ROBERT KOCH INSTITUT

Originally published as:

Klare, I., Fleige, C., Geringer, U., Witte, W., Werner, G.
Performance of three chromogenic VRE screening agars, two Etest® vancomycin protocols, and different microdilution methods in detecting vanB genotype Enterococcus faecium with varying vancomycin MICs
(2012) Diagnostic Microbiology and Infectious Disease, 74 (2), pp. 171-176.

AUTHOR MANUSCRIPT. © Elsevier (2012): This is the author's version of the work. It is posted here by permission of Elsevier for personal use, not for redistribution. Some changes resulting from the publishing process, such as editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Some changes may have been made to this work since being accepted for publication. A definitive version was subsequently published in Diagnostic Microbiology and Infectious Disease, [Volume: 74, Issue: 2, 2012 Aug 15]
DOI: 10.1016/j.diagmicrobio.2012.06.020

Performance of three chromogenic VRE screening agars, two Etest® vancomycin protocols, and different microdilution methods in detecting *vanB* genotype *Enterococcus faecium* with varying vancomycin MICs

Ingo Klare, Carola Fleige, Uta Geringer, Wolfgang Witte, Guido Werner

National Reference Centre for Staphylococci and Enterococci, Division Nosocomial Infections, Department of Infectious Diseases, Robert Koch-Institut, Wernigerode Branch, Germany

Abstract

Frequencies of vanB-type Enterococcus faecium increased in Europe during the last years. VanB enterococci show various levels of vancomycin MICs even below the susceptible breakpoint challenging a reliable diagnostics. The performance of 3 chromogenic vancomycin-resistant enterococci (VRE) screening agars, 2 Etest® vancomycin protocols, and different microdilution methods to detect 129 clinical vanB E. faecium strains was investigated. Altogether, 112 (87%) were correctly identified as VanB-type Enterococcus by microdilution MICs. An Etest® macromethod protocol was more sensitive than the standard protocol while keeping sufficient specificity in identifying 15 vanA/vanB-negative strains. Three chromogenic VRE agars performed similarly with 121 (94%), 123 (95%), and 124 (96%) vanB isolates that grew on Brilliance™ VRE Agar, CHROMagar™ VRE, and chromID™ VRE agar, respectively. Using identical media and conditions, we did not identify different growth behaviour on agar and in broth. A few vanB strains showed growth of microcolonies inside the Etest® vancomycin inhibition zones, suggesting a VanB heteroresistance phenotype.

Introduction

Enterococci are important nosocomial pathogens, and multi- and vancomycin-resistant isolates of *E. faecalis* and *E. faecium* are of special concern in the health care setting. Altogether, 8 genotypes of acquired vancomycin resistance are known in *Enterococcus* spp. worldwide, but only *vanA* and *vanB* are clinically relevant genotypes (Werner 2012). VanA-type resistance is induced by all glycopeptides and, consequently and by definition, confers resistance to vancomycin and teicoplanin. The VanB type is characterised by susceptibility to teicoplanin (no inducer) and to varying levels of vancomycin resistance expression. However, glycopeptide resistance expression in wild-type strains may vary and MIC values are ambiguous and do not allow a proper determination of the corresponding genotype based on phenotypic test results. For example, *vanA* genotype vancomycin-resistant enterococci (VRE) exhibiting a VanB (VanD) phenotype, i.e., showing susceptible or intermediate MICs for teicoplanin, are documented worldwide (Gu et al., 2009, Henrique et al., 2008, Park et al., 2008 and Song et al., 2006). In addition, diagnostics of *vanB* type strains is challenged by varying expression levels of vancomycin resistance resulting in a wide range of MICs to vancomycin from ≤ 1 to > 256 mg/L including concentrations that are defined as susceptible (≤ 4 mg/L) (Courvalin, 2006 and Depardieu et al., 2007). Expression levels do not correlate with the presence of different *vanB* allele types *vanB*1 to *vanB*3. All 3 subtypes are detected by molecular tests and *vanB*2 is the most prevalent (Courvalin, 2006 and Werner et al., 2008). Detection of *vanB* type resistance by molecular methods circumvents the complications mentioned above with respect to a phenotypical measurement of resistance gene expression but conflicts with prevalence of *vanB* among nonenterococcal, intestinal colonizers (Ballard et al., 2005, Domingo et al., 2005 and Graham et al., 2008). The *vanB* gene prevalence in nonenterococcal, intestinal colonizers did not show a geographical preference and appeared at similar rates in different parts of the world as deduced from a number of clinical studies using real-time polymerase chain reaction (PCR)–based screening assays

(Bourdon et al., 2010, Stamper et al., 2007, Usacheva et al., 2010, Werner et al., 2011b and Young et al., 2007).

