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A comparison of BHI/vancomycin agar, VITEK, and Kirby-Bauer methodologies for identifying Vancomycin resistance in enterococcal isolates from laboratory specimens

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A Comparison of BHI/vancomycin agar, VITEK, and Kirby-Bauer Methodologies for Identifying Vancomycin Resistance in Enterococcal Isolates from Laboratory Specimens

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ABSTRACT (100-200 WORDS):

A comparison of BHI/vancomycin agar, VITEK, and Kirby-Bauer disk diffusion methodologies for identifying vancomycin resistance in Enterococcal isolates was conducted. One hundred-six Enterococcal isolates were collected from several clinical sites in the Northern Illinois region and tested to determine vancomycin susceptibility by each of the three methodologies. Of the 106 isolates tested, 23 were determined by the BHI/vancomycin agar method to be vancomycin resistant and 83 were determined to be vancomycin susceptible. VITEK correctly identified all of the 83 vancomycin susceptible isolates (100%) and 20 of the 23 vancomycin resistant isolates (87%). Kirby-Bauer disk diffusion correctly identified all of the 83 vancomycin susceptible isolates (100%) and 22 of the 23 vancomycin resistant isolates (95.6%). The sensitivity and specificity of each of the three methodologies evaluated in this study are quite high; however, whether or not these values justify using only VITEK or Kirby-Bauer susceptibility testing without a confirmatory method can not be determined by this rather limited study alone. These results do confirm that incorrect susceptibility testing results occur with both VITEK and Kirby-Bauer methodologies, particularly in determining vancomycin resistance. Until additional studies confirm that automated MIC determination or Kirby-Bauer susceptibility testing consistently give reliable results, confirmatory testing with BHI/vancomycin agar plates will ensure that accurate vancomycin susceptibility results are reported for Enterococcal isolates.

Purpose The purpose of this project is to compare the following three methodologies for identifying vancomycin resistance in enterococcal isolates from laboratory specimens: BHI/vancomycin agar, VITEK, and Kirby-Bauer disk diffusion.

Background and Literature Review In 1979, U.S. Surgeon General William Stewart pronounced that it was time to "close the books on infectious diseases" (Lang, 1994). In the sixteen short years since then, however, the United States has seen a steady increase in the number of infectious disease cases (Lang, 1994). One has only to open a local newspaper or weekly news magazine to learn of infectious disease outbreaks such as *Cryptosporidium* in the Milwaukee water supply, *E. coli* 0157: find in hamburger, or hantavirus in the Southwest. These same news sources print reports from the Centers for Disease Control and Prevention which portend the emergence of "super bugs" as a consequence of microbial resistance to the antibiotic drugs we assumed would be able to treat any infection (Derlot, 1991).

Enterococci are one genus of bacteria that are emerging with multi-drug resistance. Treated effectively in the past with a combination of a beta lactam and an aminoglycoside (ampicillin and gentamicin, for example), strains of Enterococci are now resistant to beta lactams, aminoglycosides, and even to the glycopeptides (such as vancomycin) (Boyle, 1993 Handwerger, 1993 Eliopoulos, 1993). Treatment for patients infected with Vancomycin resistant Enterococci (VRE) is relegated to unproven combinations of antimicrobials or to experimental compounds (Nosocomial, 1993). The disheartening result for physicians is that most of these patients die despite antimicrobial therapy.

While media reports serve to educate the public concerning the importance of rapid identification of disease outbreaks, there is a problem facing clinical laboratories that may undermine such efforts. The problem is one of reliability. Methods currently

in use for detection of antimicrobial susceptibility have been shown to give erroneous results for some bacterial species (Centers for Disease Control, 1994). This leads to inappropriate reporting of drugs that should be effective and subsequent failure of treatment. Patients remain infected with resistant bacteria, becoming more and more critical, while other colonizing bacteria become resistant through exposure to antimicrobials. Then a downward spiral of disease spread, resistance transfer to other bacteria, and increased resistance to multiple drugs ensues.

