

Trends Associated with Glycopeptides: Heteroresistance, Tolerance, and Lack of Detection

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Introduction

The most important issues that have evolved with continued use of glycopeptides are those of heteroresistance, tolerance, and inadequate laboratory detection of vancomycin resistance. The list of organisms that have demonstrated reduced susceptibility to vancomycin is quite long. The microorganism of main concern in glycopeptide resistance is *Staphylococcus aureus*. Other microorganisms include the coagulase-negative staphylococci, particularly *S. hemolyticus*. The *Enterococcus* species have clearly been very problematic. *Leuconostoc*, *Lactobacillus*, and *Pediococcus* are innately resistant to vancomycin. Another organism, *Leifsonia aquatica* (previously called *Corynebacterium aquaticum*), has recently been reported to have decreased susceptibility to vancomycin in line-associated infections.

Case Presentation

The following case presentation addresses the issues involved with testing and detection of resistance to vancomycin and other glycopeptides. The patient was a 58-year-old gentleman with cardiovascular disease who developed a bacteremic infection associated with surgically implanted stents. *S. aureus* had been recovered from two sets of blood cultures. The isolate was reported by the laboratory in a small outlying hospital as being susceptible to oxacillin, tetracycline, rifampin, trimethoprim-sulfamethoxazole, linezolid, and vancomycin based on disk diffusion testing.

The patient's clinical condition was not improving after treatment with nafcillin and was transferred to a tertiary care center where he remained bacteremic. The first important observation in this case is that oxacillin susceptibility testing using the disk diffusion method is poor, at best.¹ Therefore, it was not surprising that the laboratory called it an oxacillin-susceptible strain based on disk diffusion, when the organism was actually PVP2A positive and, using MIC techniques, was proven to be a strain of methicillin-resistant *S. aureus* (MRSA). The patient was switched to vancomycin and blood cultures became negative after 72 hours. The decision was made not to remove the stents and the patient was transferred home on long-term intravenous vancomycin therapy.

The patient returned seven months later with back pain, fever, and an elevated sedimentation rate. Blood cultures again yielded *S. aureus* and radiographic studies suggested that he had developed a significant paraspinal abscess. Because of the proximity to the spinal cord, cultures were not performed on the abscess. Disk diffusion susceptibility studies on the blood isolate again indicated that the organism was susceptible to vancomycin.

What were the possible reasons for this patient's failure on vancomycin? Some of the factors involved may have included the following: 1) Infrequent IV removal 2), Vancomycin is slowly bactericidal compared with the beta-lactams 3) Presence of glycopeptide-intermediate *S. aureus* (GISA) or vancomycin-intermediate *S. aureus* (VISA) 4) Presence of vancomycin-resistant isolate that was not detected using standard testing in the laboratory 5) Presence of a strain of *S. aureus* that carried a heterogeneous vancomycin-resistance gene as part of a cassette of antibiotic resistance genes.

Definitions

Part of the problem with detection of glycopeptide resistance in staphylococci has to do with varied definitions of resistance in the infectious disease community around the globe. The National Committee for Clinical Laboratory Standards (NCCLS) defines VISA as an isolate for which the MIC falls between 8 and 16 µg/mL and a vancomycin-resistant *S. aureus* (VRSA) isolate as a strain for which the MIC is greater than or equal to 32 µg/mL.² In other parts of the world, however, the definitions are quite different. For example, in Japan and the United Kingdom (the British Society for Antimicrobial Chemotherapy [BSAC]), the breakpoint for vancomycin resistance in staphylococci is greater than or equal to 8 µg/mL.

The Centers for Disease Control and Prevention (CDC) has recently developed three criteria to define VISA: 1) vancomycin MICs between 8 and 16 µg/mL with a broth micro-dilution test 2) vancomycin MICs of greater than or equal to 6 µg/mL with E-testing and 3) growth within 24 hours on commercially prepared brain-heart infusion agar containing 6 µg/mL of vancomycin.