The problem of varying resistance gene expressions in VanB strains has been a matter of concern for a long time with regard to the proper identification of relevant strains (Jett et al., 1996 and Tenover et al., 1995). The supposed low overall prevalence of VanB resistance among clinical enterococci worldwide led to a comparably low recognition of this diagnostic problem until now. If and to what extent low-level *vanB*-type vancomycin resistance expression is clinically relevant could only be estimated from the few studies available so far. The described low-level resistance phenotype is linked to a weak induction of the resistance gene expression via the 2-component regulatory system $VanR_B$–$VanS_B$. Inappropriate treatment by glycopeptides led to a selection of constitutively vancomycin- and teicoplanin-resistant *vanB* strains in vitro (Depardieu et al., 2003), in an experimental rat endocarditis model (Lefort et al., 2004) and under glycopeptide therapy in patients (Hayden et al., 1993, Kawalec et al., 2001 and San Millan et al., 2009), which, as a consequence, caused treatment failures.

The dramatic increase in *vanB* type prevalence among VRE in a number of European countries (Bourdon et al., 2011, Klare et al., 2010 and Soderblom et al., 2010) requires a reliable and quick diagnostics of this resistant genotype and a differentiation from susceptible variants and other *van* genotypes. We evaluated the performance of 3 chromogenic VRE agars (*Brilliance*™ VRE Agar; chromID™ VRE, CHROMagar™ VRE) and different MIC determination procedures (microbroth dilution, standard, and macromethod Etest®) in detecting 129 clinical *E. faecium* isolates possessing *vanB*.

Materials and methods

Strain collection

Our laboratory serves as a national focal laboratory for enterococci since the beginning of the 1990s. Diagnostic and university laboratories send strains to us for (vancomycin/teicoplanin) resistance confirmation and molecular typing. Our enterococcal strain collection currently contains > 10,000 isolates, mainly from hospital surveillance and infections, but also from animals, food products, the environment, and stool colonisations in outpatients (Klare et al., 1995a, Klare et al., 1995b, Klare et al., 1999, Klare et al., 2005, Werner et al., 2007, Werner et al., 2010 and Werner et al., 2011a). Until now, we collected and identified 1060 *vanB* strains including 982 *vanB*-positive *E. faecium* and 74 *vanB*-positive *E. faecalis* which were all from hospital patients. Of the former, 129 *vanB*-type *E. faecium* isolates were selected for this study. They originated from 41 different diagnostic laboratories in Germany (which serve several hospitals), were isolated between 2005 and 2011, and showed different strain characteristics and a vancomycin MIC range of ≤ 1 to > 256 mg/L (determined by an accredited “in-house” method using broth microdilution in cation-adjusted Mueller-Hinton; see below). *E. faecium* ATCC 19434 and 15 *E. faecium vanA/vanB*-negative strains were used as negative controls.

Antibiotic susceptibility testing

For all *E. faecium* isolates, antibiotic susceptibilities were determined as MICs using a broth microdilution method using cation-adjusted Mueller-Hinton broth according to an accredited in-house procedure following the German standards of antimicrobial susceptibility testing (DIN, 2004). Classification into susceptible/intermediate/resistant (S-I-R) differs between European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical Laboratory Standard Institutes (CLSI) (CLSI, 2011); EUCAST defines MICs for vancomycin with S ≤ 4 mg/L and R > 4 mg/L (no intermediate range) and CLSI defines S ≤ 4 mg/L, I = 8–16 mg/L, and R ≥ 32 mg/L. We used the EUCAST clinical breakpoints when available; for other antibiotics, we applied breakpoints derived from CLSI, DIN, and based on other criteria, e.g., for high-level ciprofloxacin resistance defined as an MIC > 16 mg/L (Leavis et al., 2006 and Werner et al., 2010). Strains were classified as resistant with MICs (in milligrams per liter) as follows: penicillin/ampicillin > 8, teicoplanin > 2, erythromycin > 4, linezolid > 4, tetracycline > 4, rifampicin > 0.5, chloramphenicol > 16, tigecycline > 0.5, daptomycin > 4, gentamicin (high-level) > 128, streptomycin (high-level) > 512, quinupristin/dalfopristin > 4. Vancomycin MICs were also assessed by another microdilution method using VITEK® 2 (bioMérieux, Nürtingen, Germany). Samples were handled in a Smart Carrier Station (bioMérieux) and treated as recommended by the manufacturer. The correct inoculum was set using DensiCHECK™ Plus

(bioMérieux). Results were assessed as MICs leaving a preadjustment (CLSI, EUCAST, etc.) aside. The Advanced Expert System (AES™; bioMérieux) subsequently evaluated the results on the basis of default EUCAST and CLSI ("CLSI global") standards. Etest® for vancomycin was performed according to the recommendation of the manufacturer (bioMérieux). In brief, 2 different protocols were followed: first, a standard method with cation-adjusted Mueller-Hinton agar and an inoculum equivalent to McFarland standard 0.5 and, second, the so-called macromethod using brain heart infusion agar and an inoculum equivalent to McFarland standard 2.0. The latter protocol is suggested by the Etest® manufacturer (bioMérieux) for a confirmation of a supposed vancomycin resistance phenotype. Values are read after incubation at 35 °C for 24 h and again after 48 h (Etest® application sheet for *Enterococcus*/VRE and vancomycin EAS009). *E. faecium* ATCC 19434 was used as a control strain. The performance of 3 chromogenic VRE screening agars was evaluated: Oxoid *Brilliance*™ VRE Agar (Thermo Scientific Fisher, Wesel, Germany), chromID™ VRE (bioMérieux), and CHROMagar™ VRE (Mast Diagnostika, Reinfeld, Germany). Strains were streaked out on selective plates and incubated as recommended by the manufacturers. Growth as single colonies and with equivalent colours was rated as a positive result.