In light of these cases of infection with resistant bacteria, clinical laboratorians and physicians must be able to rely on the methods used for antimicrobial susceptibility testing; therefore, it is necessary to test the methods currently in use in order to modify testing methodology as necessary. In the case of vancomycin resistance in Enterococci, the federal government has recently mandated confirmation of all automated enterococcal susceptibility testing stating that, "...vancomycin resistance, in particular moderate vancomycin resistance (as manifested in the vanB phenotype), is not detected consistently with the automated methods used in many clinical laboratories" (Centers for Disease Control, 1994). According to the Hospital Infection Control Practices Advisory Committee, Vancomycin resistance must be confirmed on all enterococcal isolates from clinical specimens by one of the following methods: 1) BHI agar with 6 µg/ml vancomycin - any growth indicates resistance, or 2) Determination of Minimum Inhibitory Concentration (MIC) by agar dilution, broth macrodilution, or manual broth microdilution (Centers for Disease Control, 1994).

Although the federal government has mandated confirmatory testing for all Enterococcal isolates, there have been very few studies documenting the efficacy or inefficacy of the susceptibility determination methods commonly in use. To my

knowledge, there has been only one recent study that evaluated VITEK (software version 7.1) and Kirby-Bauer disk diffusion methodologies (Willey, 1993). Furthermore, I am not aware of any published results from studies completed on the utility of BHI /vancomycin agar, although a study is being conducted by the National Committee for Clinical Laboratory Standards (NCCLS) (Nosocomial, 1993). It is the intent of this study, then, to address the apparent lack of substantiating evidence by simultaneously determining the susceptibility of one hundred-six enterococcal isolates with VITEK, BHI/vancomycin agar, and Kirby-Bauer disk diffusion methods. This study will evaluate the reliability of the VITEK auto analyzer in identifying vancomycin resistance in enterococcal isolates and, subsequently, will examine the need for confirmatory testing.

Characteristics of Enterococci The genus *Enterococcus* is part of the family Streptococcaceae and includes members of the previous group D Streptococci (Koneman, 1992). The Enterococci are characterized as multiple drug resistant gram positive cocci which typically form gray colonies that may be alpha, beta, or gamma hemolytic on blood agar. Enterococci can be differentiated from other members of the family Streptococcaceae because they are PYR positive, bile esculin positive, and grow in 5% sodium chloride. Bacteria of the genus *Enterococcus* are found as normal flora in the human gastrointestinal tract; however, due to their increasing resistance to antibiotics, the Enterococci are becoming increasingly more important as human pathogens. The Enterococci are capable of causing both nosocomial (hospital-acquired) and community-acquired infections including endocarditis, bacteremia, gastroenteritis, neonatal sepsis, and urinary tract and wound infections (Koneman, 1992). Boyle states that the Enterococci have become the second most common cause of nosocomial infections in the past six years (Boyle, 1993).

The species most often associated with human infections are *Enterococcus faecalis* and *Enterococcus faecium* (Koneman, 1992). E. faecalis is the most common isolate and causes 80-90% of enterococcal infections, while E. faicum causes 10-15% of infections and is more resistant to both aminoglycoside and beta lactam drugs. Other enterococcal species rarely cause infections in humans but may cause infections in immunocompromised patients (Koneman, 1992).

Mechanisms of Antimicrobial Resistance in Enterococci Vancomycin belongs to a class of antimicrobial drugs known as the glycopeptides, which act to inhibit bacterial cell wall synthesis (Koneman,1992). Enterococci may display two mechanisms of antimicrobial resistance: 1) production of inactivation enzymes such as beta lactamases or 2) ribosomal resistance which alters the drug binding site on the bacterial cell wall (Koneman, 1992). The gene for vancomycin resistance is thought to be carried on a transposon and produces altered peptidoglycan precursors, the most important of which seems to be D-alanine-D-lactate. This precursor is coded for instead of the normal D-alanine-D-alanine and has a binding affinity for vancomycin which is >1000 times lower than the normal peptidoglycan (Eliopoulos, 1993). Transposons carry plasmids, which are portions of genetic material, from one bacterium to another during conjugation. This exchange of genetic material may occur between bacteria of different strain, species, or genus; thus allowing for development of vancomycin resistance in streptococci or other pathogens (Koneman, 1992).

