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One-Point Population Analysis and Effect of Osmolarity on Detection of Hetero-Vancomycin-Resistant *Staphylococcus aureus*

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The original screening test and one-point population analysis (OPPA) were compared for the detection of hetero-vancomycin-resistant *Staphylococcus aureus*, and the influence of osmolarity on those tests was evaluated. The positivity rates and the reproducibilities were similar for both tests. The addition of NaCl increased the MIC as well as the positivity rates for both tests and the reproducibility of OPPA.

From the beginning of the era of the occurrence of vancomycin (VAN)-intermediate-susceptible *Staphylococcus aureus* (VISA) in 1997 (1), there has also been concern about heterogeneous resistance to VAN. Hetero-VAN-resistant *S. aureus* (hVRSA) strains are defined as those strains that contain nonsusceptible subpopulations at a frequency of 10^{-6} or higher. Because the NCCLS MIC breakpoint for determination of nonsusceptible is ≥8 mg/liter by the twofold dilution method, nonsusceptible subcolonies can be differentiated by their growth on brain heart infusion (BHI) agar with 4 mg of VAN per liter. As hVRSA strains might be precursors of VISA strains, the need for good methods for the detection of hVRSA has been raised incrementally.

Population analysis, which determines heterogeneous VAN resistance by measurement of the number of subcolonies that grow in the presence of various concentrations of VAN, is regarded as the most reliable method for the detection of hVRSA strains. However, it is not appropriate as a screening test because of the time-consuming process required to prepare and spread many plates. The original screening test proposed by Hiramatsu et al. (2) determines heterogeneous VAN resistance if a countable number of colonies grow on a plate that contains BHI agar and 4 mg of VAN per liter and that is inoculated with 10^6 CFU of a bacterial cell suspension. Because the bacterial cell suspension is inoculated without spreading and a fixed concentration of VAN is used, the advantage of this method is its feasibility. However, the original screening test has been reported to have low levels of specificity and reproducibility. In addition, this method has been criticized because of the possibility of selection of VAN resistance (3). To confirm the heterogeneous VAN resistance status of the isolates, selection of subcolonies in the presence of high concentrations of VAN is recommended before MIC tests are conducted, as the latter tests may select for VAN resistance rather than detect it. Although several other screening methods (4, 7; H. Hanaki, S. Ohkawa, Y. Yoko, T. Hashimoto, and K. Hiramatsu, Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C-132, 1998) were proposed thereafter, a single reliable screening test has not yet been found.

Considering the definition of hVRSA, population analyses with concentrations of VAN other than 4 mg/liter do not contribute to the determination of heterogeneous VAN resistance. If population analysis with one concentration of VAN (4 mg/liter) is specific and reproducible for the detection of hVRSA, it can be used as a convenient screening method. Therefore, we planned this study to evaluate the usefulness of one-point population analysis (OPPA) by comparing it with the original screening method.

On the other hand, osmolarity is known to augment the expression of methicillin resistance in *S. aureus*, but osmolarity has not been evaluated for its influence on VAN resistance in *S. aureus*. In this study, the influences of osmolarity on both the original screening test and OPPA were also evaluated.

A total of 136 methicillin-resistant *S. aureus* (MRSA) strains isolated from a medical intensive care unit from 1996 to 1997 were tested. Among the patients from whom the tested strains were obtained, none was regarded as having a treatment failure due to the presence of VAN resistance. MICs were determined by the broth microdilution method with cation-adjusted Mueller-Hinton broth (MHB) (6) with and without 2% NaCl.

TABLE 1. MICs for original strains

NaCl content	No. of strains for which MICs (mg/liter) were:					
	Not done	1	2	4	8	Total
Without NaCl	2	17	80	37	0	136
With NaCl	2	2	52	80	0	136

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TABLE 2. Comparison of the original screening test and OPPA for detection of hVRSA

Method	Without NaCl			With NaCl		
	Total no. (%) of strains with positive results	No. (%) of strains with consistently positive results	Reproducibility[a]	Total no. (%) of strains with positive results	No. (%) of strains with consistently positive results	Reproducibility[a]
Original screening method	57 (41.9)	36 (26.5)	36/57 (63.2)	103 (75.7)	57 (41.9)	57/103 (55.3)
OPPA	55 (40.4)	25 (18.4)	25/55 (45.5)	98 (72.1)	62 (45.6)	62/98 (63.3)
P value	0.81	0.11	0.06	0.49	0.54	0.25

[a] Values are numbers of strains with consistently positive results/total numbers of strains with positive results (percent).