DNA Isolation and PCR

Genomic DNA was prepared using a DNA extraction kit (DNeasy Tissue Kit, Qiagen, Hilden, Germany) according to the manufacturer's instructions. An initial cell wall lysis step was added dissolving the cell pellet in TES buffer (10 mmol/L Tris, 0.5 mmol/L ethylenediaminetetraacetic acid, 10% sucrose [pH 8.0]) plus 10 mg/mL lysozyme (Roche Applied Science, Mannheim, Germany) followed by incubation at 37 °C for 30 min. PCR was performed with a PCR master mix (Fermentas/Thermo Fisher Scientific, St. Leon-Rot, Germany) according to the manufacturer's instructions. Approximately 0.5 µL of isolated genomic DNA (ca. 10 ng) and primers (200 nmol/L each) was added. Amplification of fragments representing the *esp*, hyl_{Efm}, and *vanA/vanB* genes was performed in a multiplex PCR as described elsewhere (Werner et al., 2011a).

Statistics

Statistical analyses were performed with the software package Ridom EpiCompare 1.0 (Ridom; http://www.ridom.de).

Results

Determination of antibiotic susceptibilities

MICs for 15 antibiotics were determined by microbroth dilution in cation-adjusted MH broth as recommended. Distribution of MIC_{50}, MIC_{90}, and the overall number of resistant strains among 129 *vanB* VRE isolates is presented in Table 1. In addition, we assessed the MICs for vancomycin and teicoplanin by an alternative microdilution method in cation-adjusted MH broth as well (VITEK® 2). All isolates were resistant to ampicillin/penicillin and ciprofloxacin (high-level), suggesting the prevalence of *vanB* resistance genes among hospital-associated clonal types. All 129 *vanB* strains revealed susceptible MICs for teicoplanin of ≤ 0.5 mg/L (VITEK® 2) and ≤ 1 mg/L (in-house method).

Standard microdilution determined a wide spectrum of vancomycin MICs ranging from ≤ 1 to > 256 mg/L (Fig. 1A). According to CLSI, only 49% of strains were classified as "resistant" to vancomycin (R ≥ 32 mg/L), and based on EUCAST criteria 86% were classified as vancomycin resistant (R > 4 mg/L); the latter is equivalent to a classification by CLSI combining "intermediate" and "resistant". Resistances to antibiotics of last resort remained rare. Altogether, 17 *vanB* strains (13%) revealed MICs for vancomycin of ≤ 4 mg/L equivalent to a susceptible range defined by EUCAST and CLSI. Forty-eight (37%) exhibited MICs of 8 or 16 mg/L, which are intermediate according to CLSI. According to EUCAST definitions, altogether, 111 *vanB* strains could be classified as "resistant *vanB*-like" (86%; MICs > 4 mg/L). Analyses by VITEK® 2 revealed vancomycin MICs in the susceptible range for 16 strains (12 %) and additional 64 isolates (49%) showed an MIC of 8 mg/L (intermediate according to CLSI; Fig. 1B). However, VITEK 2 AES™ evaluation was independent of the corresponding standard set, also classifying strains with a vancomycin MIC of 8 mg/L (intermediate according to CLSI) correctly as "resistant* (*vanB*-like)". All 15 *vanA/vanB*-negative *E. faecium* and the ATCC control strain revealed MICs for vancomycin and teicoplanin of ≤ 1 mg/L (not shown).

The standard Etest® protocol using an inoculum of McFarland standard 0.5 and MH agar detected 21 (16%) after 24 h and 18 (14%) of *vanB* strains with MICs of ≤ 4 mg/L after 48 h of incubation, respectively (Fig. 1C). All 15 *vanA/vanB*-negative *E. faecium* and the ATCC *E. faecium* control strain revealed vancomycin Etest® MICs of ≤ 1.5 mg/L (24/48 h), and 13 of them had an identical MIC after 24 and 48 h. In contrast, *vanB* strains generally showed an elevated 48-h MIC versus the 24-h MIC (Fig. 1C). Etest® inhibition zones for *vanA/vanB*-negative *E. faecium* and the ATCC *E. faecium* control strain showed a sharp edge of growth inhibition, whereas most *vanB* strains expressed a specific phenotype with a shady growth at lower vancomycin concentrations (Fig. 2B and C), most probably related to the resistance phenotype induced after vancomycin exposure. This zone of weaker growth could span the range from susceptible to resistant MICs (Fig. 2B). Altogether, 2 *vanB E. faecium* strains showed microcolonies inside the inhibition zone ("heteroresistance phenotype"), 1 had an MIC of 32 mg/L and the other isolate was initially rated as susceptible with an MIC of 3 mg/L (Fig. 2D). According to the Etest® application recommendations, these strains should be classified as resistant with MIC > 256 mg/L. Microcolonies taken from inside the inhibition zone, grown overnight on MH agar plates, and tested again for Etest® vancomycin MICs revealed similar phenotypes as assessed before. The modified Etest® macromethod using McFarland 2.0 and BHI agar revealed altogether higher MICs for the 129 *vanB E. faecium* isolates (Fig. 1D), confirming the higher sensitivity of this alternative protocol. Altogether, 13 (10%; 24-h incubation) and 7 (5%; 48-h incubation) of the *vanB* strains showed MICs of ≤ 4 mg/L, respectively. With this protocol, all 15 *vanA/vanB*-negative *E. faecium* and the ATCC *E. faecium* control strain revealed vancomycin Etest® MICs of 3 mg/L after 24 h of incubation and in all but 1 cases an identical value after 48 h (1 isolate had an MIC of 4 mg/L). Six isolates showed microcolony growth inside the Etest inhibition zone, suggesting a heteroresistance phenotype; 5 of them already possessed MICs of > 4 mg/L which would have been classified as resistant anyway (not shown in detail).

