hVISA detection (14). A new modified Etest, called Etest glycopep-tide resistant detection (GRD), has recently been developed and demonstrated to have high sensitivity and specificity in detecting hVISA (24, 55).

The purpose of this study was to determine if the MIC to vancomycin had increased significantly among clinical MRSA isolates that were collected over a 9-year period and to deter-mine the prevalence of hVISA within this population using the Etest GRD methodology and vancomycin population analysis profile.

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MATERIALS AND METHODS

Bacterial strains. A random selection of 167 clinical MRSA bloodstream isolates collected from 2000 to 2008 at a 689-bed academic medical center was

used in this study. All isolates were processed at the time of collection and stored at $-80°C$ until they were tested. *S. aureus* Mu3 (ATCC 700698; hVISA) and a non-methicillin-resistant *S. aureus* strain (ATCC 29213) were used as reference organisms for the antimicrobial susceptibility tests and PAP-AUC analysis. *S. aureus* strains NRS382 (USA100), NRS383 (USA200), NRS384 (USA300-0114), and NRS123 (USA400) were acquired from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA).

PFGE. To determine the epidemiological relatedness of the MRSA isolates, pulsed-field gel electrophoresis (PFGE) was performed using SmaI DNA digestion as previously described (32). All gels were analyzed using BioNumerics software (Applied Maths, Austin, TX) and normalized with NCTC 8325 (19). Seventeen to 20 unique isolates, as assessed by PFGE, were selected from each year (2000 to 2008) for vancomycin susceptibility testing and evaluation for the presence of hVISA. Some isolates that were indistinguishable by PFGE and that appeared in multiple years were included in the analysis. *S. aureus* isolates NRS382, NRS383, NRS384, and NRS123 were used as standard controls for PFGE types USA100, USA200, USA300, and USA400, respectively.

Antimicrobial susceptibility tests. Vancomycin MICs were measured and compared by Etest (AB bioMérieux, Solna, Sweden) and two broth microdilution tests, Sensititre (TREK Diagnostic Systems, Cleveland, OH) and MicroScan (Siemens Healthcare Diagnostics, Deerfield, IL). Each of the susceptibility tests utilized a 0.5 McFarland standard inoculum in sterile water and was performed according to the manufacturers' instructions. Results from the broth microdilution tests were read manually.

Etest GRD. Etest GRD was performed on all isolates according to the manufacturer's instructions (AB bioMérieux). Briefly, a bacterial suspension of a 0.5 McFarland standard inoculum in sterile water was spread on a Mueller-Hinton agar plate with 5% sheep blood (Remel, Lenexa, KS). Next, a GRD strip consisting of a double-sided gradient with vancomycin and teicoplanin was applied to the plate. The plate was incubated at $37°C$, and the elliptical zones of the Etest GRD strip were read at 24 and 48 h. Isolates defined to be positive for hVISA had a GRD value of ≥ 8 $\mu g/ml$ for either vancomycin or teicoplanin and a standard vancomycin Etest MIC of <4 $\mu g/ml$. Two or more microcolonies present in the zone of inhibition at ≥ 8 $\mu g/ml$ also were an indicator for a positive GRD test (55).

PAP-AUC. PAP-AUC was done as described by Wootton et al. (53). Briefly, overnight cultures grown in Trypticase soy broth (BD Diagnostics, Sparks, MD) on an orbital shaker at $37°C$ and 250 rpm were serially diluted onto brain heart infusion agar (BD Diagnostics) plates containing 0, 0.5, 1, 1.5, 2, 4, and 8 $\mu g/ml$ of vancomycin. The numbers of CFU were counted after 48 h of incubation at $37°C$. The number of CFU/ml was plotted against the aforementioned vancomycin concentrations using GraphPad Prism software (San Diego, CA). The AUC was computed by the trapezoidal method for each test isolate and *S. aureus* Mu3. A ratio was calculated by dividing the AUC of the test isolate by the AUC of Mu3; a ratio of between ≥ 0.9 and <1.3 was considered positive for hVISA.

Statistical analysis. Statistical software (SAS, version 9.2; SAS Institute, Inc., Cary, NC) was utilized to analyze the MICs over time for each antimicrobial susceptibility test and to compare the MICs among the different tests for each year. The summary statistics for the MICs from the different tests for each year were presented by the geometric mean values. For comparison, the MIC values were sorted into four categories: ≤ 0.75, 1, 1.5, and ≥ 2. The generalized linear mixed models (GLIMMIX) procedure was used to fit a multinomial model with fixed effects for the type of test, year, and the interaction of the two and a random effect for different bacterial isolates nested within each year. A P value of <0.05 was considered significant for all statistical tests.