For ease in description, the vancomycin resistance patterns of Enterococci have been divided into the following three groups: 1) vanA - high level, inducible resistance to both vancomycin and teicoplanin (both glycopeptide antimicrobials) which is carried on a transposon; 2) vanB - moderate to high level resistance to vancomycin, also carried on a chromosome, but susceptibility to teicoplanin; and 3) vanC - intrinsic low

level vancomycin resistance (Boyce, 1994). E. faecalis and E. faecium may display either vanA or vanB resistance while vanC resistance is more typical of E. gallinarum and E. casseliflavus (Boyce, 1994). Let us now examine some manifestations of vancomycin resistance in enterococcal infections in the United States.

The CDC's National Nosocomial Infection Surveillance system (NNIS) has compiled some statistics outlining the rapid increase in the number of VRE infections in the United States. These numbers demonstrate the magnitude of the crisis physicians face in treating patients with VRE and also support the need for accurate susceptibility testing in order to impede the spread of resistance. The percent of nosocomial VRE infections in the general patient population reported to the CDC increased from 0.3% in 1989 to 7.9% in 1993, while the percent of VRE cases among intensive care unit patients increased from 0.4% in 1989 to 13.6% in 1993 (Nosocomial, 1993). [Table 1] The mortality figures for patients with VRE septicemia versus patients with vancomycin susceptible enterococcal septicemia were 36.6% and 16.4% mortality, respectively (Nosocomial, 1993). Interestingly, higher numbers of nosocomial VRE infections were reported from university affiliated hospitals than from non-teaching sites (Nosocomial, 1993).

Incidents of nosocomial VRE outbreaks are well documented (Handwerger, 1993 Boyce, 1994). One particularly interesting incident will illustrate the rapid dissemination of vancomycin resistance among patient groups. Chow, et al., evaluated the DNA of thirty-eight VRE isolates from patients in five hospitals in three states and found one strain to be common among four patients in hospital B, located in Chicago, Illinois, three patients in hospital A, also in Chicago, and two patients in hospital C, located in Detroit, Michigan (Chow, 1993). [Figure 1] With evidence of enterococcal spread as inconceivable as that presented in this study, the need for

accurate detection of enterococcal susceptibility patterns is apparent. Let us now look briefly at the principles of susceptibility testing methods commonly in use in clinical laboratories.

Antimicrobial Susceptibility Testing Methodologies The methodology most commonly in use today is an automated detection system. The VITEK system is currently being used for antimicrobial susceptibility testing at Saint Anthony Medical Center. VITEK is an automated microbiology system used for rapid organism identification and susceptibility testing. VITEK GPI cards are used for the identification of gram positive organisms to the species level while GPS-TA cards are used for determining MIC values of catalase negative gram positive cocci. The VITEK system operates by utilizing the principles of nephelometry (measurement of light scatter due to microbial growth) and colorimetry (detection of microbial metabolism by measuring colored end products or indicators). The VITEK Filler/Sealer fills tests cards containing either antibiotics or reagents for specific biochemical tests with the prepared 0.5 McFarland Standard broth suspension of the organism to be tested. The filled card is then placed into the Reader/Incubator where a 35 degree Celsius incubation temperature is maintained. VITEK measures the percentage of change in light readin gs as compared to an initial zero base line reading. The identification or MIC for the organism is then determined according to its biochemical reactions or its growth rate in the presence of varying antibiotic concentrations, respectively. Inoculation of the appropriate VITEK card determines what testing will be performed by the instrument.

BHI/vancomycin agar is one of the confirmatory methods recommended by the federal government for confirming automated susceptibility testing. BHI/vancomycin agar plates are a solid, enriched culture media incorporating 6 mg of the antibiotic

Vancomycin. The plates are inoculated with a McFarland 0.5 Standard broth suspension of the enterococcal isolate and are read for growth after aerobic incubation at 37 degrees Celsius for 24 hours. Growth of more than one individual colony indicates vancomycin resistance while lack of growth indicates susceptibility to vancomycin.

Finally, Kirby-Bauer disk diffusion has also been modified by NCCLS for determining vancomycin susceptibility. The Kirby-Bauer Method is a way to measure the *in vitro* susceptibility of bacteria to antimicrobial agents. Filter- paper disks containing 30 µg vancomycin are applied to the moist Mueller-Hinton agar surface after appropriate inoculation of the agar with a 0.5 McFarland Standard broth suspension of the enterococcal isolate. The vancomycin diffuses into the surrounding medium, presenting a gradient of vancomycin concentration as the Enterococci are multiplying logarithmically on the agar surface. The diameter of the zone of inhibition relates linearly to the MIC of the enterococcal isolate.