Growth on screening agars

We assessed growth of the 129 *vanB* strains and the 15 *vanA/vanB*-negative strains on 3 chromogenic VRE agars: *Brilliance*™ VRE Agar, chromID™ VRE, and CHROMagar™ VRE. Performance was similar on all 3 media (Table 2). In general, ≥ 86% of all *vanB E. faecium* grew on the tested screening agars, 4–5% grew in small colonies only (also rated as positive), and 4–6% did not grow at all. Growth behaviour was not always congruent on all 3 media but isolates that did not grow mainly possessed susceptible MICs as determined by the in-house microdilution, VITEK® 2, and Etest® method. Of the 15 non-*vanA/vanB E. faecium* strains, 14 did not grow on all media and 1 strain grew on 2 agar plates (*Brilliance*™ VRE Agar and chromID™ VRE). The susceptible ATCC *E. faecium* control strain did not grow on all chromogenic media.

4. Discussion

The numbers of infections and colonizations with *vanB* type VRE increased in a number of European countries during recent years (Bourdon et al., 2011, Klare et al., 2010 and Soderblom et al., 2010). The supposed growing incidence of *vanB* type enterococci may have different reasons and could only be partly explained by an increased incidence of *vanB* type resistant strains. It cannot be excluded that the increased reporting of *vanB* type resistant enterococci may, at least in part, be attributed to the improved diagnostic capabilities of genotypic assays in targeting the corresponding resistance genes and to the introduction of sensitive chromogenic agar media in diagnosing corresponding *vanB* type enterococci. In addition, it is not known so far whether the reservoir of *vanB* resistance genes in nonenterococcal species may feed the resistance gene pool of *Enterococcus* spp. (Ballard et al., 2005). The problem of low-level vancomycin resistance expression in *vanB* type enterococci has been known for a long time but has not been extensively studied or addressed until now, most probably due to its supposed low prevalence and thus moderate clinical significance (Adler et al., 2010, Raponi et al., 2010 and Rathe et al., 2010). The low predictive value for *vanB* VRE according to the results of VRE screening studies performed in countries of different continents was mainly addressed to the wide prevalence of *vanB* genes in nonenterococcal, intestinal colonizers (Rathe et al., 2010, Stamper et al., 2007, Usacheva et al., 2010 and Young et al., 2007). It has to be considered that a substantial number of *vanB* enterococci may have been missed in previous studies comparing the performance of real-time PCR-based screening assays against phenotypic comparator assays with a supposed low sensitivity in diagnosing *vanB* VRE with low-level vancomycin resistance expression (Werner et al., 2011b).

The strains in our study may not represent a common and naïve *vanB* genotype *E. faecium* population and thus the investigated strain collection may be biased by the following reasons: all strains were from clinical samples and represented hospital-associated strains of *E. faecium* (MLST CC17) according to their ampicillin/penicillin and high-level ciprofloxacin resistance phenotypes. In addition, markers only prevalent among hospital strains such as *esp* and hyl_{Efm} were widely prevalent among the tested 129 isolates (86% and 64%, respectively; not shown in details). Additionally, strains from the present study were preselected in laboratories performing primary diagnostics and sent to us for confirmation and further analysis, a number of them was suspicious in respect of the unsecured or discrepant diagnostic result for VanB when comparing genotypic and phenotypic assay results.

The growth disadvantage of enterococci with an inducible *vanB* type resistance in MH broth has been described (Jett et al., 1996), and, correspondingly, several protocols including the Etest® macromethod suggest using rich media (BHI) for VRE confirmation. Thus far, results of our study were in line with results of previous reports; using rich media (BHI instead of MH) and a higher inoculum (McFarland 2.0 instead of 0.5) was also superior here (more sensitive) in identifying *vanB* type *E. faecium* strains with a low-level vancomycin resistance expression (Fig. 1C and D). As a negative control, all 15 *vanA/vanB*-negative *E. faecium* and the ATCC *E. faecium* control strain revealed susceptible vancomycin Etest® MICs of ≤ 4 mg/L (24/48 h). However, the macromethod protocol revealed comparably high values of 3 (4) mg/L near the susceptible breakpoint of ≤ 4 mg/L. It has to be emphasized that, due to unknown reasons, MICs for *E. faecalis* isolates appear 1–2 twofold dilutions higher than those for *E. faecium* (not shown) and, consequently, these assumptions are only suitable for *vanB* type *E. faecium*.