CDC Recommendations for VISA Detection in the Laboratory

The CDC has made recommendations for better detection of these rare organisms because of their clinical importance. Clinicians should be quite suspicious of an organism for which the vancomycin MIC is greater than or equal to 4 µg/ml and/or the vancomycin zone diameter is less than or equal to 14 mm. Disk diffusion testing is clearly contraindicated for the detection of VISA because this method is not sufficiently sensitive to detect decreased susceptibility to vancomycin in staphylococci.³ If a VISA isolate is suspected, laboratory technicians should look for small colony variants since these organisms are stressed and do not grow as rapidly. Technicians should also confirm the identity of the isolate to ensure that it is *S. aureus* and not, for example, *Leukonostoc*, which is innately resistant to vancomycin. The results should be confirmed with a quantitative method such as broth microdilution or E-test and incubated for a full 24 hours again at 35 degrees Celsius. These organisms grow very slowly and if they are not incubated for a full 24 hours, they may not be detected. Finally, any staphylococcal isolate determined to have an elevated MIC for vancomycin (MIC greater than or equal to 4.0 µg/mL) should be reported to a state laboratory or to the CDC for confirmation.

Vancomycin-Resistant *S. aureus* (VRSA)

The first VRSA clinical isolate was reported in Michigan in June 2002 and another in Pennsylvania in October 2002. This isolate contained a *vanA* determinant that mediates high-level vancomycin resistance in *Enterococcus*. The MIC for this strain was 1024 µg/mL. Conjugative transfer of a gene from a co-infecting vancomycin-resistant enterococcus was the probable explanation for the origin of these VRSA isolates. Because enterococci produce sexual pheromones, there has always been a concern that enterococci might attract staphylococci and transfer their vancomycin-resistant genes. In fact, it appears that this activity occurred with these two isolates.

The third confirmed isolate of VRSA was in New York State in April 2004 and also contained the *vanA* gene. However, this isolate was not initially identified by the commonly used automated systems in the U.S.

(Microscan® and Vitek®). When this organism was initially tested using a Microscan®, it was considered to be susceptible with an MIC of 4 µg/mL. Follow-up testing with E-test and broth microdilution revealed that the organism was vancomycin-resistant. The E-test MIC was greater than 256 µg/mL but the MIC by broth microdilution using standard techniques was 64

µg/mL. As a result of the failure of these automated systems to detect vancomycin resistance in the laboratory, the CDC has issued specific recommendations regarding testing for these isolates.⁴ When performing automated susceptibility testing, strains of *S. aureus*, particularly MRSA, should include a vancomycin agar screening plate containing 6 µg/mL of vancomycin. Laboratories should examine the plate for growth of small colonies after 24 hours of incubation. We have implemented this recommendation in our laboratory at the Carolinas Medical Center in Charlotte, NC. Unfortunately, it is somewhat time-consuming and adds to the cost of detection. In addition, with only three reported VRSA isolates, the probability of detecting one of these organisms is very low. As researchers and clinicians recognize the overall clinical importance of maintaining vigilance of these procedures, more and more laboratories are investing an enormous amount of time and resources to pick up these incredibly rare strains.

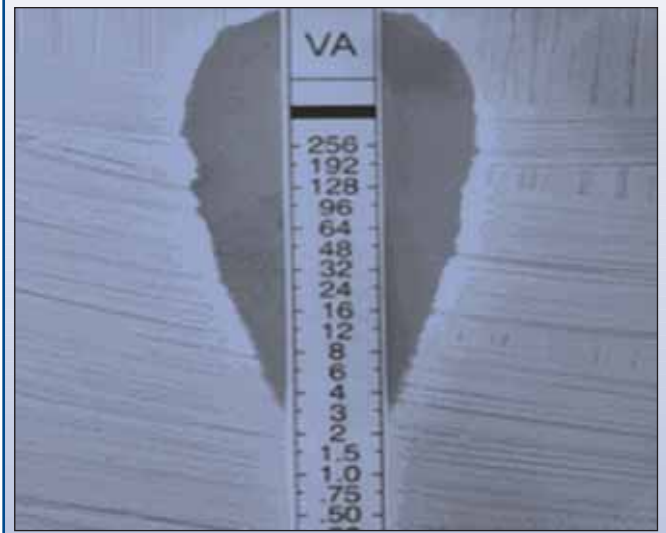
Heterogenous VISA (hVISA)