The three methodologies discussed, VITEK, BHI/vancomycin agar plates, and Kirby-Bauer disk diffusion, will be evaluated in this study to determine their ability to give accurate susceptibility testing results for vancomycin with respect to enterococcal isolates from patient specimens.

As stated previously, there has been little past research concerning the efficacy or inefficacy of these three susceptibility detection methods. One published test conducted by the CDC in 1993 evaluated VITEK, disk diffusion, and another automated technology by sending five enterococcal strains of known identity and resistance to participating clinical laboratories in New Jersey (Tenover, 1993). The results indicated that 96% of the laboratories correctly identified a vanA strain as highly resistant regardless of the susceptibility testing methodology they used

(Tenover, 1993). In contrast, only 29% of laboratories correctly identified the vanB strain as vancomycin resistant. 63% of VITEK users reported incorrect results as did two of four labs using disk diffusion (which is approved as a confirmatory method) (Tenover, 1993). With respect to the vanC isolate, none of the labs participating identified the strain as resistant, however, 97% of VITEK users identified the resistance as intermediate as did the CDC lab itself (Tenover, 1993). Furthermore, another group of researchers who evaluated only the VITEK GPI card with version 7.1 software concluded that, "Detection of vancomycin resistance by the VITEK GPS-TA card in Enterococci has been markedly improved and should now be considered acceptable" (Willey, 1993). In light of the ambiguous results of these two studies and the lack of further substantiating evidence that one method of vancomycin susceptibility testing is superior with respect to Enterococcal isolates, let us now look at the methods and results of the current study conducted at Saint Anthony Medical Center.

Methods One hundred-six Enterococcal isolates from clinical specimens were obtained from several medical centers in the northern Illinois region. Vancomycin resistant Enterococcal isolates were procured from several medical centers in order to obtain a greater number of resistant isolates for testing. Each of the enterococcal isolates obtained were initially frozen from pure cultures in thioglycollate broth with 10% glycerine in order to allow batch testing at a later date. Each specimen was then thawed and streaked onto a blood agar plate to obtain fresh 24 hour growth while ensuring the viability of the organism and purity of the inoculum. After 24 hours of incubation at 37 degrees Celsius in 5-9% CO_2, colonies from each plate were inoculated to a BHI/vancomycin agar screening plate, Mueller-Hinton plate (Kirby-Bauer method), and VITEK GPS-TA card for determining susceptibility to vancomycin.

Inoculation to the BHI/vancomycin agar plate was done by making a 0.5

McFarland standard inoculation of each isolate in a separate tube of sterile Mueller-Hinton broth. One streak of this inoculum was then made on the surface of the agar with several isolates being tested in different areas of one plate. The plates were then incubated at 35 degrees Celsius in an ambient air incubator for 24 hours and examined for growth. Any visible growth on the BHI/vancomycin plates was interpreted as vancomycin resistance. The Mueller-Hinton plates for Kirby-Bauer testing were inoculated for confluent growth with the same 0.05 McFarland standard in Mueller-Hinton broth that was used for each isolate for the BHI/vancomycin screening plate. A vancomycin (30 µg) sensitivity disk was pressed onto each agar plate within 15 minutes of inoculation and the plates were incubated for 18 hours at 35 degrees Celsius in an ambient air incubator. Inhibition zone diameters were measured with sliding caliper and susceptibility determined according to NCCLS tables as follows: isolates with a zone of inhibition ≤14 mm vancomycin resistant , 15-16 mm intermediate, and ≥17 mm vancomycin susceptible. The VITEK GPS-TA cards (and GPI cards for speciating vancomycin resistant isolates in order to estimate vancomycin resistant phenotype) were inoculated according to the manufacturer' recommendations and were evaluated with VITEK software version 8.0.

