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Novel Screening Agar for Detection of Vancomycin-Nonsusceptible *Staphylococcus aureus*▽

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In January 2006, the Clinical and Laboratory Standards Institute (CLSI) updated breakpoints for vancomycin susceptibility testing for *Staphylococcus aureus* such that an MIC greater than 2 mg/liter was considered to represent nonsusceptibility to vancomycin. Previously, an MIC of 4 mg/liter had been considered to represent susceptibility. Additionally, in 2009, the CLSI altered the guidelines for staphylococci such that disk diffusion was no longer an acceptable means for testing vancomycin susceptibility in these organisms. To accommodate the change in breakpoints and methodological updates, we designed a medium consisting of brain heart infusion agar with 3 mg/liter vancomycin (BHI-V3) to screen for isolates of *S. aureus* with reduced susceptibility to vancomycin. We challenged this medium using a previously characterized collection of 100 isolates of *S. aureus*, including 55 vancomycin-susceptible isolates and 45 isolates representing vancomycin-intermediate strains of *S. aureus* (VISA) (with vancomycin MICs of 4 mg/liter or greater). All of the VISA isolates grew on the agar, for 100% sensitivity. Nineteen vancomycin-susceptible isolates also grew on the agar, for 65% specificity. We then incorporated BHI-V3 into clinical practice. In the first 60 days postimplementation, we identified 17 potential VISA isolates out of 421 *S. aureus* isolates tested. Thirteen out of the 17 were confirmed to represent VISA isolates. In light of the excellent sensitivity of this medium, we recommend that clinical laboratories incorporate BHI-V3 to screen for vancomycin-nonsusceptible isolates of *S. aureus*.

At the site of this study, 60 to 70% of infections in which *Staphylococcus aureus* is the etiologic agent are caused by methicillin-resistant *S. aureus* (MRSA). For these infections, vancomycin is considered the mainstay of antimicrobial therapy. The ability to accurately identify isolates of *S. aureus* with reduced susceptibility to vancomyin is paramount for a successful clinical outcome.

Until recently, strains of S. *aureus* with reduced susceptibility to vancomycin could be detected using a screening medium consisting of brain heart infusion agar (BHI) supplemented with 6 mg/liter vancomycin (BHI-V6). The medium is inoculated with 10 μl of a 0.5 McFarland standard suspension of the organism and incubated at 35 to 37°C for 24 h. Strains with reduced susceptibility to vancomycin demonstrate growth of one or more colonies after 24 h of incubation (1, 2, 8, 9). However, as of January 2006, the Clinical and Laboratory Standard Institute (CLSI) breakpoints for vancomycin susceptibility for *S. aureus* were updated such that strains with an MIC greater than 2 mg/liter were considered to be nonsusceptible to vancomycin, where those with an MIC of 4 mg/liter had previously been considered to be vancomycin susceptible (1–4). As a vancomycin MIC of 4 to 8 mg/liter is considered to represent intermediate susceptibility, the use of an agar medium such as BHI-V6 as a means to screen for vancomycin-intermediate strains of *S. aureus* (VISA) is not adequate for this purpose, as those strains having a vancomycin MIC greater than 2 but less than 6 mg/liter are not detected by this method.

To accommodate the change in breakpoints, we designed a screening medium consisting of BHI with 3 mg/liter vancomycin (BHI-V3). BHI-V3 was manufactured for our laboratory by Remel (Lenexa, KS). A noninhibitory pink dye, amaranth, was added to the agar to assist in rapid visual differentiation of it from other screening agars in the laboratory, such as the 6 mg/liter vancomycin screening agar (yellow) and/or the oxacillin screening agar (clear). The agar is inoculated using the same method as for the BHI-V6 medium: 10 μl of a 0.5 McFarland standard suspension of organism is added to the agar and incubated for 24 h at 35 to 37°C. Several isolates can be placed onto one plate. For quality control purposes, *Enterococcus faecalis* ATCC 29212 was used as a positive growth control. This organism is the negative growth control for the 6 mg/liter vancomycin screening agar and has an MIC of 4 mg/liter by Etest in our laboratory. *S. aureus* ATCC 29213 was used as a negative growth control and has an MIC of 2 mg/liter by Etest in our laboratory. These organisms were chosen as quality control isolates because they bracket the desired concentration range of the agar. Any growth on BHI-V3 was interpreted as positive after confirmation that the isolate was *S. aureus*. Any isolate that grew on BHI-V3 was tested directly from this medium for vancomycin MIC confirmation, or, if there was not sufficient growth to do so, the isolate was subcultured to another 3 mg/liter BHI-V3 medium prior to performance of susceptibility testing to maintain vancomycin selective pressure.

In the initial evaluation, we challenged the BHI V3 with eight clinical isolates of *E. casseliflavus* and *E. gallinarum*, which intrinsically have a vancomycin-intermediate phenotype. All of these isolates grew on the screening agar and had a vancomycin MIC of 6 or 8 mg/liter, as determined by Etest.