RESULTS

PFGE profiles of MRSA isolates. PFGE was utilized to identify the genetic backgrounds of 167 MRSA isolates, and the pulsed-field profiles were compared to those of standard USA100 to USA400 strains (see Fig. S1 in the supplemental material). Forty-four (26%), 1 (0.6%), 12 (7%), and 8 (5%) of the isolates had profiles that were within two band differences of those for USA100, USA200, USA300, and USA400, respectively. The remaining 102 (61%) isolates were distinguishable from the USA100 to USA400 strains. Fifty-four percent of the isolates appeared more than once between 2000 and 2008, including one USA100-like isolate that was present in 7 of the 9 years.
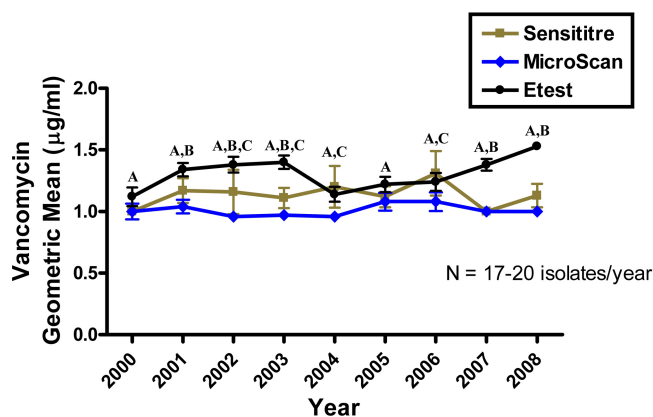

FIG. 1. Vancomycin MICs of MRSA, displayed as geometric means ± standard errors of the means, were measured by Sensititre, MicroScan, and Etest for isolates recovered from 2000 to 2008. Etest reported significantly higher MICs than MicroScan (indicated by A) and Sensititre (indicated by B). Sensititre results were also significantly higher than MicroScan results for certain years (indicated by C) ($P < 0.05$).

Vancomycin MICs from Sensititre, MicroScan, and Etest. The vancomycin geometric mean MICs from the three different antimicrobial susceptibility tests ranged from 1.00 to 1.31 $\mu g/ml$ for Sensititre, 0.96 to 1.08 $\mu g/ml$ for MicroScan, and 1.12 to 1.53 $\mu g/ml$ for Etest (Fig. 1). The Sensititre MIC results fluctuated the most from year to year, yet there were no significant differences between the years due to the large variability among the isolates from each year. MicroScan displayed the lowest MICs and remained stable throughout the study. When the MIC values from Etest were compared, there was a significant increase between 2000 and 2008 ($P < 0.01$); however, this was not a gradual increase over time (Fig. 1). There was a significant increase in the MIC between 2000 and 2001 ($P = 0.04$), but the MIC did not significantly change for the next 2 years. In 2003 and 2004, the MIC significantly dropped ($P = 0.01$) and did not rise again until 2007 ($P = 0.02$).

The vancomycin MIC modes remained fixed throughout the study period, with overall modes of 1 $\mu g/ml$ for Sensititre and MicroScan and 1.5 $\mu g/ml$ for Etest (Table 1). Utilizing the Sensititre and MicroScan methodologies, 81% and 94% of the isolates, respectively, had a MIC of 1 $\mu g/ml$, while using Etest, 31% and 63% of the isolates had MICs of 1 $\mu g/ml$ and 1.5 $\mu g/ml$, respectively. The overall frequency of MRSA isolates for which the MIC was ≥ 2 $\mu g/ml$ varied from 17% by Sensititre to 4% by MicroScan and 2% by Etest. Etest results were significantly elevated compared with those of MicroScan for every year, and this was also true when the Etest MICs were compared to the Sensititre MICs for 5 of the 9 years (Fig. 1; $P < 0.05$). For 2002 to 2004 and 2006, the Sensititre results were statistically higher than the MicroScan results (Fig. 1; $P < 0.05$).

Etest GRD for detecting hVISA. Of the 167 isolates tested by Etest GRD, 6 were positive for hVISA by having a teicoplanin MIC value of ≥ 8 $\mu g/ml$ and a standard Etest vancomycin MIC of <4 $\mu g/ml$ (Table 2). No consistent relationship existed for the year that the isolate was obtained or the clonal pattern and the presence of hVISA.

