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ORIGINAL ARTICLE

# Pitfalls in the identification of *Enterococcus* species and the detection of *vanA* and *vanB* genes

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**Significance and Impact of the Study:** The study shows that even though the performance of Vitek 2 Advanced Expert System was good in identifying enterococci to species level, it is important to verify results by a molecular method when phenotypic findings are discordant with epidemiologic patterns. Furthermore, GeneXpert® *vanA/vanB* PCR and ChromID VRE combined with in-house PCR were applied in rectal samples for the detection of VRE colonization among critically ill patients. GeneXpert® showed an excellent performance in detecting *vanA*-positive enterococci, but false-positive results for *vanB*-gene detection render its application problematic in departments with high incidence of *vanB*-positive enterococci.

**Keywords**
enterococci, GeneXpert®, identification, vancomycin-resistant enterococci, Vitek 2.

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## Abstract

The aims were to assess the performance of Vitek 2 in identifying enterococcal species and the implementation of GeneXpert® *vanA/vanB* PCR for the detection of vancomycin-resistant enterococci (VRE). Gram-positive cocci from clinical and environmental specimens ($n = 431$) suspicious of being enterococci by conventional methods were evaluated by Vitek 2. This system identified 296 *Enterococcus faecium*, 87 *Enterococcus faecalis*, 10 *Enterococcus villorum*, 9 *Enterococcus gallinarum*, 9 *Enterococcus durans*, 5 *Enterococcus casseliflavus*, 1 *Enterococcus* spp. and 14 isolates as Non-*Enterococcus*. All strains were submitted to pulsed field gel electrophoresis (PFGE) analysis showing 64 banding patterns. Representative strains from each banding pattern were further characterized to species level by 16S rDNA sequencing. The misidentification rate by Vitek 2 to species level among 429 molecularly identified enterococci was 6% (26 isolates). Additionally, 372 rectal swabs were obtained from critically ill patients. They were evaluated for the presence of VRE by ChromID VRE combined with in-house PCR *vs* GeneXpert®. GeneXpert® showed high (>92%) sensitivity, specificity, accuracy for *vanA*-positive *Enterococcus* detection, as well as, sensitivity and specificity for *vanB*-positive strains. Positive predictive value for detection of *vanB*-positive enterococci by GeneXpert® *vanA/vanB* was low (30%). GeneXpert® showed the same efficacy as ChromID VRE in detecting *vanA*-positive enterococci, but lower for *vanB*-gene detection.

## Introduction

Enterococci remain important causes of infections especially in the hospital environment (Werner *et al.* 2008; Van Tyne and Gilmore 2014). The most commonly isolated enterococcal species responsible for the majority of health care-associated infections are *Enterococcus faecalis* and *Enterococcus faecium*, while *Enterococcus durans*, *Enterococcus casseliflavus* and *Enterococcus gallinarum* cause only sporadic cases (Van Tyne and Gilmore 2014). Enterococcal infections are difficult to treat since these bacteria are intrinsically resistant to multiple antimicrobial agents (Van Tyne and Gilmore 2014). Moreover, strains isolated from hospitalized patients are commonly resistant to beta-lactams, aminoglycosides and vancomycin (Bell *et al.* 1998; Papadimitriou-Olivgeris *et al.* 2014a; Van Tyne and Gilmore 2014; Vazquez-Guillamet and Kollef 2014). One of the most commonly used automated systems in clinical microbiology laboratories is the Vitek 2 Advanced Expert System which provides accurate and rapid identification of bacteria (Fang *et al.* 2012). However, certain reports state misidentification of enterococcal species using this apparatus, a fact that may lead to serious clinical misapprehension since each species has different resistance patterns (Singer *et al.* 1996; Garcia-Garrote *et al.* 2000; Ligozzi *et al.* 2002).