Results The overall results of the study are shown in Tables 2, 3, and 4. The BHI/vancomycin agar method identified 23 of the 106 enterococcal isolates as vancomycin resistant. The remainder of the clinical isolates failed to grow on these plates and were therefore identified as vancomycin susceptible. Since BHI/Vancomycin agar plates are recommended by the CDC for confirming laboratory determination of vancomycin resistance in Enterococci, the results obtained with Kirby-Bauer and VITEK methodologies were evaluated in reference to the results described

above (Centers for Disease Control, 1994). Testing using Kirby-Bauer methodology correctly identified 22 of the 23 vancomycin resistant isolates (96%) and all of the 83 vancomycin susceptible isolates (100%). The isolate misidentified as vancomycin sensitive by the Kirby-Bauer methodology was presumptively identified as the vanC phenotype. VITEK GPS-TA MIC determination correctly identified 20 of the 23 vancomycin resistant isolates (87%) and all of the 83 vancomycin susceptible isolates (100%). The three isolates incorrectly identified as vancomycin sensitive by VITEK may be either the vanA or the vanB phenotype.

Discussion The sensitivity and specificity of each method tested are shown in Table 5. As stated previously, there has been little past research concerning the efficacy or inefficacy of the three susceptibility testing methods evaluated in this study. The one published test I was able to find was conducted by the CDC in 1993 and evaluated VITEK, disk diffusion, and another automated technology by sending five enterococcal strains of known identity and susceptibility pattern to participating clinical laboratories in New Jersey (Tenover, 1993). The results of this study indicated that 96% of the laboratories correctly identified the vanA strain as highly resistant regardless of the susceptibility testing methodology used. In contrast, only 29% of the laboratories correctly identified the vanB strain as vancomycin resistant. 63% of VITEK users reported incorrect results as did two of four labs using disk diffusion. With respect to the vanC isolate, none of the labs participating identified the strain as resistant; however, 97% of VITEK users identified the resistance as intermediate.

Although the present study was conducted in a different format than the CDC study, the results of the two studies seem to correlate. Only four enterococcal isolates were incorrectly identified in the present study, and there seems to be little significant difference in the ability of VITEK and Kirby-Bauer methodologies to correctly identify

vancomycin resistance in Enterococci. Although I was unable to definitively determine the resistance phenotype of the VRE isolates using methods available to me, I was able to base resistance phenotypes on those commonly associated with each enterococcal species. In agreement with the CDC study, 15% of isolates possibly displaying either vanA or vanB resistance phenotypes were incorrectly identified as vancomycin sensitive by VITEK. Similarly, one of three isolates expected to display the vanC phenotype was incorrectly identified as vancomycin sensitive by Kirby-Bauer disk diffusion. However, in contrast to the results of the CDC study, VITEK correctly identified all three of the enterococcal isolates which presumptively express the vanC phenotype.

The sensitivity and specificity of each of the three methodologies evaluated in this study are quite high. [Table 5] Whether or not these values justify using only VITEK automated MIC determination or Kirby-Bauer susceptibility testing without a confirmatory method can not be determined by this rather limited study alone. However, these results do confirm that incorrect susceptibility testing results occur with both VITEK and Kirby-Bauer methodologies, particularly in determining vancomycin resistance. This data does support the reliability of BHI/vancomycin agar screening plates as a confirmatory method for determining both vancomycin susceptibility and resistance. Additionally, it should be noted that the frequency of recovering VRE in the northern Illinois region can not be estimated on the basis of this data since resistant isolates were collected from several medical centers and not all vancomycin susceptible isolates recovered during the same time period were tested.

In conclusion, before clinical laboratories can rely on either VITEK or Kirby-Bauer susceptibility testing methods alone, a larger study must be conducted. It may be found that these two methods are unable to reliably detect certain phenotypes of

vancomycin resistance, or that the problem in detecting enterococcal resistance to vancomycin is more sporadic, varying with each enterococcal strain. Until additional studies confirm that automated MIC determination or Kirby-Bauer susceptibility testing consistently give reliable results, confirmatory testing with BHI/vancomycin agar plates will ensure that accurate vancomycin susceptibility results are reported for enterococcal isolates.

Regardless of the results of this and future studies, the striking spread of antimicrobial resistance in Enterococci and other pathogens will continue to plague the treatment of infectious disease. Vancomycin resistant Enterococci are not just a problem for large hospitals or select patient populations; rather, this type of resistance is a concern for all medical centers due to the increase in number of nosocomial infections caused by VRE. From the standpoint of healthcare professionals, the most disheartening result of vancomycin resistance in Enterococci reaches beyond the threat of spreading antimicrobial resistance to the struggle of managing patients infected with organisms that can not be treated effectively with any available antibiotic. As one physician unambiguously stated, these patients will most likely die.

