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Glycopeptide-Intermediate *Staphylococcus aureus*: Evaluation of a Novel Screening Method and Results of a Survey of Selected U.S. Hospitals

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Isolates of *Staphylococcus aureus* with decreased susceptibilities to glycopeptide antimicrobial agents, such as vancomycin and teicoplanin, have emerged in the United States and elsewhere. Commercially prepared brain heart infusion agar (BHIA) supplemented with 6 μg of vancomycin per ml was shown in a previous study to detect glycopeptide-intermediate *S. aureus* (GISA) with high sensitivity and specificity; however, this medium, when prepared in-house, occasionally showed growth of vancomycin-susceptible control organisms. This limitation could significantly impact laboratories that prepare media in-house, particularly if they wished to conduct large surveillance studies for GISA. Therefore, a pilot study to detect GISA was performed with vancomycin-containing Mueller-Hinton agar (MHA) prepared in-house in place of commercially prepared BHIA. MHA was selected for this study because this medium is widely available and well standardized. The results of the pilot study showed that supplementation of MHA with 5 μg of vancomycin per ml was both a sensitive and a specific method for screening for GISA isolates. This method was used to screen for GISA among 630 clinical isolates of methicillin-resistant *S. aureus* collected during 1997 from 33 U.S. hospitals. Although 14 *S. aureus* isolates grew on the screening agar, all were vancomycin susceptible (MICs were ≤1 μg/ml) by broth microdilution testing. Population analyses of five isolates revealed two with a subpopulation for which vancomycin MICs were 8 μg/ml. In summary, the MHA screen plate containing 5 μg of vancomycin per ml prepared in-house provides a sensitive and cost-effective method for large-scale screening for GISA for which vancomycin MICs are 8 μg/ml. However, confirmation of isolates as vancomycin resistant is critical. This study suggests that GISA was not a widespread problem in the United States in 1997.

Isolates of *Staphylococcus aureus* with decreased susceptibilities to glycopeptide antimicrobial agents, such as vancomycin and teicoplanin, have been documented in Japan (2, 5, 6), New Jersey (3, 16), Michigan (3, 16), New York (14), the United Kingdom (7), and France (13). Currently, no standardized method to screen for glycopeptide-intermediate *S. aureus* (GISA) in the clinical microbiology laboratory exists. In a previous study, commercially prepared brain heart infusion agar (BHIA) plates supplemented with 6 μg of vancomycin per ml, i.e., the screening plates used to detect vancomycin-resistant enterococci, were shown to work well as a screen for GISA, but the same medium prepared in-house occasionally showed growth of vancomycin-susceptible control organisms (17). The lack of specificity of the in-house-prepared medium posed problems for laboratories interested in initiating large-scale surveillance projects for GISA since the commercially prepared medium was considerably more expensive than that prepared in-house.

The primary goal of this study was to identify an alternative medium to BHIA that could be prepared in-house for use in a screening test for GISA. We selected Mueller-Hinton agar (MHA) because it readily supports the growth of staphylococci and it is well standardized, commercially available, and easily prepared in-house. After determining the optimal concentration of vancomycin needed to detect GISA, this screening medium was used to search for GISA among clinical isolates of methicillin-resistant *S. aureus* (MRSA) collected from 33 hospitals participating in Project ICARE (Intensive Care Antimicrobial Resistance Epidemiology) (9).

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MATERIALS AND METHODS

Pilot study. To determine the optimal concentration of vancomycin to use in MHA to detect GISA, nine well-characterized strains of GISA were selected for testing (Table 1). In addition, *S. aureus* ATCC 29213 (vancomycin susceptible), *S. aureus* ATCC 25923 (vancomycin susceptible), *Enterococcus faecalis* ATCC 29212 (vancomycin susceptible), and *E. faecalis* ATCC 51299 (vancomycin resistant) were included as quality-control organisms (17). Two lots each of MHA from five different manufacturers (Accumedia, Baltimore, Md.; BBL/Becton Dickinson Microbiology Systems, Cockeysville, Md.; Difco, Cockeysville, Md.; Oxoid, Basingstoke, Hampshire, England; and Remel, Lenexa, Kans.) were prepared according to the manufacturers' instructions and used to prepare sets of plates with concentrations of vancomycin ranging from 3 to 6 μg/ml. BHIA plates supplemented with 6 μg of vancomycin per ml were purchased from Remel. All MHA and BHIA plates were inoculated with 10 μl of a suspension of the test isolate equivalent in density to a 0.5 McFarland standard (approximately 10^8 CFU/ml). Test plates were incubated with no more than two in a stack in ambient air at 35°C and examined for growth after 24 and 48 h.