When comparing vancomycin Etest results on MH agar using the standard protocol to the MIC results of VITEK® 2 (Fig. 1B) and of the in-house DIN protocol (Fig. 1A), it became obvious that resistance expression is not different on agar and in broth (Table 2). The performance of chromogenic VRE agars was superior to the standard Etest protocol results (24 h) and the MIC assays in liquid broth, but similar to the results of the Etest® macromethod (Table 2). Nevertheless, these assays have different applications (screening versus MIC determination) and a head-to-head comparison is, maybe, less reasonable.

We describe here for the first time a heteroresistance phenotype for vancomycin for a few VanB isolates. The heteroresistance phenotype has only been described for a clinical *vanA* VRE and for in vitro–generated descendents of *vanA*-positive *E. faecium* strain ATCC 51559 (Alam et al., 2001 and Khan et al., 2008). Also, teicoplanin heteroresistance in *vanA* VRE strains was described recently (Qu et al., 2009). Several genomic rearrangements and deletions within the *vanA* gene cluster elements and mutational changes within the 2-component regulator genes *vanS* and *vanR* were identified in glycopeptide-heteroresistant strains, but whether these changes were functionally linked to the described heteroresistance phenotype has not been experimentally proven so far. In our hands, the high-level resistant colonies grown inside the inhibition zone were not stable after passage and showed the same phenomenon again.

In conclusion, altogether, 112 *vanB*-type isolates (87%) were correctly identified as VanB type VRE by both microbroth dilution methods and when using interpretation criteria set as recommended by the manufacturer. The Etest® macromethod protocol using a higher inoculum of McFarland standard 2.0 and 24/48 h of incubation on BHI agar was superior to the standard protocol with McFarland standard 0.5 and MH agar in identifying VanB *E. faecium* strains while keeping sufficient specificity in correctly diagnosing 15 *vanA/vanB*-negative test and reference strains. A few *vanB* isolates showed growth of microcolonies inside the vancomycin inhibition zone, suggesting a VanB heteroresistance phenotype only detectable by Etest® vancomycin. Three commercial chromogenic VRE agars performed similarly with 121 (94%), 123 (95%), and 124 (96%) *vanB* isolates that grew on *Brilliance*™ VRE Agar, CHROMagar™ VRE, and chromID™ VRE agar, respectively.

Acknowledgments

The authors thank Dr. Knauf and Mrs. Flagmeier (bioMérieux, Nuertingen, Germany), Dr. Hensiek (Mast Diagnostika, Reinfeld, Germany), and Drs. Scholz and Scopes (Thermo Scientific Fisher, Wesel, Germany) for application advices concerning their products. None of the company representatives exerted influence on the study design, results interpretation, and conclusive evaluations.