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TABLE 1. Performance of BHI-V3 with previously characterized strains of *S. aureus*

Vancomycin MIC (mg/liter)[a]	Growth on BHI-V3 (no. of isolates)	No growth on BHI-V3 (no. of isolates)	Total
≥8	10	0	10
4	35	0	35
2	17	7	24
1	1	27	28
0.5	1	2	3

[a] Vancomycin MIC determined by CLSI broth reference microdilution (BMD) using Difco Mueller-Hinton broth.

TABLE 2. Results of testing with other susceptibility methods for previously characterized strains of *S. aureus* with a vancomycin MIC of 2 mg/liter and growth on BHI-V3

No. of isolates	Vancomycin MIC (mg/liter)			
	BMD BBL[a]	BMD Difco[a]	Agar dilution[a]	Etest[b]
13	2	2	2	2
2	2	2	2	3
1	4	2	4	3
1	2	2	4	2

[a] Vancomycin MIC determined at the CDC.
[b] Vancomycin MIC determined at Washington University.

Then, the ability of BHI-V3 medium to detect isolates of *S. aureus* with reduced vancomycin susceptibility was verified using a previously characterized collection of *S. aureus* isolates with MICs to vancomycin ranging from 0.5 to 8 mg/liter (7). The vancomycin MIC for these strains was determined by the Centers for Disease Control and Prevention (CDC) using CLSI reference broth microdilution (7). The MIC that was used for the purpose of deciding whether these isolates exhibited full or intermediate susceptibility to vancomycin was based on the Difco broth microdilution result. The collection included 55 vancomycin-susceptible isolates (i.e., with a vancomycin MIC of 2 mg/liter or less) and 45 vancomycin-intermediate isolates (VISA) with a vancomycin MIC of 4 or 8 mg/liter. For the purpose of this analysis, the MIC results of the strain collection were coded and not revealed until the testing was complete.

All of the VISA isolates grew on the BHI-V3 agar, for 100% sensitivity for this screening method (Table 1). This is a significant improvement in sensitivity over what has been reported for several other methods for the detection of VISA isolates. For example, Swenson and colleagues have previously reported that BHI-V6 agar failed to detect 33% (12 of 36) of VISA isolates with an MIC of 4 mg/liter (7). Other methods that have shown poor sensitivity for the detection of VISA include the use of Kirby-Bauer disk diffusion, the Trek Sensititre broth microdilution panel, the Vitek Legacy, and the Vitek2 GP61 and GP67 antimicrobial susceptibility cards (7, 10). The Etest has previously been demonstrated to be sensitive for detection of VISA, but it did categorize one of 45 VISA isolates as vancomycin susceptible in a recent study (7).

Although BHI-V3 has the excellent sensitivity that is desirable in a screening assay, there were 19 vancomycin-susceptible isolates that grew on the medium, corresponding to 65% specificity (Table 1). One of the susceptible isolates growing on BHI-V3 had a vancomycin MIC of 0.5 mg/liter, another had an MIC of 1 mg/liter, and 17 of the discrepant isolates had an MIC of 2 mg/liter. However, as the agar has a vancomycin concentration of 3 mg/liter, we postulated that since MIC methods are performed using doubling dilutions, it was possible that some of the isolates with a broth microdilution MIC of 2 mg/liter may actually have an MIC greater than 2 mg/liter but less than 4 mg/liter, the next highest doubling dilution. To examine this question, we performed MIC analysis for the 17 discrepant isolates by the use of Etest (bioMérieux, Durham, NC) to identify isolates having a vancomycin MIC between 2 and 4 mg/liter. Of the 17 isolates, three had a vancomycin MIC of 3 mg/liter by Etest, and one of these had a vancomycin MIC of 3 mg/liter by Etest and an MIC of 4 mg/liter by broth reference microdilution (BMD) (BBL) and agar dilution (Table 2). Of the remaining 14 isolates, one had an MIC of 4 mg/liter by agar dilution as performed by Swenson et al. (7; Table 2). If these four isolates are considered to represent "true positives," the specificity of BHI-V3 increases to 71%. As we and others have previously demonstrated, MIC testing for vancomycin is method dependent (6, 7) and so it is difficult to determine the true specificity of BHI-V3 for isolates with a vancomycin MIC very close to the breakpoint between susceptible and intermediate, but it does appear that this is the range where BHI-V3 performs most poorly with regard to specificity. It is also possible that the poor specificity of the medium reflects the detection of heteroresistant *S. aureus* (hVISA) strains that are not readily detected by the comparator methods.