Collection of MRSA isolates. Six hundred thirty isolates previously reported to be MRSA were collected over a period of 6 months in 1997 from 33 ICARE hospitals representing different geographical regions of the United States. Upon receipt, the isolates were subcultured twice onto blood agar plates and then frozen at −70°C in sterile defibrinated sheep blood until needed. Of the isolates tested, 83% were confirmed as methicillin (oxacillin) resistant by disk diffusion testing according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines (11) with 1-μg-oxacillin disks while 17% were either intermediate or susceptible by disk diffusion testing.

Screening clinical MRSA isolates for vancomycin resistance. MHA was prepared according to the manufacturers' instructions with one lot each of MHA from two different manufacturers (Difco and Oxoid). The two sources of the medium were chosen at random from among those used during the pilot phase of the study. The plates were supplemented with 5 μg of vancomycin per ml and

TABLE 1. *S. aureus* isolates used in the GISA screen pilot study[a]

Isolate	Vancomycin MIC or range (μg/ml)	Interpretation
GISA isolates		
S. aureus Japan strain Mu3	2	S
S. aureus Florida strain 800	2	S
S. aureus Japan strain N20	4	S
S. aureus Michigan strain 966	4	S
S. aureus Florida strain 803	4	S
S. aureus Japan strain Mu50	8	I
S. aureus Japan strain Mu3-8R	8	I
S. aureus Michigan strain 963sm	8	I
S. aureus New Jersey strain 992	8	I
Quality-control isolates		
S. aureus ATCC 29213	0.5–2	S
S. aureus ATCC 25923	2	S
E. faecalis ATCC 29212	1–4	S
E. faecalis ATCC 51299	16–32	R

[a] Abbreviations in the interpretation column reflect NCCLS interpretive categories (S, susceptible; I, intermediate; and R, resistant). Vancomycin MICs (for staphylococci) of ≤4 μg/ml indicate susceptibility, while vancomycin MICs (for staphylococci) of ≥32 μg/ml indicate resistance.

inoculated with the 630 clinical isolates and quality-control organisms by the method described for the pilot study. For comparison, 109 of the clinical isolates were randomly selected and also inoculated onto commercially prepared BHIA plates supplemented with 6 μg of vancomycin (Remel) per ml. Any isolate that grew on either screening agar was confirmed to be *S. aureus* by Gram staining, Staphaurex latex agglutination testing (Murex Diagnostics, Atlanta, Ga.), and catalase testing (8). The isolates that grew on the initial Difco and Oxoid lots were retested on a different lot of the Difco and Oxoid screening agar.

Broth microdilution testing. The vancomycin MICs for the clinical isolates were confirmed by the broth microdilution method on custom MIC panels (Accumed International/Sensititre, Westlake, Ohio) according to NCCLS guidelines (10, 12).

Population analysis. Five randomly selected isolates of MRSA that grew on at least one of the four lots of MHA screening agar were subjected to population analysis as previously described (5) with the following modifications. Both MHA (Difco) and BHIA (Remel) plates supplemented with vancomycin in doubling concentrations ranging from 0.25 to 8 μg/ml (plus an extra plate containing 6 μg of vancomycin per ml) were prepared. Each test isolate and a quality-control organism (*S. aureus* Mu50 [MIC, 8 μg/ml] [6]) were suspended in Mueller-Hinton Broth (MHB) to an optical density equivalent to a 0.5 McFarland standard. This inoculum was serially diluted in MHB, and 100 μl was plated in duplicate on the vancomycin-containing test plates. After inoculation, the plates were inverted and incubated at 35°C for 24 h. Individual colonies growing on the plates were enumerated for each dilution. Colonies of each isolate were counted in order to confirm the number of viable organisms in the inoculum.

PFGE. Pulsed-field gel electrophoresis (PFGE) of macrorestriction fragments produced by *Sma*I digestion of DNAs obtained from the 14 isolates that grew on the screening plates was performed with a CHEF DRIII system (Bio-Rad, Hercules, Calif.) (1). Interpretation of the fragment patterns was accomplished by using published criteria (18).

RESULTS

Pilot study. MHA plates containing one of four concentrations of vancomycin were tested for their ability to differentiate between *S. aureus* strains that were intermediate to vancomycin (MIC, 8 μg/ml) and those strains that were susceptible to vancomycin (MIC, ≤4 μg/ml). MHA containing 5 μg of vancomycin per ml showed the best sensitivity and specificity for detecting GISA (Tables 2 and 3), and results agreed with the results of the vancomycin-resistant enterococcus screening plates (BHIA containing 6 μg of vancomycin per ml) (data not shown). Results at 24 h for the 5-μg/ml screening plate were similar to those at 48 h, and all MHA lots from all manufacturers tested gave comparable results. MHA containing 3 and 4 μg of vancomycin per ml showed growth of vancomycin-susceptible quality-control strains. At 6 μg/ml, the growth of some isolates for which vancomycin MICs of 8 μg/ml were known was suppressed. One *S. aureus* strain for which the MIC was 4 μg/ml, the Japanese strain N20, did not grow on plates supplemented with 3 μg of vancomycin per ml, while *S. aureus* strains 966 and 803, for which vancomycin MICs were also 4 μg/ml, grew on all MHA plates supplemented with ≤6 μg/ml.