With the unbridled resurgence of many of the bacterial pathogens that formerly posed little challenge or threat to modern antibiotic treatment, healthcare professionals are becoming acutely aware of the increasing demand for susceptibility testing of clinical isolates. Until a new generation of antimicrobials is developed, the battle against spreading bacterial resistance and treatment failure will continue and it is the role of the clinical microbiology laboratory to provide prompt and reliable antimicrobial susceptibility results.

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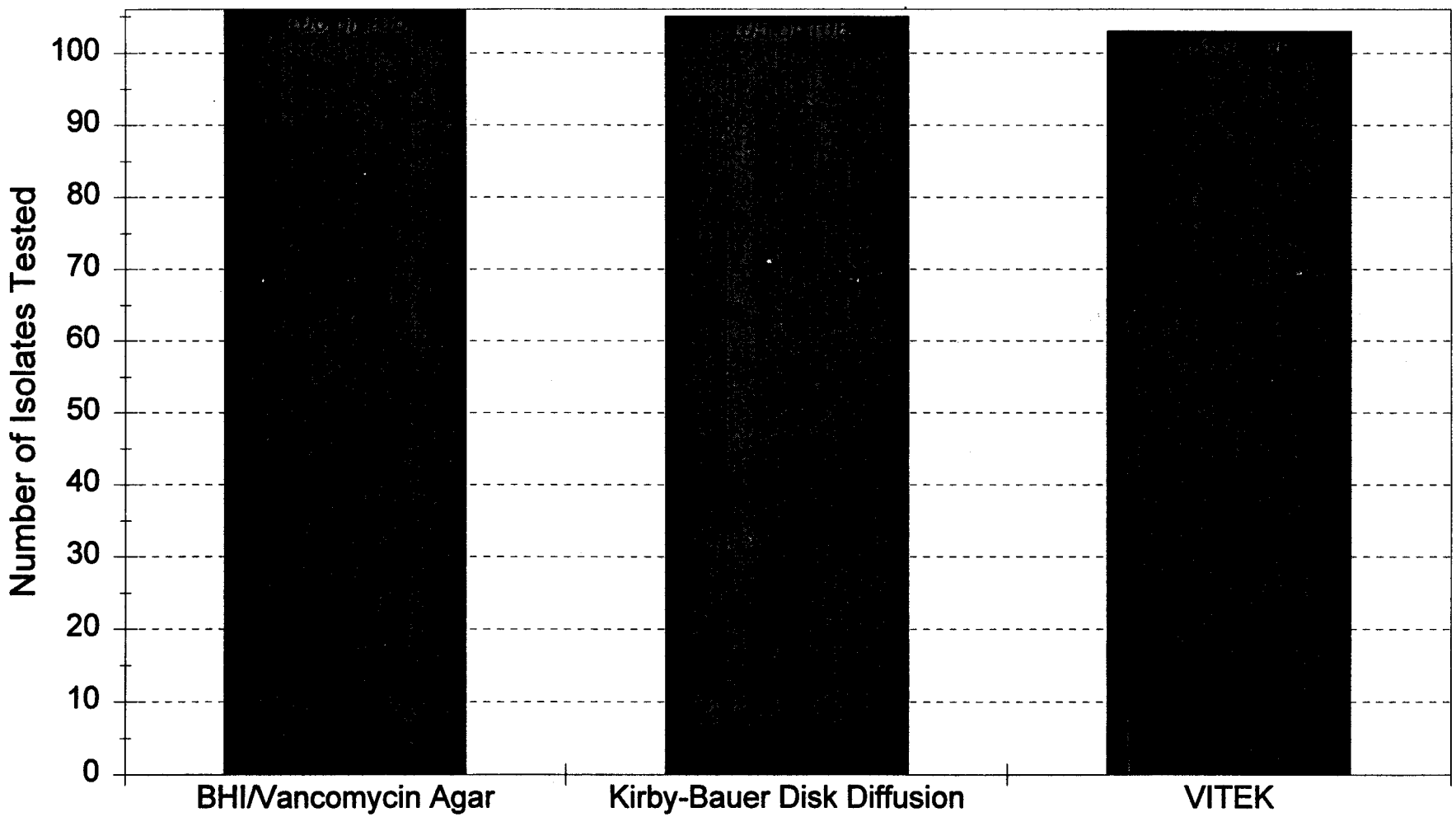