Subsequent to verifying the performance of BHI-V3 agar on a characterized set of isolates, we incorporated the medium into routine use in our laboratory to test all clinical isolates of *S. aureus* for vancomycin nonsusceptibility. During the first 60 days of use, we identified 17 potential VISA out of 421 *S. aureus* isolates tested using BHI-V3. Those isolates that grew on BHI-V3 were characterized using Kirby-Bauer disk diffusion with a 30-μg vancomycin disk (Becton Dickinson BBL, Sparks, MD), vancomycin Etest strips, Sensititre GPALL (Trek Diagnostic Systems, Cleveland OH) (vancomycin MIC range from 0.25 to 32 mg/liter), Microscan Pos MIC panel type 26 (Siemens Healthcare Diagnostics, Deerfield, IL) (vancomycin MIC range from 0.25 to 16 mg/liter), and the Vitek2 AST-GP67 card (bioMérieux) (MIC range from 0.25 to 32 mg/liter) (Table 3). We observed variable sensitivity for each of the methods with regard to VISA detection. For the purpose of calculating sensitivity and specificity, isolates that had an MIC of 4 or 8 by broth microdilution or of >2 to ≤8 by Etest were considered to represent VISA. The previous CLSI disk diffusion susceptibility breakpoint for vancomycin of 15 mm or greater would have misclassified all of the VISA isolates as being vancomycin susceptible. Thirteen out of the 17 isolates were confirmed to be VISA strains when BHI-V3 was implemented into routine clinical use, for an overall specificity of 99%. Microscan had the highest sensitivity (92%), failing to detect only one VISA strain, followed by Etest (85% sensitive) and then Sensititre (54% sensitive). Vitek2 GP67 had the worst sensitivity, detecting only 1 of the 13 VISA isolates. It should be noted, however, that the VISA isolates did have a GP67 vancomycin MIC of 2 mg/liter, whereas in our experience *S. aureus* isolates typically have a vancomycin MIC of ≤0.5 mg/liter when tested

TABLE 3. *S. aureus* isolates recovered from the 3 mg/liter vancomycin screening agar

Source	Methicillin susceptibility	Kirby Bauer zone diam (mm)	MIC (mg/liter)			
			Etest	Microscan	Sensititre	Vitek
Blood	MRSA	17	3	4	4	2
Blood	MRSA	17	3	2	2	2
Blood	MRSA	16	2	1	2	2
Catheter tip	MRSA	17	4	4	1	≤0.5
Blood	MRSA	18	2	2	2	1
Scalp wound	MSSA[a]	18	3	4	4	2
Hip wound	MRSA	17	4	4	2	2
Nares	MRSA	22	4	4	2	1
Chest wound	MRSA	18	4	4	2	2
Blood	MSSA	16	2	4	2	2
Blood	MSSA	24	2	2	2	2
Urine	MRSA	18	4	4	4	2
Sinus	MSSA	19	4	4	4	2
Tracheal aspirate	MRSA	18	6	4	4	8
Blood	MRSA	19	2	4	2	2
Blood	MSSA	19	2	2	1	1
BAL[b]	MRSA	18	4	4	8	2

[a] MSSA, methicillin-susceptible *S. aureus*.
[b] BAL, bronchoalveolar lavage specimen.

with this methodology (personal observation). This would suggest that laboratories using the GP67 AST card for vancomycin susceptibility testing of *S. aureus* should consider additional testing to rule out VISA when an MIC of 2 mg/liter is generated and/or the concomitant use of a screening medium such as BHI-V3 to ensure detection of VISA isolates.

In light of the excellent sensitivity of BHI-V3 for detection of VISA isolates, we recommend that clinical laboratories use this medium to screen all isolates of *S. aureus* for nonsusceptibility to vancomycin. If the isolate does not grow on this medium, one can be assured that the isolate is vancomycin susceptible and can report it as such. Any isolate that does grow on the agar should have confirmatory testing performed to determine its vancomycin susceptibility. For confirmatory analysis, the isolate should be either tested directly with the BHI-V3 agar or, if there is not adequate growth to do so, subcultured to a second BHI-V3 agar plate to maintain vancomycin selective pressure. We feel that this is a cost-effective algorithm that is easy to incorporate into a typical laboratory workflow, with excellent performance for detection of VISA isolates. Further, the use of a medium such as BHI-V3 would nicely supplement disk diffusion testing where vancomycin interpretive zone size breakpoints (other than ≤6-mm diameter) are currently unavailable.

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ADDENDUM

Following the acceptance of this note, we discovered a publication by Kosowska-Shick et al. (5) that had eluded our search efforts. In that publication, the authors describe the use of a BHI containing 3 mg/liter vancomycin to screen for MRSA with increased vancomycin MIC levels. While the medium was prepared in-house instead of commercially and did not contain the dye amaranth, it was effectively the same design as ours. Similarly, the medium previously reported by this group had a sensitivity of 100% for the detection of hVISA and VISA isolates among the collection of 982 MRSA strains evaluated. However, in their study, this screening medium carried a false positivity rate of 94% that was reduced upon repeat testing, whereas in our study the false positivity rate was 29.6%. This might be due to differences in the incubation period or to the low number (5 of 982) of VISA and hVISA strains found in the collection tested at the Hershey Medical Center. Our plates were read after 24 hour of incubation, whereas the previous study evaluated growth on the screening medium after 48 hours of incubation. In addition, our collection contained 45 of 100 isolates with a vancomycin MIC ≥ 4 mg/liter. We did not evaluate strains for the presence of the hVISA phenotype by the use of a standardized method, but, had any hVISA strains been identified, our false positivity rate would have declined. We regret not having discovered this report earlier, but both groups came independently to the same conclusion, i.e., that it is reasonable to include a screening medium such as BHI with 3 mg/liter of vancomycin to test MRSA for evidence of reduced susceptibility to vancomycin.

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