In addition to VISA and VRSA, there are other isolates that have reduced susceptibility to vancomycin, which is very difficult to detect. These strains are called heterogenous VISA (hVISA). They appear to be the stage that precedes development of VISA. These are strains of *S. aureus* that contain sub-populations of vancomycin-intermediate daughter cells and the larger population of parent cells that are fully susceptible to the glycopeptides. The MICs of these parent strains characteristically fall within the susceptible range of 1 and 4 µg/mL. It appears that vancomycin is imparting some type of selective pressure that favors the outgrowth of these very small, rare sub-populations of vancomycin-resistant clones that lead to hVISA. Continued stress from vancomycin leads to a uniform population of VISA.

Figure 1 is the first test of *S. aureus* from the case presentation. Clearly, there is something different about this organism. It is not a VRSA, but the MIC is 3µg/mL, which is a little high for *S. aureus*. Upon further testing, the organism was identified as hVISA.

Figure 1

Organism identified as hVISA with MIC of 3µg/mL



Detection of hVISA / VISA (GISA)

There are no standardized methods for the detection of hVISA which was first reported in Japan in 1996. Interestingly, several months later in the same hospital, a VISA was reported. A PFGE analysis on this VISA found that this organism probably evolved from the hVISA strain that had been detected earlier. Historically, heteroresistance among *S. aureus* to teicoplanin, a glycopeptide that is widely used outside of the U.S., was actually reported much earlier than resistance to vancomycin. There are MRSA strains in existence that are resistant to teicoplanin but are still vancomycin susceptible, therefore, cross-resistance does not necessarily occur between teicoplanin and vancomycin. However, all VISA tested thus far have shown reduced susceptibility to teicoplanin, which makes it a nice marker for VISA.

Most strains of VISA/GISA are susceptible by disk diffusion with zone diameters typically between 17 and 18 mm, but their colony morphologies are different. Because they are very slow growing, the colonies are tiny. These colonies can be encouraged to express themselves better either by diluting out the inoculum and allowing the smaller colonies to grow for a longer period of time or by growing the small colonies on a very rich medium, like brain-heart infusion supplemented with vitamin K or hemin. The NCCLS recommends that all staphylococci with zone diameters of 14 mm or less should be tested by an MIC method. The disk diffusion procedure will not differentiate strains with reduced susceptibility to vancomycin (MICs of 4 to 8 µg/mL) from susceptible strains (MICs of 0.5 to 2 µg/mL), even when incubated for a full 24 hours.²

Another method that has worked quite well for detection of VISA/GISA is the gradient effusion technique or the E-test. Walsh *et al* showed that the E-test is probably the best test to use in terms of high sensitivity (96%) and specificity (97%).⁵ The E-Test is a very straight-forward procedure using 0.2mL of McFarland 2.0 onto brain heart Infusion agar, using both vancomycin and teicoplanin E-test strips. Incubate at 35 degrees Celsius for 24 and 48 hours. The VISA/GISA is typically defined as a strain that has a teicoplanin MIC of 12 µg/mL or greater or a vancomycin MIC of 8 µg/mL and a teicoplanin MIC of 8 µg/mL.

Figure 2 shows an example of the E-test. On the right hand side, the standard MIC is done with 0.5 McFarland. The organism appears susceptible with an MIC of around 0.75 µg/mL. When streaked out with the same organism using a 2.0 McFarland, the MIC comes all the way up to 12 µg/mL, which identified the isolate as hVISA. The E-test detects these strains that are otherwise very difficult to detect using other methods in the laboratory.

Mechanism of resistance in VISA

The mechanism of resistance in VISA has been reported by Sieradzki *et al* to involve some alterations in the bacterial cell wall.⁶ The glycopeptide molecules appear to be sequestered away from the actual site of synthesis of the cell wall of these bacteria. There is an increase in the cell wall turnover that results in an excess of the non-cross-linked D-Ala-D-Ala. Vancomycin eventually disappears from the culture media and the cell wall becomes thickened. These organisms do not replicate quickly and they lose something in terms of fitness when they become VISA.