For screening tests, a bacterial cell suspension was adjusted to a McFarland 0.5 standard (10^8 CFU/ml). BHI agar plates containing 4 mg of VAN per liter with and without 4% NaCl were prepared. For the original screening test, 10 μl of the suspension was inoculated, and for OPPA, 50 μl of the cell suspension was spread with a bent glass rod. The results were considered positive when any signs of growth were found by the original screening method after incubation for 48 h at 37°C and when five or more colonies were found by OPPA to be growing under the same conditions. Two colonies, a small colony and a large colony, from among the subcolonies grown for OPPA were selected, and then MIC tests were done with cation-adjusted MHB with and without 2% NaCl. We used VAN-free agar for the subcultures of subcolonies for MIC tests to avoid the selection of resistance. Tests for confirmation of the results, such as population analysis with various VAN concentrations or MIC tests with subcolonies after selection in the presence of high concentrations of VAN, were not attempted in this study. The concentrations of NaCl were chosen according to the recommendations of NCCLS for susceptibility tests with MRSA: 4% for the agar dilution method and 2% for the broth dilution method (6). All tests were repeated twice. Chi-square tests were used for statistical comparisons.

A VAN MIC of 8 mg/liter or more was not detected for any of the strains tested (Table 1). The addition of 2% NaCl to the growth medium increased the VAN MICs for the original strains ($P < 0.01$).

Although the positivity rate and the reproducibility of the original screening test were higher than those of OPPA when the tests were performed without NaCl, the difference was not significant statistically (Table 2). On the contrary, the proportions of strains with consistently positive results and the reproducibilities were higher by OPPA when BHI agar was used with NaCl; however, the difference was also not significant statistically.

The number of positive strains increased greatly in both tests when NaCl was added to the growth media (Table 3). The reproducibility of OPPA increased significantly with the addition of NaCl.

While VAN MICs were ≥8 mg/liter for subcolonies of 12 strains when the strains were tested without NaCl, those for 65 strains revealed that they were not susceptible to VAN when MIC tests were performed with NaCl (Table 4).

In this study, OPPA was not superior to the original screening test in regard to the reproducibility. Sensitivity and specificity could not be evaluated because a confirmatory test was not attempted.

This study is the first to report on the influence of NaCl on the detection of hVRSA. The addition of NaCl to the growth medium increased the positivity rates for strains that could grow on BHI agar with VAN. We do not understand the mechanism of this phenomenon. Although a similar phenomenon is well known in the expression of oxacillin resistance in *S. aureus*, the exact mechanism of the phenomenon has not been clarified (5). The mechanism and the meaning of the influence of NaCl on antibiotic resistance in *S. aureus* need to be determined. The possibility of a change in the activities of antibiotics by the addition of NaCl needs to be evaluated. The relationship between NaCl and the activation of known resis-

TABLE 3. Influence of NaCl on results of screening tests for detection of hVRSA

NaCl content	Original screening method			OPPA		
	Total no. (%) of strains with positive results	No. (%) of strains with consistently positive results	Reproducibility[a]	Total no. (%) of strains with positive results	No. (%) of strains with consistently positive results	Reproducibility[a]
Without NaCl	57 (41.9)	36 (26.5)	36/57 (63.2)	55 (40.4)	25 (18.4)	25/55 (45.5)
With NaCl	103 (75.7)	57 (41.9)	57/103 (55.3)	98 (72.1)	62 (45.6)	62/98 (63.3)
P value	<0.01	<0.01	0.33	<0.01	<0.01	0.03

[a] See footnote *a* of Table 2.

TABLE 4. MICs of substrains

Colony size and NaCl content	No. of strains for which MICs (mg/liter) were:					
	0.5	1	2	4	8	16
Small colony						
Without NaCl	0	9	42	38	4[a]	0
With NaCl	0	0	10	37	40[b]	6[b]
Large colony						
Without NaCl	1	5	40	37	10[a]	0
With NaCl	0	1	7	34	49[b]	2[b]

[a] For 2 strains in both small and large colonies, the MICs were ≥8 mg/liter.
[b] For 28 strains in both small and large colonies, the MICs were ≥8 mg/liter.

tance mechanisms, the *mecA* gene in MRSA and cell wall augmentation in hVRSA, also needs to be evaluated.

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