In the majority of cases, vancomycin resistance is related to the presence of *vanA* and *vanB* genes which render different levels of resistance to glycopeptides (Werner *et al.* 2008; Papadimitriou-Olivgeris *et al.* 2014a). Nowadays, vancomycin-resistant enterococci (VRE) are endemic globally (ECDC 2012). VRE percentage among European countries shows important variability ranging from 2 to 34·9%; among them Greece reports a rate of 23·1%, while in USA hospitals 33% of enterococci are resistant to vancomycin (Hidron *et al.* 2008; ECDC 2012). In order to implement an effective infection control programme, rapid and accurate VRE identification of infected and colonized patients is imperative (Tacconelli *et al.* 2004; Humphreys 2014). Moreover, accurate species identification is essential for the interpretation of antibiotic resistance patterns (Singer *et al.* 1996; Garcia-Garrote *et al.* 2000). For these reasons, either of two approaches is usually applied in diagnostic laboratories: chromogenic agar or Real-Time PCR (RT-PCR) directly in the clinical specimen.

In this study, the performance of Vitek 2 Advanced Expert System towards evaluation of enterococci identification to species level was assessed. Furthermore, GeneXpert® *vanA/vanB* PCR and ChromID VRE combined with in-house PCR were applied for the detection of VRE.

## Results and discussion

### Group I (evaluation of Vitek 2 system by molecular methods)

In order to evaluate the accuracy of Vitek 2 Advanced Expert System in the identification of enterococci, Vitek 2 was compared with 16S rDNA sequencing combined with the respective pulsed field gel electrophoresis (PFGE) banding patterns (applied for clonal identification). Among a total of 431 isolates tested, Vitek 2 Advanced Expert System identified 417 (97%) as enterococci (296 *Ent. faecium*, 87 *Ent. faecalis*, 10 *Enterococcus villorum*, 9 *Ent. gallinarum*, 9 *Ent. durans*, 5 *Ent. casseliflavus* and 1 *Enterococcus* spp.), whereas 14 isolates were either unidentified (six strains) or belonged to other genuses. PFGE analysis identified 52 main clonal types and 12 subtypes resulting to a total of 64 DNA banding patterns. The 16S rDNA sequencing of 64 strains classified 20 clonal types as *Ent. faecium* (299 strains), 18 as *Ent. faecalis* (95), 7 as *Ent. villorum* (11), 3 as *Ent. gallinarum* (14), 1 as *Ent. casseliflavus* (6), 1 as *Ent. durans* (4), 1 as *Aerococcus* spp. (1) and 1 as *Bacillus* spp. (1 strain). 16S rDNA sequencing combined with the corresponding PFGE pattern identified 429 (99·5%) enterococci, as presented in Table 1. Only two strains were Non-*Enterococcus* by 16S rDNA sequencing analysis. The clinical importance of the correct knowledge of species is underlined by the fact that each one has different epidemiologic and antibiotic resistance characteristics which have consequences in the treatment and outcome of enterococcal infections. More specifically, *Ent. faecalis*, which is the most commonly encountered species in community-acquired infections, is usually susceptible to ampicillin and glycopeptides, whereas *Ent. faecium*, which is more often recovered from hospitalized patients, is usually ampicillin-resistant and may carry *vanA* or *vanB* genes to confer glycopeptides-resistance (Werner *et al.* 2008).

Vitek's 2 misidentification rate to species level as revealed by molecular methodology was 6% (26 isolates). More specifically, among 95 *Ent. faecalis,* six were falsely identified as *Ent. faecium,* one as *Ent. durans* and two as Non-*Enterococcus* by Vitek 2 Advanced Expert; among 299 *Ent. faecium* four were falsely characterized as *Ent. durans*, one as *Enterococcus* spp. and four as Non-*Enterococcus*. They were followed by *Ent. gallinarum* (six were ultimately found to be Non-*Enterococcus*), *Ent. villorum* (one was *Ent. faecalis)* and *Ent. casseliflavus* (one was characterized as *Ent. gallinarum*). Moreover, five isolates were falsely identified as *Ent. durans* by Vitek 2 system (Table 1).