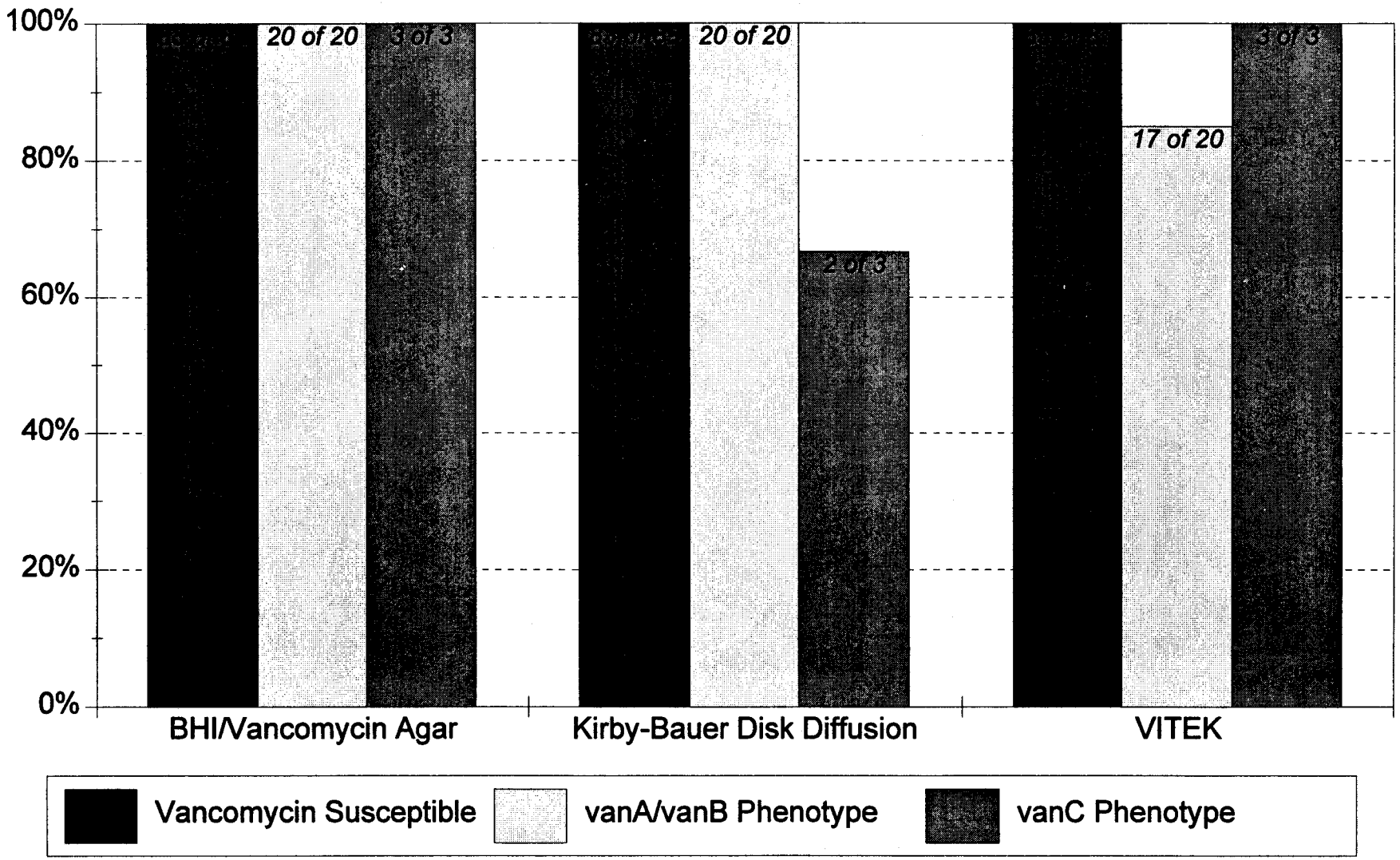
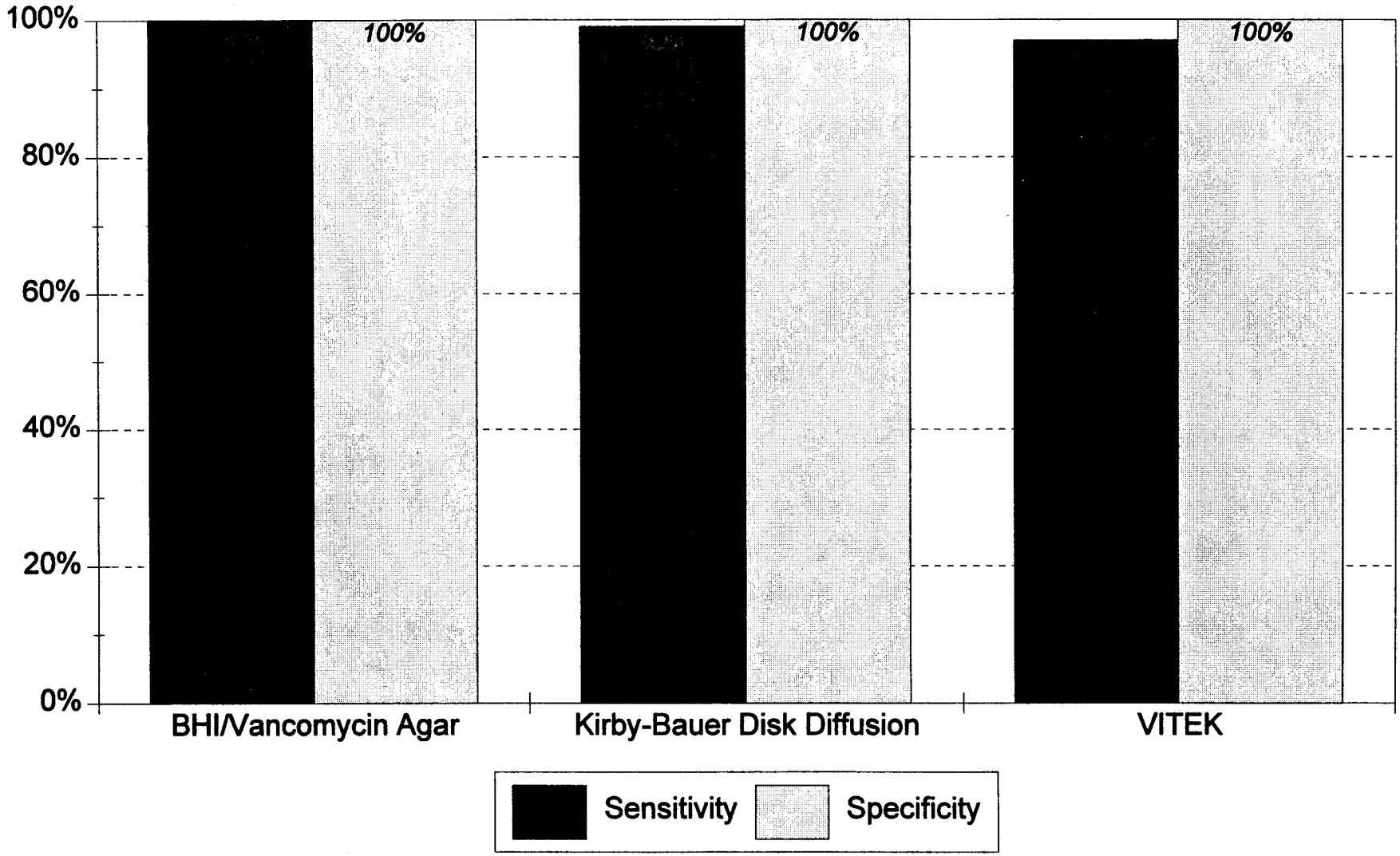
Isolates Correctly Identified (Table 2)



Number of Isolates Correctly Identified (Table 3)



Sensitivity and Specificity of Methods (Table 5)



Isolates Correctly Identified as Vancomycin Resistant (Table 4)