References

Adler H, Oezcan S, Frei R. Vancomycin-resistant enterococci of vanB genotype may pose problems for screening with highly selective media. J Clin Microbiol 2010;48:2323.
Alam MR, Donabedian S, Brown W, Gordon J, Chow JW, Zervos MJ, et al. Heteroresistance to vancomycin in Enterococcus faecium. J Clin Microbiol 2001;39:3379–81.
Ballard SA, Pertile KK, Lim M, Johnson PD, Grayson ML. Molecular characterization of vanB elements in naturally occurring gut anaerobes. Antimicrob Agents Chemother 2005;49:1688–94.
Bourdon N, Berenger R, Lepoultier R, Mouet A, Lesteven C, Borgey F, et al. Rapid detection of vancomycin-resistant enterococci from rectal swabs by the Cepheid Xpert vanA/vanB assay. Diagn Microbiol Infect Dis 2010;67:291–3.
Bourdon N, Fines-Guyon M, Thiolet JM, Maugat S, Coignard B, Leclercq R, et al. Changing trends in vancomycin-resistant enterococci in French hospitals, 2001–2008. J Antimicrob Chemother 2011;66:713–21.
Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing; Table Enterococcus spp. M02 and M07 (M100-S21). CLSI; 2011.
Courvalin P. Vancomycin resistance in Gram-positive cocci. Clin Infect Dis 2006;42(Suppl. 1):S25–34.
Depardieu F, Courvalin P, Msadek T. A six amino acid deletion, partially overlapping the VanSB G2 ATP-binding motif, leads to constitutive glycopeptide resistance in VanBtype Enterococcus faecium. Mol Microbiol 2003;50:1069–83.
Depardieu F, Podglajen I, Leclercq R, Collatz E, Courvalin P. Modes and modulations of antibiotic resistance gene expression. Clin Microbiol Rev 2007;20:79-114.
DIN, Deutsches Institut für Normung. DIN 58940, Medical Microbiology – Susceptibility testing of pathogens to antimicrobial agents. Part 8: microdilution. General method specific requirements. In: Deutsches Institut für Normung e.V., editor. DIN Taschenbuch 222: Medizinische Mikrobiologie und Immunologie – Diagnostische Verfahren. Berlin: Beuth-Verlag; 2004. p. 342–55.
Domingo MC, Huletsky A, Giroux R, Boissinot K, Picard FJ, Lebel P, et al. High prevalence of glycopeptide resistance genes vanB, vanD, and vanG not associated with enterococci in human fecal flora. Antimicrob Agents Chemother 2005;49:4784–6.
Graham M, Ballard SA, Grabsch EA, Johnson PDR, Grayson ML. High rates of fecal carriage of nonenterococcal vanB in both children and adults. Antimicrob Agents Chemother 2008;52:1195–7.
Gu L, Cao B, Liu Y, Guo P, Song S, Li R, et al. A new Tn1546 type of VanB phenotype-vanA genotype vancomycin-resistant Enterococcus faecium isolates in mainland China. Diagn Microbiol Infect Dis 2009;63:70–5.
Hayden MK, Trenholme GM, Schultz JE, Sahm DF. In vivo development of teicoplanin resistance in a VanB Enterococcus faecium isolate. J Infect Dis 1993;167:1224–7.
Henrique PM, Palazzo IC, Zanella RC, Darini AL. Molecular characterization of enterococci harboring genotype and phenotype incongruence related to glycopeptide resistance isolated in Brazilian hospitals. Mem Inst Oswaldo Cruz 2008;103:301–5.
Jett B, Free L, Sahm DF. Factors influencing the Vitek Gram-positive susceptibility system's detection of vanB-encoded vancomycin resistance among enterococci. J Clin Microbiol 1996;34:701–6.
Kawalec M, Gniadkowski M, Kedzierska J, Skotnicki A, Fiett J, Hryniewicz W. Selection of a teicoplanin-resistant Enterococcus faecium mutant during an outbreak caused by vancomycin-resistant enterococci with the vanB phenotype. J Clin Microbiol 2001;39:4274–82.
Khan SA, Sung K, Layton S, Nawaz MS. Heteroresistance to vancomycin and novel point mutations in Tn1546 of Enterococcus faecium ATCC 51559. Int J Antimicrob Agents 2008;31:27–36.
Klare I, Heier H, Claus H, Böhme G, Marin S, Seltmann G, et al. Enterococcus faecium strains with vanA-mediated high-level glycopeptide resistance isolated from animal foodstuffs and fecal samples of humans in the community. Microb Drug Resist 1995a;1:265–72.
Klare I, Heier H, Claus H, Reissbrodt R, Witte W. vanA-mediated high-level glycopeptide resistance in Enterococcus faecium from animal husbandry. FEMS Microbiol Lett 1995b;125:165–71.
Klare I, Badstubner D, Konstabel C, Böhme G, Claus H, Witte W. Decreased incidence of VanA-type vancomycin-resistant enterococci isolated from poultry meat and from fecal samples of humans in the