Vancomycin Treatment- Risk of Treatment Failures

Sakoulas *et al* have shown that the risks for vancomycin treatment failure in MRSA bacteremia begins to emerge with increasing vancomycin MICs well within the susceptible range (>0.5 µg/mL).⁷ These data indicate that when the MIC for vancomycin is greater than or equal to 0.5 µg/mL, the number of vancomycin failures increases. However, most laboratories in the U.S. use systems that only test down to 2 µg/mL. These data suggest that laboratories should adopt methods that detect these organisms in lower concentrations than we currently test. Since we are beginning to see increases in clinical failures with isolates that have MICs of 1 µg/mL, perhaps testing lower than the current 2 µg/mL in current automated systems should be considered.

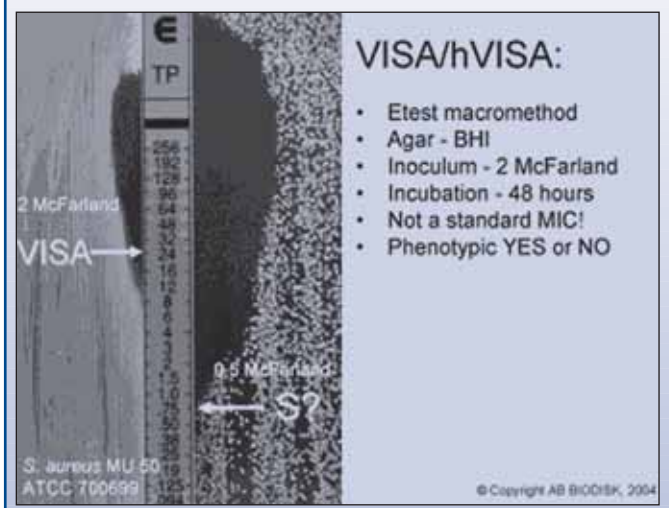
These researchers also determined that testing the bactericidal activity or potency of vancomycin in vitro could determine whether a *S. aureus* was likely to respond optimally to vancomycin. The problem with bactericidal testing is that it is extremely labor-intensive. Conducted properly, the assay could take one technician an entire day to complete, which is not a feasible routine task in most clinical laboratories.

Role of AGR in MRSA

The accessory gene regulator operon (*agr*), appears to coordinate many different critical virulence factors in MRSA including exoproteins, exotoxins, and adhesin expression. Typically the *agr* upregulates production of secreted virulence factors such as hemolysins and proteases, while downregulating production of virulence factors expressed on the staphylococcal cell surface. Certain DNA sequence polymorphisms at this locus comprise four *S. aureus agr* groups. For example, many of the community-associated MRSA fall into the *agr* group III polymorphism group. The GISA are highly enriched for the *agr* group II polymorphisms and the exfoliative toxin producing strains are associated more with *agr* group IV polymorphisms. There is also some suggestive evidence that the *agr* group II polymorphisms impart some intrinsic selective advantage to some MRSA clones under vancomycin selective pressure.⁸

Figure 2

Isolate identified as hVISA on the E-Test with MIC of 12 µg/mL



Community-Acquired MRSA

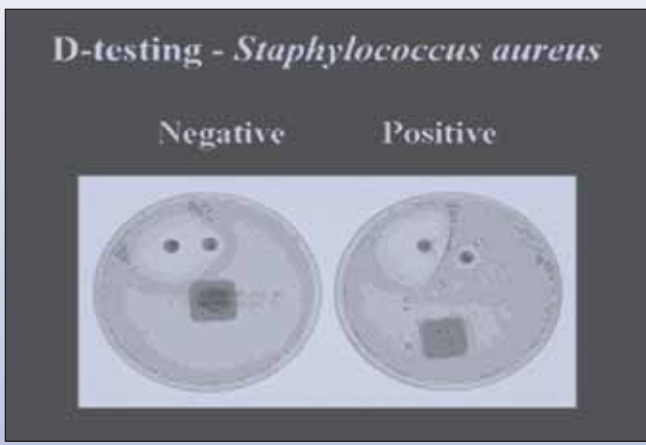
Community-acquired MRSA outbreaks are occurring throughout the country. They are very difficult to treat and as a result, are becoming problematic. The Carolinas Medical Center in Charlotte treated an outbreak that started in a high school wrestling team, spilled over into the football team of a major university, and continued on into their significant others. There were over 116 admissions within 12 months with significant community-associated MRSA infections. Fortunately, these isolates are characteristically susceptible to a variety of other agents such as trimethoprim-sulfamethoxazole, rifampin, tetracyclines, linezolid, quinupristin-dalfopristin, vancomycin, and often clindamycin and the fluoroquinolones. It is strongly recommended that isolates with discordant erythromycin and clindamycin susceptibility results be tested for inducible macrolide-lincosamide-streptogramin B (MLS_B) resistance.