TABLE 1. Annual frequencies and percentages for each test for vancomycin

| Yr (no. of isolates) and test	No. (%) of isolates for which vancomycin MIC (μg/ml) was:			
	≤0.75	1	1.5	≥2
2000 (18)				
Sensititre	1 (6)	16 (88)		1 (6)
MicroScan	1 (6)	16 (88)		1 (6)
Etest	4 (22)	6 (33)	8 (44)	0 (0)
2001 (18)				
Sensititre	0 (0)	14 (78)		4 (22)
MicroScan	0 (0)	17 (94)		1 (6)
Etest	0 (0)	5 (28)	13 (72)	0 (0)
2002 (19)				
Sensititre	2 (11)	12 (63)		5 (26)
MicroScan	1 (5)	18 (95)		0 (0)
Etest	1 (5)	3 (16)	14 (74)	1 (5)
2003 (20)				
Sensititre	0 (0)	17 (85)		3 (15)
MicroScan	1 (5)	19 (95)		0 (0)
Etest	0 (0)	4 (20)	15 (75)	1 (5)
2004 (19)				
Sensititre	0 (0)	15 (79)		4 (21)
MicroScan	1 (5)	18 (95)		0 (0)
Etest	1 (5)	11 (58)	7 (37)	0 (0)
2005 (19)				
Sensititre	0 (0)	16 (84)		3 (16)
MicroScan	0 (0)	17 (89)		2 (11)
Etest	1 (5)	8 (42)	10 (53)	0 (0)
2006 (18)				
Sensititre	0 (0)	12 (67)		6 (33)
MicroScan	0 (0)	16 (89)		2 (11)
Etest	0 (0)	10 (56)	7 (39)	1 (5)
2007 (19)				
Sensititre	0 (0)	19 (100)		0 (0)
MicroScan	0 (0)	19 (100)		0 (0)
Etest	0 (0)	4 (21)	15 (79)	0 (0)
2008 (17)				
Sensititre	0 (0)	14 (82)		3 (18)
MicroScan	0 (0)	17 (100)		0 (0)
Etest	0 (0)	0 (0)	16 (94)	1 (6)
Total (167)				
Sensititre	3 (2)	135 (81)		29 (17)
MicroScan	4 (2)	157 (94)		6 (4)
Etest	7 (4)	51 (31)	105 (63)	4 (2)
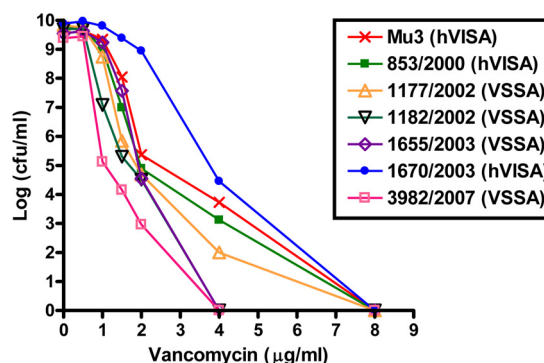
FIG. 2. Population analysis profile curves of the six isolates that were positive for hVISA by Etest GRD. Two isolates were identified to be hVISA and four isolates were defined to be vancomycin-susceptible *S. aureus* (VSSA) compared to the susceptibility of the Mu3 reference strain.

PAP-AUC for detecting hVISA. PAP-AUC was performed to confirm the positive results from the GRD Etest, and two isolates were identified to be hVISA using this analysis (Fig. 2). The two hVISA isolates from 2000 and 2003 had PAP-AUC ratios of 0.91 and 1.23, respectively. Both hVISAs had vancomycin MICs of 1.5 to 2 μg/ml according to Sensititre and Etest, while MicroScan reported a 1-μg/ml MIC for both isolates (Table 2).

DISCUSSION

Reduced susceptibility to vancomycin in *S. aureus* has been a major medical concern for over a decade. Several studies have reported elevated vancomycin MICs in MRSA isolates where the MICs are at the upper end of the susceptibility range (18, 27, 38, 40–43, 50, 52). However, not all medical institutions are reporting an increase in vancomycin MICs (1, 11, 20, 33). One study measured vancomycin MICs in MRSA isolates collected from 2002 to 2006 from nine different U.S. medical centers using a reference broth microdilution assay with a broad range of precise incremental dilutions (39). Their results showed that they did not detect a reduction in vancomycin susceptibility at any of the test sites, which are similar to our results using three separate antimicrobial susceptibility tests. Although the MIC by Etest did increase from 2000 to 2008, it fluctuated over time and was clinically insignificant to support a consistent decline in vancomycin susceptibility. More specifically, the majority of the isolates in this study had a vancomy-

TABLE 2. Isolates positive for hVISA by Etest GRD

Isolate	Yr	Clonal pattern	GRD values (μg/ml) for VAN/TEC[a]	Vancomycin MIC (μg/ml) by Sensititre, MicroScan, Etest	PAP-AUC result
853	2000	Not defined	1/16	2, 1, 1.5	hVISA
1177	2002	Not defined	2/32	2, 1, 2	VSSA[b]
1182	2002	USA400	1.5/24	1, 1, 1.5	VSSA
1655	2003	USA100	1.5/8	1, 1, 1.5	VSSA
1670	2003	Not defined	2/12	2, 1, 2	hVISA
3982	2007	Not defined	1/≥32	1, 1, 1.5	VSSA

[a] VAN, vancomycin; TEC, teicoplanin.
[b] VSSA, vancomycin-susceptible *S. aureus*.

cin MIC of 1 or 1.5 μg/ml, and the difference between the two Etest MICs from 2000 and 2008 was <1.5-fold, whereas the other studies have detected a ≥1.5-fold MIC increase or a significant increase in the frequency of isolates having vancomycin MICs of ≥1 μg/ml (41, 43, 52). None of these trends were found in our study.