These results highlight the importance of verifying the phenotypic identification by a molecular method, when

**Table 1** Identification of enterococci by phenotypic (Vitek 2 Advanced Expert System) and molecular methods (16S rDNA sequencing and PFGE banding patterns)

| | Molecular methods | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Enterococcus faecium* (299) | *Enterococcus faecalis* (95) | *Enterococcus villorum* (11) | *Enterococcus gallinarum* (14) | *Enterococcus casseliflavus* (6) | *Enterococcus durans* (4) | Other† (2) |
| Phenotypic | | | | | | | |
| *Ent. faecium* (296) | 290 | 6 | 0 | 0 | 0 | 0 | 0 |
| *Ent. faecalis* (87) | 0 | 86 | 1 | 0 | 0 | 0 | 0 |
| *Ent. villorum* (10) | 0 | 0 | 10 | 0 | 0 | 0 | 0 |
| *Ent. gallinarum* (9) | 0 | 0 | 0 | 8 | 1 | 0 | 0 |
| *Ent. casseliflavus* (5) | 0 | 0 | 0 | 0 | 5 | 0 | 0 |
| *Ent. durans* (9) | 4 | 1 | 0 | 0 | 0 | 4 | 0 |
| *Enterococcus* spp. (1) | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Non-*Enterococcus** (14) | 4 | 2 | 0 | 6 | 0 | 0 | 2 |

PFGE, pulsed field gel electrophoresis.

*Unidentified (6), *Alloiococcus otitis* (3), *Leuconostoc mesenteroides* (2), *Staphylococcus chromogenes* (1), *Vagococcus fluvialis* (1), *Gemella* spp. (1).

†*Bacillus* spp. (1), *Aerococcus* spp. (1).

phenotypic laboratory findings are discordant with expected clinical or epidemiologic patterns. In this study, phenotypic identification did not correspond to the resulted PFGE banding patterns for some strains. Even though MALDI-TOF Mass Spectrometry has shown better performance than Vitek 2 for bacterial identification and is recommended, if available, 16S rDNA sequencing remains the method of reference (Guo *et al.* 2014). It has also been reported that closely related species such as *Ent. casseliflavus* and *Ent. gallinarum*, may have identical 16S rDNA sequences and that sequencing of *sodA* gene encoding the manganese-dependent superoxide dismutase has been proved to be accurate for molecular identification of enterococci (Poyart *et al.* 2000). In this collection we have used the 16S rDNA sequencing approach since 14 isolates were phenotypically characterized as 'non-*Enterococcus*' and *sodA* typing perhaps could not identify them. Moreover, the obtained sequencing results were of 99–100% homology with the reference strains.

Misidentification rate of enterococci was lower in this study (6%), as compared to previous reports (8–17%), probably due to technological progress in the identification panels (Garcia-Garrote *et al.* 2000; Ligozzi *et al.* 2002; Fang *et al.* 2012). A lower rate of correct identification was observed with *Ent. faecalis* (91%) as compared to *Ent. faecium* (97%), a finding contradicting previous studies showing a reduced accuracy in *Ent. faecium* identification (Garcia-Garrote *et al.* 2000; Ligozzi *et al.* 2002). A possible explanation may be the fact that *Ent. faecalis* in our study originated mainly from environmental samples, while Vitek 2 Advanced Expert System was validated using clinical enterococcal isolates. Singer *et al.* (1996) reported an outbreak of VRE in a hospital in Indianapolis in which 31 of 84 isolates (37%) were identified by the Vitek Gram-positive identification (GP ID, bioMerieux, Marcy l'Etoile, France) software as being *Ent. durans* which usually causes sporadic infections in humans. Analysis of these isolates by the Centers for Disease Control and Prevention with PFGE and 16S rDNA sequencing showed that they were actually *Ent. faecium* (Singer *et al.* 1996). Among our nine isolates that were identified as *Ent. durans* by Vitek 2 Advanced Expert System, four were *Ent. faecium* and one was *Ent. faecalis* by molecular methods, a finding indicating that Vitek 2 still encounters limitations in identifying *Ent. faecium* and *Ent. faecalis* correctly to the species level.