D-Testing

MLS_B resistance is either constitutive or inducible following exposure to a macrolide. The D-test identifies inducible resistance that might predict mutational clindamycin constitutive resistance. The D-test is performed by placing clindamycin and erythromycin disks at an edge-to-edge distance of 15 to 20 mm and looking for flattening of the clindamycin zone nearest the erythromycin disk. A positive D-test suggests the presence of an *erm* gene that could result in constitutive clindamycin resistance and clinical failure.⁹ On the left of **Figure 3**, there is a strain of MRSA that is clearly erythromycin-resistant and clindamycin-susceptible. After incubation of this organism on a plate with the disks in close approximation, there is no truncation of the zone of inhibition around clindamycin, indicating that this organism does not harbor an inducible *erm* gene. By comparison, on the right side, there is an erythromycin-resistant, clindamycin susceptible strain, but the zone of inhibition has clearly been impacted with the diffusion of erythromycin through the median. Erythromycin has turned on that gene which is normally turned off and the organism is now resistant to clindamycin. The recommendation from the NCCLS is that these organisms be reported as clindamycin-resistant.

Figure 3

A positive D-test suggests the presence of an *erm* gene that could result in constitutive clindamycin resistance and clinical failure



The data in **Figure 4** are from the Carolinas Medical Center in Charlotte from 2003-2004. Over 1000 strains of staphylococci have now been identified as clindamycin-erythromycin discordant. Approximately 75% of strains of methicillin susceptible *S. aureus* (MSSA) for which D-tests were conducted demonstrated a positive D-test, indicating these organisms had an inducible *erm* gene. By comparison, the inducible *erm* gene was far less common in MRSA. Out of the 963 isolates of MRSA tested, less than 50% of the inpatient MRSA isolates and only 25% of the community-associated MRSA isolates harbored an inducible *erm* gene, which is good news in terms of treatment options for these infections.

MIC Interpretive Criteria (Breakpoints)

How do we determine, with MIC testing, whether an organism is susceptible or resistant in the laboratory? There are a number of factors that come into play in developing breakpoints. First, look at the population distribution of MICs of the organism. Drawing a breakpoint through that bell-shaped curve is not optimal because it may incorrectly identify an organism as resistant or susceptible. Second, breakpoints are based on the pharmacokinetics and the pharmacodynamic parameters of the compound in the organism. Third, the impact of specific recognized mechanisms of resistance on MICs has to be taken into account. Most importantly, what was the clinical outcome after treating an organism with a certain MIC with a particular antibiotic? Was it eradicated? Does the patient show clinical improvement and cure, or not? All of these factors come into play in determining breakpoints.

Based on several historical factors, the NCCLS may want to reconsider the breakpoints that we currently use for susceptibility testing, at least for *S. aureus*. Vancomycin breakpoints were established for staphylococci years ago, prior to the recognition of resistance mechanisms, prior to the recognition of strains exhibiting decreased susceptibility to the compound, and prior to correlation of higher MIC (susceptible) values with clinical failures among patients treated with vancomycin.

Other Treatments for Glycopeptide-resistant *S. aureus*: Daptomycin and Linezolid

Daptomycin is an acidic glycopeptide that was recently approved by the FDA. The MICs for daptomycin are typically less than or equal to 1 µg/mL for MRSA. It is more rapidly bactericidal against MRSA than vancomycin and it has *in vitro* activity against VISA, VRSA and many enterococcal strains.