The conflicting results in the literature documenting elevated vancomycin MICs in MRSA may partly be due to failure of epidemiologically typing the MRSA isolates. Some MRSA clones have higher levels of dissemination than others and possess elevated vancomycin MICs, which may cause false perceptions of an overall reduction in vancomycin susceptibility for all strains of MRSA (8, 26, 31, 48). The purpose of our study was to assess vancomycin susceptibility in the population of clinically encountered MRSA clonal types and not to define the overall MRSA susceptibility profile for clinical purposes in the associated patient population. PFGE was performed to eliminate clonal strains within each year that could otherwise influence the results. This study identified a highly diverse sample of MRSA isolates. Twenty-six percent of the isolates were USA100-like, a common MRSA strain found in hospitals, whereas 7% and 5% were similar to the community-acquired strains USA300 and USA400, respectively.

The type of antimicrobial susceptibility test that is performed to measure the vancomycin MICs may also affect the assessment of whether susceptibility within the population has changed. In this study, three standard susceptibility tests that are used in the clinical setting were chosen to measure the MICs. Although elevated vancomycin MICs could not be demonstrated by the three different tests, there were differences among the assays. In agreement with a previous study, Sensititre panels had the highest variability (47). It is unclear why MicroScan reported the lowest vancomycin MICs. Other studies found MicroScan to report vancomycin MICs in MRSA similar to or higher than those that Etest and Sensititre report (17, 45, 47). Etest reported vancomycin MICs higher than those reported by the two broth microdilution assays. This was expected and has been described elsewhere (33, 36). Etest strips contain a gradient of vancomycin concentrations, including an intermediate reading of 1.5 μg/ml, as opposed to the broth microdilution assays, where the concentrations are in 2-fold increments. Most of the isolates had a MIC of 1.5 μg/ml, causing the Etest results to be more elevated than the results of the other two assays. Etest also measures the MIC for a larger proportion of bacteria at ~10^8 CFU than the broth microdilution assays at ~10^4 CFU, making Etest more sensitive in detecting resistant mutants and MIC increases (11, 33, 43).

In addition to assessing a reduction in vancomycin susceptibility, the prevalence of hVISA was determined by the newly developed Etest GRD. Two studies have verified that the Etest GRD has high sensitivity (93 to 94%) in detecting hVISA compared to PAP-AUC but variable specificity (82% and 95%) (24, 55). Nonetheless, the specificity of the Etest GRD is similar to or higher than the specificities of other screening tests, such as the Etest macrodilution method, the vancomycin screening plate method with brain heart infusion agar, and the teicoplanin screening plate method with Mueller-Hinton agar (14). According to the Etest GRD, 3.5% of the MRSA isolates in our sample were positive for hVISA. All of the positive isolates had teicoplanin MIC values ≥8 μg/ml. Teicoplanin is more sensitive in the detection of the hVISA phenotype than vancomycin (55). It was revealed by PAP-AUC that only two of the six isolates were confirmed to be hVISA. The small sample size precludes any conclusion regarding the disparity observed between Etest GRD and PAP-AUC but does suggest that additional study is warranted. The two hVISA isolates had vancomycin MICs of 1.5 to 2 μg/ml by Etest and Sensititre. Vancomycin MICs of >1 μg/ml have been associated with hVISA (2, 12, 33), but this is not a strong indicator for hVISA because Etest and Sensititre reported equally high MICs in the vancomycin-susceptible isolates. The incidence of hVISA in other hospitals has varied, ranging from 0 to 50% (3, 4, 7, 13, 21, 22, 23, 25, 28, 29, 34, 35, 50, 52, 54). The type of screening test used to detect hVISA and the number of isolates screened are responsible for this wide range. A standard screening test with high sensitivity and specificity for detecting hVISA needs to be implemented to better understand the prevalence and clinical impact of these organisms.

In conclusion, a decrease in vancomycin susceptibility in MRSA isolates from our institution over a 9-year period was not detected by three standard antimicrobial susceptibility tests. The vancomycin MICs differed among the three susceptibility tests, in which Etest resulted in the highest MICs and MicroScan reported the lowest MICs. Finally, the GRD Etest and PAP-AUC defined a low prevalence of hVISA at 1.2%.

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