Screening of MRSA isolates for the GISA phenotype. Of the 630 clinical MRSA isolates, 14 grew on the vancomycin-MHA screening plates. All 14 were confirmed to be *S. aureus* by latex agglutination and catalase testing (8), but vancomycin MICs for these isolates ranged from 0.5 to 1 μg/ml by broth microdilution testing. The 14 *S. aureus* isolates were retested with a different lot of MHA agar (Difco). Only 4 of the 14 *S. aureus* isolates grew on the second lot of screening plates. All of the remaining 616 isolates were tested by broth microdilution testing, and vancomycin MICs for them, including those that grew on the screening medium, were ≤2 μg/ml at 24 h. At 48 h, the vancomycin MIC for one isolate (strain 2569) increased from 2

TABLE 2. Test isolate results of MHA-vancomycin agar pilot screen testing after 24 h

Vancomycin concn (μg/ml)	MIC (μg/ml) for isolates (*n*)	No. of positive plates/total no. of plates tested with medium from[a]:				
		Accumedia	BBL	Difco	Oxoid	Remel
6	8 (4)	2/8	2/8	2/8	2/8	3/8[b]
	4 (3)	4/6	4/6	4/6	4/6	4/6
	2 (2)	0/4	0/4	0/4	0/4	0/4
5	8 (4)	8/8	8/8	8/8	8/8	8/8
	4 (3)	4/6	4/6	4/6	4/6	4/6
	2 (2)	0/4	0/4	0/4	0/4	0/4
4	8 (4)	8/8	8/8	8/8	8/8	8/8
	4 (3)	5/6[c]	4/6	4/6	4/6	4/6
	2 (2)	0/4	0/4	0/4	0/4	1/4
3	8 (4)	8/8	8/8	8/8	8/8	8/8
	4 (3)	4/6	4/6	4/6	4/6	4/6
	2 (2)	2/4	0/4	0/4	0/4	0/4

[a] Sources of the media used for the screening plates. Two lots from each manufacturer were tested.
[b] Mu3-8R, Mu50, and 963sm grew on one lot of the Remel media.
[c] Japan strain N20 grew on one of the lots of the Accumedia media. This was the only screening plate for which N20 was positive for growth.

TABLE 3. Quality-control results of MHA-vancomycin agar pilot screen testing after 24 h

Vancomycin concn (μg/ml)	Quality-control organism	No. of positive plates/total no. of plates tested with medium from[a]:				
		Accumedia	BBL	Difco	Oxoid	Remel
6	*E. faecalis* ATCC 51299	2/2	2/2	2/2	2/2	2/2
	E. faecalis ATCC 29212	0/2	0/2	0/2	0/2	0/2
	S. aureus ATCC 29213	0/2	0/2	0/2	0/2	0/2
	S. aureus ATCC 25923	0/2	0/2	0/2	0/2	0/2
5	*E. faecalis* ATCC 51299	2/2	2/2	2/2	2/2	2/2
	E. faecalis ATCC 29212	0/2	0/2	0/2	0/2	0/2
	S. aureus ATCC 29213	0/2	0/2	0/2	0/2	0/2
	S. aureus ATCC 25923	0/2	0/2	0/2	0/2	0/2
4	*E. faecalis* ATCC 51299	2/2	2/2	2/2	2/2	2/2
	E. faecalis ATCC 29212	1/2	0/2	0/2	2/2	1/2
	S. aureus ATCC 29213	0/2	0/2	0/2	0/2	0/2
	S. aureus ATCC 25923	0/2	0/2	0/2	0/2	0/2
3	*E. faecalis* ATCC 51299	2/2	2/2	2/2	2/2	2/2
	E. faecalis ATCC 29212	2/2	2/2	2/2	2/2	0/2
	S. aureus ATCC 29213	0/2	0/2	0/2	0/2	0/2
	S. aureus ATCC 25923	0/2	0/2	0/2	2/2	0/2

[a] Sources of the media used for the screening plates. Two lots from each manufacturer were tested.

to 4 μg/ml; the MICs for the remaining isolates were unchanged.