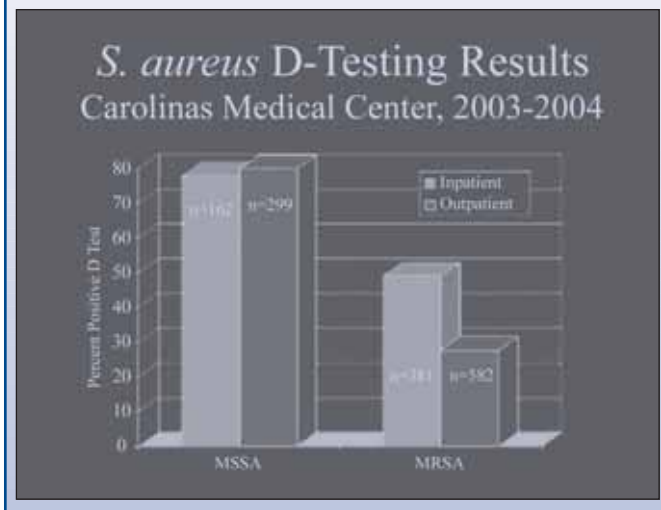
Linezolid is a compound that has been used successfully for treatment of infections due to MRSA and also vancomycin-resistant enterococcus. Reports of resistance to linezolid among staphylococci are rare, but, there have been reports of resistance in Boston and Charlotte. Linezolid resistance in *S. aureus* isolates has been associated with G2576T mutations in domain V of the 23S rRNA gene. Reversion to susceptibility *in vitro* when only one of the five copies of the gene was mutated suggests that resistance is unstable in the absence of antibiotic pressure.¹⁰

Vancomycin-Resistant Enterococci (VRE)

The initial reports of emergence of VRE came out of France and England in 1988 and in the U.S. in 1989.

Figure 4

Clindamycin-erythromycin discordant *S. aureus*. Positive D-tests indicating the presence of an inducible erm gene



Resistance is seen in both *E. faecalis* and *E. faecium*. There are at least five phenotypes of VRE that have been described: vanA, vanB, vanC, vanD, and vanE. These phenotypes correspond with the genotypes *vanA*, *vanB*, *vanC*, *vanD*, and *vanE*. The vancomycin MICs for vanA (>64 µg/mL) and vanB (4-1024 µg/mL) are quite high while the MICs for teicoplanin are very high for vanA (16-512 µg/mL) and very low for vanB (≤ 0.5 µg/mL). The species that usually express vanA and vanB are *E. faecalis* and *E. faecium*. The genes are typically acquired and they can be transferred.

In comparison, the vancomycin MICs for the vanC (2-32 µg/mL), vanD (128 µg/mL), and vanE (16 µg/mL) phenotypes are somewhat lower, although they are fairly high for vanD. Teicoplanin MICs are very low for vanC (≤ 0.5 µg/mL) and vanE (0.5 µg/mL) and somewhat higher for vanD (4.0 µg/mL). The vanC phenotype is innately carried by enterococci (*E. gallinarum*, *E. casseliflavus*, and *E. flavescence*) but they are not that significant in terms of clinical activity and infection control. The vanC phenotype is not acquired and is not transferable. The vanD and vanE phenotypes are acquired, but are also not transferable.

Quinupristin-dalfopristin (Synercid®) is a drug used to treat vancomycin-resistant *E. faecium* (VREF). It is only active against *E. faecium*, not *E. faecalis*. Resistant strains of *E. faecium* have been described, but they are rare. Synergy against many strains of VREF has been observed in vitro between quinupristin-dalfopristin and ampicillin, linezolid, vancomycin, gentamycin, and doxycycline.

Linezolid inhibits the initiation of protein synthesis in VREF. It is generally considered bacteriostatic and is active against *E. faecium* and *E. faecalis*. When combined with quinupristin-dalfopristin or vancomycin, linezolid demonstrates enhanced activity against MRSA. Synergy with linezolid and doxycycline, at least in the laboratory, has also been shown against VREF.