community after discontinuation of avoparcin usage in animal husbandry. Microb Drug Resist 1999;5:45–52.
Klare I, Konstabel C, Mueller-Bertling S, Werner G, Strommenger B, Kettlitz C, et al. Spread of ampicillin/vancomycin-resistant Enterococcus faecium of the epidemicvirulent clonal complex-17 carrying the genes esp and hyl in German hospitals. Eur J Clin Microbiol Inf Dis 2005;24:815–25.
Klare I, Werner G, Witte W. Enterococci with vancomycin resistance from German hospitals in 2008/2009 [German]. Epidemiol Bull 2010;44:427–37.
Leavis HL, Willems RJL, Top J, Bonten MJM. High-Level ciprofloxacin resistance from point mutations in gyrA and parC confined to global hospital-adapted clonal lineage CC17 of Enterococcus faecium. J Clin Microbiol 2006;44:1059–64.
Lefort A, Arthur M, Depardieu F, Chau F, Pouzet C, Courvalin P, Fantin B. Expression of glycopeptide-resistance gene in response to vancomycin and teicoplanin in the cardiac vegetations of rabbits infected with VanB-type Enterococcus faecalis. J Infect Dis 2004;189:90–7.
Park IJ, Lee WG, Shin JH, Lee KW, Woo GJ. VanB phenotype-vanA genotype Enterococcus faecium with heterogeneous expression of teicoplanin resistance. J Clin Microbiol 2008;46:3091–3.
Qu TT, Zhang JL, Zhou ZH, Wei ZQ, Yu YS, Chen YG, et al. Heteroresistance to teicoplanin in Enterococcus faecium harboring the vanA gene. J Clin Microbiol 2009;47:4194–6.
Raponi G, Ghezzi MC, Gherardi G, Lorino G, Dicuonzo G. Analysis of methods commonly used for glycopeptide and oxazolidinone susceptibility testing in Enterococcus faecium isolates. J Med Microbiol 2010;59:672–8.
Rathe M, Kristensen L, Ellermann-Eriksen S, Thomsen MK, Schumacher H. Vancomycinresistant Enterococcus spp.: validation of susceptibility testing and in vitro activity of vancomycin, linezolid, tigecycline and daptomycin. APMIS 2010;118:66–73.
San Millan A, Depardieu F, Godreuil S, Courvalin P. VanB-type Enterococcus faecium clinical isolate successively inducibly resistant to, dependent on, and constitutively resistant to vancomycin. Antimicrob Agents Chemother 2009;53:1974–82.
Soderblom T, Aspevall O, Erntell M, Hedin G, Heimer D, Hokeberg I, et al. Alarming spread of vancomycin resistant enterococci in Sweden since 2007. Euro Surveill 2010;15:19620.
Song JH, Ko KS, Oh WS, Park S, Heo ST, Kwon KT, et al. High frequency of vancomycinresistant Enterococcus faecium isolates with VanB phenotype and vanA genotype in Korean hospitals. Diagn Microbiol Inf Dis 2006;56:401–6.
Stamper PD, Cai M, Lema C, Eskey K, Carroll KC. Comparison of the BD GeneOhm VanR Assay to culture for identification of vancomycin-resistant enterococci in rectal and stool specimens. J Clin Microbiol 2007;45:3360–5.
Tenover FC, Swenson JM, O'Hara CM, Stocker SA. Ability of commercial and reference antimicrobial susceptibility testing methods to detect vancomycin resistance in enterococci. J Clin Microbiol 1995;33:1524–7.
Usacheva EA, Ginocchio CC, Morgan M, Maglanoc G, Mehta MS, Tremblay S, et al. Prospective, multicenter evaluation of the BD GeneOhm VanR assay for direct, rapid detection of vancomycin-resistant Enterococcus species in perianal and rectal specimens. Am J Clin Pathol 2010;134:219–26.
Werner G, Klare I, Fleige C, Witte W. Increasing rates of vancomycin resistance among Enterococcus faecium isolated from German hospitals between 2004 and 2006 are due to wide clonal dissemination of vancomycin-resistant enterococci and horizontal spread of vanA clusters. Int J Med Microbiol 2007;298:515–27.
Werner G, Strommenger B, Witte W. Acquired vancomycin resistance in clinically relevant pathogens. Future Microbiol 2008;3:547–62.
Werner G, Fleige C, Ewert B, Laverde-Gomez JA, Klare I, Witte W. High-level ciprofloxacin resistance among hospital-adapted Enterococcus faecium (CC17). Int J Antimicrob Agents 2010;35:119–25.
Werner G, Fleige C, Geringer U, van Schaik W, Klare I, Witte W. IS element IS16 as a molecular screening tool to identify hospital-associated strains of Enterococcus faecium. BMC Infect Dis 2011a;11:e80.
Werner G, Serr A, Schütt S, Schneider C, Klare I, Witte W, et al. Comparison of direct cultivation on a selective solid medium, polymerase chain reaction from an enrichment broth, and the BD GeneOhm™ VanR Assay for identification of vancomycin-resistant enterococci in screening specimens. Diagn Microbiol Inf Dis 2011b;70:512–21.
Werner G. Surveillance of antimicrobial resistance among Enterococcus faecium and Enterococcus faecalis isolated from human (clinical/commensal), food animal, meat and environmental samples. In: Semedo-Lemsaddek T, Barreto-Crespo MT, Tenreiro R (Eds), Enterococcus and safety. 2012. Nova Science Publishers Inc., Hauppage, NY.
Young HL, Ballard SA, Roffey P, Grayson ML. Direct detection of vanB2 using the Roche LightCycler vanA/B detection assay to indicate vancomycin-resistant enterococcal carriage — sensitive but not specific. J Antimicrob Chemother 2007;59:809–10.

Tables and Figures

Table 1. Antibiotic susceptibilities of 129 *vanB* type *Enterococcus faecium*.

	MIC_{60}	MIC_{90}	% Resistant
Vancomycin	16	64	86 [a]/49 [b]
Teicoplanin	≤ 1	≤ 1	0
Ampicillin	> 32	> 32	100
Penicillin	> 32	> 32	100
Ciprofloxacin[c]	> 128	> 128	100 (HL)
Gentamicin	8	> 2048	41 (HL)
Streptomycin	1024	> 2048	58 (HL)
Erythromycin	> 8	> 8	97
Tetracyclin[c]	≤ 0.5	> 16	14
Rifampicin	> 4	> 4	92
Daptomycin[c]	2	4	0
Quinupristin/dalfopristin[c]	2	8	11
Linezolid[d]	1	2	1
Tigecycline[c]	≤ 0.06	0.13	5
Chloramphenicol	≤ 4	8	0

a R according to EUCAST (> 4 mg/L; identical to I+R according to CLSI).
b R according to CLSI (≥ 32 mg/L).
c Only 110 VRE isolates were tested.
d Only 105 VRE isolates were tested.

Table 2. Results of MIC determinations and growth on chromogenic selective VRE agars of 129 *vanB* type *E. faecium*.