### Group II (evaluation of GeneXpert® *vanA*/*vanB* assay)

The performance of GeneXpert® *vanA*/*vanB* assay as compared to ChromID VRE agar with *vanA*/*vanB* PCR for the presence of VRE from 372 rectal samples is depicted in Table 2. ChromID VRE was shown in previous studies to have high accuracy VRE identification from rectal samples (Ledeboer *et al.* 2007; Klare *et al.* 2012). GeneXpert® *vanA*/*vanB* PCR identified 40 *vanA*-positive and 37 *vanB*-positive rectal swabs. ChromID VRE agar combined with *vanA*/*vanB* in-house PCR identified 38 *Ent. faecium* (31 *vanA* and 7 *vanB*-positive) and 12 *Ent. faecalis* (eight *vanA* and four *vanB*-positive). The vancomycin $MIC_{50}$ of *vanA*-positive and *vanB*-positive *Enterococcus* were 256 mg $l^{-1}$ (range: 128–256 mg $l^{-1}$) and 4 mg $l^{-1}$ (range: 1·5–16 mg $l^{-1}$) respectively. The teicoplanin $MIC_{50}$ of *vanA*-positive was 24 mg $l^{-1}$ (range: 12–64 mg $l^{-1}$) and of *vanB*-positive strains 0·5 mg $l^{-1}$ (range: 0·125–1 mg $l^{-1}$).

**Table 2** Performance of GeneXpert® *vanA*/*vanB* PCR as compared to ChromID VRE combined with *vanA*/*vanB* PCR

| GeneXpert® PCR result Parameters | Number of results | | | | Diagnostic test evaluation | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | TP | FP | TN | FN | Sensitivity | Specificity | PPV | NPV | Accuracy |
| *vanA* | 39 | 1 | 332 | 0 | 100% | 99·7% | 99·7% | 100% | 0·987 |
| *vanB* | 11 | 26 | 335 | 0 | 100% | 92·8% | 29·7% | 100% | 0·930 |

TP, true positive; FP, false positive; TN, true negative; FN, false negative; PPV, positive predictive value; NPV, negative predictive value.

To the best of our knowledge, this is the first study in Greece evaluating the accuracy of GeneXpert® *vanA*/*vanB* assay for the direct detection of VRE from rectal swabs among patients hospitalized in ICUs where VRE are endemic (Papadimitriou-Olivgeris *et al.* 2014a). All parameters for the detection of *vanA*-positive *Enterococcus* were high (>92%), as well as, the sensitivity, specificity, negative predictive value (NPV) and accuracy for the detection of *vanB*-positive *Enterococcus* (Table 2). On the contrary, positive predictive value (PPV) for the detection of *vanB*-positive strains was low (30%). The high performance for the detection of *vanA* by the GeneXpert® is in accordance with the literature (Bourdon *et al.* 2010; Babady *et al.* 2012). When the identification of *vanB* was evaluated, the PPV was low (29·7%) since only 11 samples (30%) out of 37 *vanB*-positive ones detected by the GeneXpert® were also identified as VRE by ChromID. The results concerning the detection of *vanB* are similar to those of Bourdon *et al.* (2010) that reported a PPV of 2·6%. The high false-positive results for *vanB* presence in rectal samples have been justified by the fact that this gene has been also identified in a variety of intestinal bacteria, other than enterococci (Bourdon *et al.* 2010; Marner *et al.* 2011).