One hundred nine of the 630 isolates were selected at random and tested on commercially prepared BHIA plates containing 6 μg of vancomycin per ml. Of nine isolates initially detected by the MHA screening plates, eight again grew on MHA but not on BHI and one (strain 2349) grew on both the BHIA and MHA plates. Two isolates that were not initially detected by the MHA plates grew on the BHIA plates. All of these isolates were subsequently shown to be susceptible to vancomycin by broth microdilution testing.

Population analyses. Population analysis was performed on the three clinical isolates that grew on two lots of MHA media and the two isolates that grew on both MHA and BHIA to determine the presence of subpopulations resistant to vancomycin. Three of the five isolates grew only on media containing ≤1 μg of vancomycin per ml. Isolate 2248 showed a few colonies on BHIA plates containing 6 and 8 μg of vancomycin per ml, although individual colonies were difficult to detect since growth appeared as a thin haze at these concentrations of vancomycin. Isolate 2349 showed growth on plates containing up to 4 μg of vancomycin per ml. The colonies from isolates 2248 and 2349 that grew on the plates containing higher concentrations of vancomycin were subcultured onto blood agar plates and retested by broth microdilution testing; however, the MICs for each of the colonies reverted to 1 μg/ml in broth.

PFGE. All 14 *S. aureus* isolates were examined by PFGE. Each isolate had a unique PFGE pattern that was different from those of known GISA strains by published criteria (18).

DISCUSSION

Isolates of GISA have emerged in many parts of the world, suggesting that broader surveillance for such organisms should be undertaken (2–5, 7, 13). In a previously reported study, the agar screening test used to detect vancomycin-resistant enterococci (which used commercially prepared BHIA supplemented with 6 μg of vancomycin per ml), was shown to be a sensitive and specific screening test for detecting *S. aureus* strains for which vancomycin MICs were ≥4 μg/ml. However, BHIA screening plates prepared in-house occasionally showed growth of vancomycin-susceptible isolates (17). Thus, laboratories interested in initiating large-scale surveillance programs for GISA were faced with either purchasing large quantities of media, which might prove to be expensive, or preparing media in-house, which might show specificity problems. In the present study, we explored the possibility of substituting in-house-prepared MHA, a well-characterized and highly standardized medium, for commercially prepared BHIA in the vancomycin agar screen test. MHA screening plates containing 3 or 4 μg of vancomycin per ml detected all isolates for which vancomycin MICs were 4 to 8 μg/ml; however, growth of vancomycin-susceptible isolates was also common. Thus, the specificity of the plates with these vancomycin concentrations was not optimal. On the other hand, screening plates containing 6 μg/ml lacked sensitivity, suppressing the growth of some isolates for which vancomycin MICs were 8 μg/ml. MHA supplemented with 5 μg of vancomycin per ml prepared in-house appeared to be an acceptable and cost-effective alternative to the commercially prepared vancomycin-resistant enterococcus screening plates for detecting staphylococci with decreased susceptibilities to vancomycin. While BHIA provides slightly better growth of GISA isolates, supplemented MHA should be an acceptable alternative for clinical laboratories that prepare media in-house, particularly for large-scale surveillance studies for GISA.

In this study, there was not much difference among the growth patterns of the staphylococcal isolates on different lots of MHA or between the results observed after 24 and 48 h of incubation. However, growth of several *S. aureus* isolates that ultimately were shown by broth microdilution testing to be susceptible to vancomycin was observed on MHA plates containing 5 μg of vancomycin per ml. Therefore, isolates that grow on these screening plates should be tested by a broth microdilution or Etest method (17). It appears from the reports from the United Kingdom (7) and France (13) that laboratories should expect to see such occasional growth of *S. aureus* strains regardless of the screening medium used.

Among the clinical isolates of MRSA in this study, only 14 organisms grew on the MHA screening media. None were confirmed as GISA by reference broth microdilution MIC test-

ing, although two isolates showed resistant subpopulations in the population analysis experiments. Hiramatsu et al. (5) and Sieradzki et al. (15) have noted such resistant subpopulations in staphylococcal isolates before. The clinical significance of such strains remains unproven, and they should still be reported as vancomycin susceptible until further clinical data that suggest otherwise are available. However, it is recommended that *S. aureus* strains for which vancomycin MICs are found to be ≥4 μg/ml be reported to the state health department and the Centers for Disease Control and Prevention (15).

GISA have been reported to occur in U.S. hospitals, but none were found among the 33 hospitals that participated in this study. This suggested that undetected GISA was not a widespread problem among U.S. hospitals in 1997. None have been identified in subsequent surveillance studies conducted in Project ICARE hospitals through June 1999.

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