	% MIC <=4 mg/L	% MIC>=4 mg/L	% Grown	% Grown (small CFU)	% Not grown
MBD MIC	14.0	86.0			
VITEK® 2 MIC	12.4	87.6			
Etest MH 24 h	16.3	83.7			
Etest MH 48 h	14.0	86.0			
Etest BHI 24 h	10.1	89.9			
Etest BHI 48 h	5.4	94.6			
Brilliance™ VRE Agar			88.4	5.4	6.2
chromID™ VRE			87.6	8.5	3.9
CHROMagar™ VRE			86.0	9.3	4.7

MBD = In-house microdilution method in MH broth; MH = cation-adjusted Mueller-Hinton; BHI = brain heart infusion; CFU = colony-forming unit. Strains were classified as VanB phenotype when they were resistant to vancomycin according to EUCAST (R > 4 mg/L; identical to I+R according to CLSI. All 129 strains were susceptible to teicoplanin (MIC ≤ 1 mg/L).

Figure 1. Distribution of MICs for vancomycin determined by different test methods at 35–37 °C. (A) Results of in-house DIN microdilution in cation-adjusted Mueller-Hinton broth; (B) results of VITEK® 2 (cation-adjusted Mueller-Hinton broth; bioMérieux); (C) Coventional Etest® Vancomycin on cation-adjusted Mueller-Hinton agar after 24 and 48 h of incubation; and (D) Etest® Vancomycin macromethod on brain heart infusion agar after 24 and 48 h of incubation.

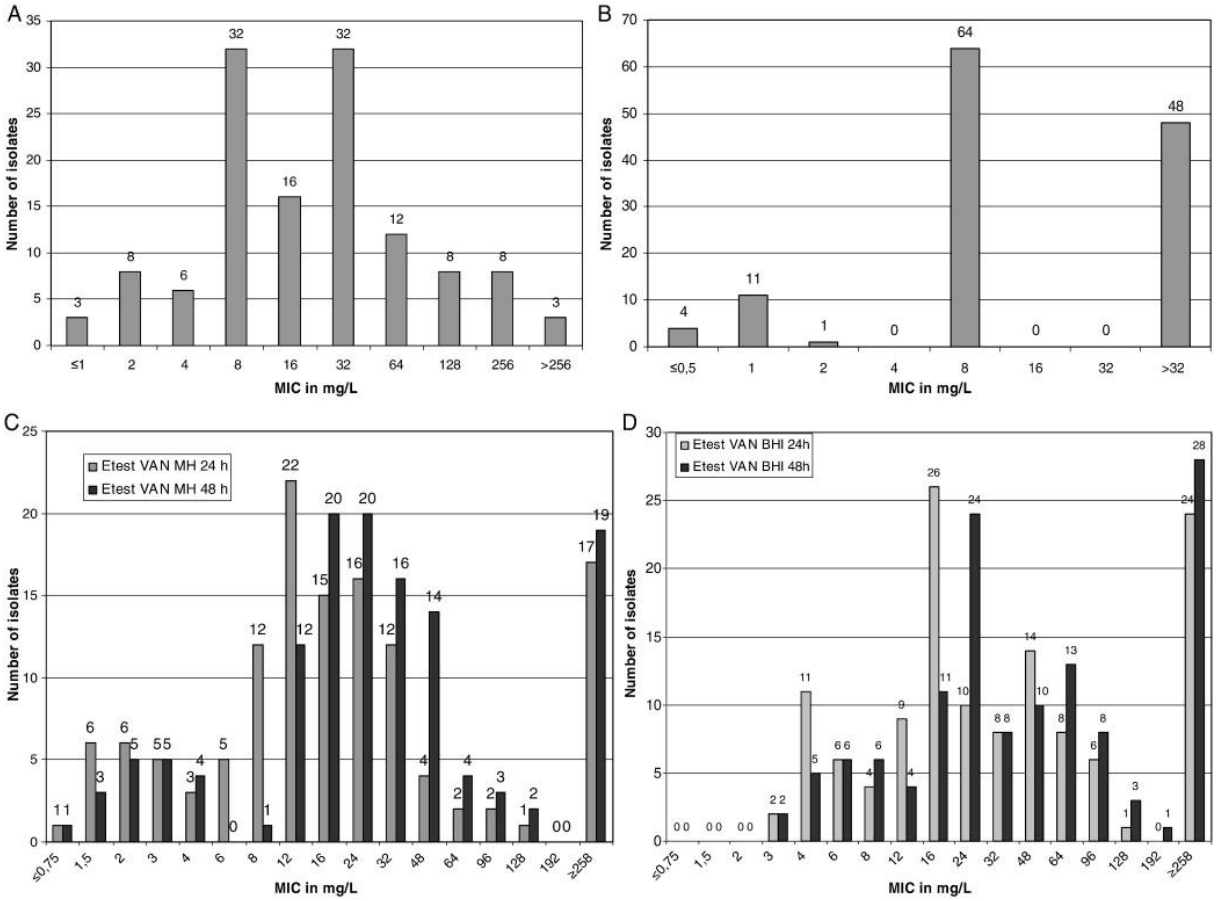

Figure 2. Etest® Vancomycin phenotypes of *Entercoccus faecium* isolates on Mueller-Hinton agar. (A) Sharp inhibition zone of *vanA/vanB*-negative, vancomycin-susceptible strains; (B/C) atypical inhibition zone with shady growth showing resistant (B) and susceptible MICs (C), both *vanB*-type *E. faecium*; (D) growth of microcolonies inside the inhibition zone in some *vanB*-type *E. faecium* (heteroresistance phenotype).