Another possible explanation for the high false-positives for *vanB* gene detection by the GeneXpert® is the fact that ChromID VRE contains vancomycin to a concentration of 8 mg $l^{-1}$, higher than the susceptibility cut-off of CLSI (4 mg $l^{-1}$) (CLSI 2015), meaning that isolates carrying *vanB* with MIC <4 mg $l^{-1}$ might have been inhibited and did not grow in ChromID VRE. In order to overcome this obstacle, we included an enrichment step of 24 h incubation into Brain-Heart-Infusion Broth before inoculation onto ChromID VRE. This step increased the inoculum for *vanB*-enterococci and resulted in the isolation of seven *vanB*-positive enterococci with vancomycin MIC of 1·5–6 mg $l^{-1}$, lower than that of the agar concentration. As reported by Delmas *et al.* (2007), the inclusion of an enrichment step significantly increases the sensitivity of ChromID VRE, however, it also increases the possibility of nonenterococcal colony growth, most commonly Gram-negative bacilli or yeasts. Since Greek ICU patients are enterically colonized with carbapenemase-producing bacteria and *Candida* spp. (Papadimitriou-Olivgeris *et al.* 2014b), during the enrichment step we used colistin and caspofungin into the Brain-Heart-Infusion Broth in order to inhibit growth of aforementioned micro-organisms.

The use of either methodology for VRE surveillance depends on many factors. In ICUs where *vanB*-positive enterococci predominate, it may be prudent to use the ChromID VRE with an enrichment step, since GeneXpert® gives many false-positive results regarding *vanB* detection, a fact that may lead to bed isolation of not actually colonized patients (Delmas *et al.* 2007). In cases of outbreaks where the dissemination of VRE is rapid, GeneXpert® has the advantage of detecting colonized patients in <2 h, as compared to 48–72 h of ChromID VRE (Delmas *et al.* 2007; Marner *et al.* 2011). On the contrary, in nonoutbreak periods ChromID VRE may be used, since mean duration of ICU hospitalization for a patient to be colonized by VRE surpasses 10 days (Papadimitriou-Olivgeris *et al.* 2014a). Calculated cost for ChromID VRE combined with *vanA* and *vanB* in-house PCR was 25 Euros per sample, whereas the respective one for GeneXpert® *vanA*/*vanB* was approx. 100 Euros. Thus, the cost of GeneXpert® is significantly higher than that of the comparator which renders its wide application controversial. Moreover, another advantage of ChromID VRE application is that it allows further epidemiologic analyses of the isolates (Papadimitriou-Olivgeris *et al.* 2014a).

In conclusion, even though Vitek 2 Advanced Expert System appears to be a reliable method for identification of enterococci in a diagnostic laboratory, it is important to verify results by a molecular method when phenotypic findings are discordant with epidemiologic patterns. ChromID VRE and GeneXpert® *vanA*/*vanB* have comparable efficacy in detecting *vanA*-positive enterococci. With regard to *vanB*-gene detection, GeneXpert® shows many false-positive results that renders its use problematic in departments with high incidence of *vanB*-positive enterococci.

## Materials and methods

### Samples and isolates

This is a retrospective study dealing with two groups of isolates. Group I included 431 Gram-positive cocci,

suspicious of being enterococci by conventional means (Gram-positive cocci, catalase-negative, positive for esculin hydrolysis and growth in 6·5% NaCl Tryptic Soy Broth; Becton Dickinson and Company, BD, Franklin Lakes, NJ) collected by the Departments of Microbiology and Hygiene (Environmental Unit) of the Medical School, University of Patras, Greece. Two hundred and ninety-five isolates were recovered between November 2009 to December 2011 in the context of a screening programme for VRE rectal colonization and infection of patients hospitalized in two Intensive Care Units (ICUs) and 136 were collected from November 2009 to July 2010 from seashore and river water sources of Achaia Prefecture (Southwestern Greece). Aforementioned collections of isolates were investigated by phenotypic and genotypic methods.

Group II was comprised of 372 rectal samples recovered during the 'Hospital Surveillance Programme for multidrug-resistant bacterial colonization of patients at risk and Healthcare Workers', approved by the University Hospital Ethics Committee that waived the need for informed consent (HEC No: 571) for VRE colonization. Direct molecular identification by real-time PCR was compared with conventional culture and PCRs for the respective genes (*vanA* and *vanB*).