Detection of VRE in the Laboratory

The NCCLS provides recommendations for *in vitro* detection of VRE.² When testing for VRE via the M2-Disc diffusion method, plates again should be held for a full 24 hours and examined using transmitted light. These organisms grow more slowly. The presence of a haze or any growth within the zone of inhibition indicates resistance and organisms with intermediate zones should be retested by an MIC method. According to the M7- MIC recommendations, when testing for VRE, plates should be held again for a full 24 hours for accurate detection of isolate resistance. For isolates with vancomycin MICs of 8-16 µg/mL, perform biochemical tests to confirm identification of an enterococcus species. It is easy to be fooled by *Leuconostoc* and *Pediococcus* species. Vancomycin screening agar should consist of brain heart infusion agar with vancomycin 6 µg/mL and 0.5 McFarland inoculum incubated at 35 degrees centigrade in ambient air for a full 24 hours. Even one colony on this plate would be considered presumptive evidence of VRE.

A vancomycin MIC test, along with tests for motility and pigment production, should be performed on all isolates that are presumed to be resistant based on growth of vancomycin screen agar. These tests will distinguish between the clinically important species with acquired resistance to vancomycin (i.e., vanA and vanB, *E. faecalis* and *E. faecium*) from those species with intrinsic resistance to vancomycin (vanC), such as *E. gallinarum* (a non-pathogen) and *E. casseliflavus*, both of which often grow on the screening plate. Both *E. gallinarum* and *E. casseliflavus* are motile and *E. casseliflavus* has a yellow pigment. In contrast to other enterococci, *E. casseliflavus* and *E. gallinarum*, with vancomycin MICs of 8-16 µg/mL (intermediate), differ from clinically relevant VRE from an infection control perspective. Patients that are infected with *E. casseliflavus* and *E. gallinarum* do not require any type of infection control precautions despite the fact these organisms might be considered vancomycin-resistant.

Conclusions

In summary, laboratory detection of antibiotic resistance among staphylococci to a variety of antibiotics including vancomycin is clearly problematic. Vancomycin resistance among enterococci is commonplace and clinically associated primarily with *E. faecium*. A number of therapeutic alternatives exist for the treatment of infections caused by vancomycin-resistant gram-positive pathogens.

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Target Audience: Physicians; clinical microbiologists; pharmacists; hospital epidemiologists; public health authorities; health care professionals in training; and others interested in the epidemiology, recognition, and management of infections due to resistant staphylococci.

Learning Objective: After reading this publication, the reader should be able to understand the problem of detection of heteroresistance in the laboratory.

CME Self Assessment Examination

Volume VIII, Issue 2

(At least three of the five answers must be correct in order to obtain a CME certificate)

See mailing instructions and other pertinent information on the reverse side.

- 1) All of the following species are innately resistant to vancomycin except:
 - a) *Leuconostoc*
 - b) *Pediococcus*
 - c) *Peptostreptococcus*
 - d) *Lactobacillus*Answer: _____
- 2) With regard to vancomycin-intermediate *Staphylococcus aureus* (VISA), which statement is false?
 - a) The presence of small colony variants on culture plates may be a clue to the presence of VISA
 - b) Colonies which are white in color suggest VISA
 - c) Incubation for less than 24 hours may reduce the likelihood that VISA is detected in culture
 - d) Disk diffusion testing is inadequate for detection of VISAAnswer: _____
- 3) In order to detect inducible clindamycin resistance among isolates of community-associated MRSA, which test should be performed?
 - a) The E-test
 - b) Broth microdilution assays to determine the MIC of clindamycin
 - c) Kirby-Bauer testing
 - d) The D-testAnswer: _____
- 3) Among community-associated MRSA isolates, the presence of clindamycin-erythromycin discordance on susceptibility results suggests the presence of:
 - a) *erm*
 - b) *env*
 - c) *pol*
 - d) *agr*Answer: _____
- 3) Which of the following antimicrobial agents does not demonstrate *in vitro* activity against VISA, VRSA and VREF?
 - a) Quinupristin/dalfopristin
 - b) Daptomycin
 - c) Clindamycin
 - d) LinezolidAnswer: _____

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