## Group I

*Phenotypic identification*

Environmental isolates ($n = 136$) recovered from water samples (sea and river) according to ISO standard methods for the detection and enumeration of *Enterococcus* spp. (ISO 7899-02:2000) and clinical isolates ($n = 295$) were phenotypically identified by Gram stain, catalase test and Vitek 2 Advanced Expert System (bioMerieux, Marçy l'Etoile, France).

*Molecular identification*

All catalase-negative Gram-positive cocci ($n = 431$) were investigated for clonal identification by PFGE of chromosomal *Sma*I DNA digests performed in a CHEF DR III apparatus (Bio-Rad Laboratories, Richmond, CA), and categorized in PFGE types and subtypes according to established criteria (Tenover *et al.* 1995; Morrison *et al.* 1999). A total of 64 representative strains from all 52 different PFGE clonal types (showing more than six DNA band differences) and 12 subtypes (showing 1–6 DNA band differences) were further characterized to species level by 16S rDNA sequencing using two pairs of universal primers (forward: 5′-AGAGTTTGATCATGGCTCA-3′ and reverse: 5′-ACGGCGACTGCTGCTGGCAC-3′) (Gatselis *et al.* 2006). Sequencing of amplified products was performed in the ABI PRISM 310 apparatus (ABI PRISM® 310 Genetic Analyzer; Applied Biosystems, Foster City, CA) and compared with existing universal microbial genes' sequencing data. Molecular identification was based on 99–100% homology with the respective bacterial species (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

## Group II

Three hundred and seventy-two double rectal swabs (Stuart agar gel double swabs, Copan, Brescia, Italy) were obtained from ICU patients for VRE colonization screening.

*ChromID VRE and PCR (vanA, vanB)*

One swab was inoculated into Brain-Heart-Infusion Broth (bioMerieux) with colistin (750 mg $l^{-1}$) and caspofungin (500 mg $l^{-1}$) for 24 h in order to inhibit growth of multidrug-resistant Gram-negative bacteria and fungi. Subcultures were performed onto chromogenic agar (ChromID VRE; bioMerieux) and Trypticase agar plates with 5% sheep blood (TSAB; bioMerieux). MICs of vancomycin and teicoplanin were determined by a gradient method (Etest bioMerieux). Results were interpreted according to CLSI (2015). In order to assess the presence of *vanA* and *vanB* genes from isolated enterococci, in-house PCR was performed using amplification primers (for *vanA* gene the forward: 5′-ATGGCAAGTCAGGTGAAGATGG-3′ and the reverse: 5′- TCCACCTCGCCAACAACTAACG-3′, and for *vanB* gene the forward; 5′- TCTGTTTGAATTGTCTGGTAT and the reverse 5′-GACCTCGTTTAGAACGATG-3′) as previously described (Woodford *et al.* 1993; Damani *et al.* 2010). The PCR cycling conditions were as follows: denaturation 5 min at 94°C, amplification 30 cycles of 25 s at 94°C, 40 s at 52°C and 50 s at 72°C and a final extension step of 10 min at 72°C. The PCR products were detected by 2% agarose gel electrophoresis with ethidium bromide staining. Since culture methods remain the gold standard in the identification of VRE from rectal swabs, in the present study ChromID VRE combined with in-house PCR was considered as such.

*GeneXpert® vanA/vanB*

The second swab was evaluated directly with GeneXpert® *vanA/vanB* (Cepheid, Sunnyvale, CA) real-time PCR according to the manufacturer's instructions. Cost per sample of each methodology was calculated in Euros.

## Statistical analysis

SPSS vers. 19.0 (SPSS, Chicago, IL) software was used for data analysis. Specificity, sensitivity, PPV and NPV were calculated in order to assess the performance of GeneXpert® *vanA/vanB* PCR in the identification of VRE. Its

accuracy was investigated using receiver operating characteristic (ROC) analysis.

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## Conflict of Interest

No conflict of interest declared.

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