Determination of a Tentative Epidemiological Cut-Off Value (ECOFF) for Dalbavancin and Enterococcus faecium

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Abstract: Dalbavancin is a lipoglycopeptide antibiotic that shows potent activity against Gram-positive bacteria. It circumvents vanB-type glycopeptide resistance mechanisms; however, data on the in vitro activity of dalbavancin for Enterococcus faecium (E. faecium) are scarce, and thus, no breakpoints are provided. In recent years, there has been a continuing shift from vanA-type to vanB-type vancomycin-resistance in enterococci in Central Europe. Therefore, we aimed to investigate the in vitro activity of dalbavancin against different van-genotypes, with particular focus on vanB-type E. faecium. Dalbavancin susceptibility was determined for 25 vanB-negative, 50 vanA-positive, and 101 vanB-positive clinical E. faecium isolates (typed by cgMLST). Epidemiological Cut-Off Values (ECOFFs) were determined using ECOFFinder. For vanB-type E. faecium isolates, dalbavancin MICs were similar to those of vancomycin-susceptible isolates reaching values no higher than 0.125 mg/L. ECOFFs for vanB-negative and vanB-positive isolates were 0.5 mg/l and 0.25 mg/L respectively. In contrast, E. faecium possessing vanA predominantly showed dalbavancin MICs >8 mg/L, therefore preventing the determination of an ECOFF. We demonstrated the potent in vitro activity of dalbavancin against vancomycin-susceptible and vanB-type E. faecium. On the basis of the observed wildtype distribution, a dalbavancin MIC of 0.25 mg/L can be suggested as a tentative ECOFF for E. faecium.

Keywords: dalbavancin; E. faecium; antibiotic resistance; ECOFF; MIC determination

1. Introduction

Enterococci are intestinal commensals in many animals and humans. However, few members of this genus are also important nosocomial pathogens that are capable of causing severe infections in critically ill and immunocompromised patients. In particular, Enterococcus faecium isolates have received increased public health attention due to a dramatic increase of related infections and the accumulation of antibiotic resistance determinants [1]. The European Antimicrobial Resistance Surveillance Network (EARS-Net) has shown an overall increasing trend in the proportion of vancomycin-resistant enterococci (VRE = E. faecium) over the last few years [2,3]. However, no distinct geographical patterns could be observed across Europe, and even in countries with similar VRE rates, trends may differ with regard to the dissemination of different strain backgrounds and van-type genotype dynamics. Countries such as Denmark and Switzerland showed rising VRE trends, mainly due to an increased incidence of vanA-type E. faecium clonal types like ST203 and ST796 [4–6]. In contrast, other countries such as Germany and Poland reported a...
high prevalence of \textit{vanB}-type resistance, which was preferably found among the \textit{E. faecium} isolates of ST78 or ST117 [7–9].

Most VRE remain susceptible against last resort antibiotics such as linezolid, daptomycin (high dose), and tigecycline, but their use for patient treatment is constrained by several factors [10]. In addition to pharmacological and regulatory limitations, resistances to these novel antibacterials are increasingly reported [11–15]. In the case of \textit{vanB}-type VRE, teicoplanin may be considered an effective treatment alternative. However, at least in Germany, the substance is not widely used, and published experience reports warn against the development of a resistance to teicoplanin under therapy [16].

For lipoglycopeptides such as telavancin, dalbavancin, and oritavancin, different in vitro activities against VRE have been published [17,18]. Oritavancin is the only lipoglycopeptide that is active against all VRE, regardless of the \textit{van}-genotype that is present [19]. The antibiotic has been approved by the FDA and EMA for the treatment of acute bacterial skin and skin-structure infections (ABSSSI) but is only available on request or as an import in Europe. Telavancin and dalbavancin seem to be only effective against \textit{vanB}-type VRE. Additionally, telavancin has been approved for nosocomial and ventilator-associated pneumonias and as such, is not of interest as an VRE agent.

Dalbavancin (Xydalba®) is approved in several European countries, including Germany, for treating ABSSSI and has been included in a clinical trial to demonstrate its efficacy in the treatment of invasive infections such as infective endocarditis and complicated bacteremia. However, the trial was terminated, and the results have not yet been published (trial identifier: NCT03148756; study ID: DAL-MD-09).

With the present study, we investigated the in vitro activity of dalbavancin, especially against \textit{vanB}-type VRE. Furthermore, we aimed to determine a tentative ECOFF for \textit{E. faecium} isolates, since EUCAST provides neither an ECOFF for \textit{E. faecium} nor distinguishes between vancomycin-susceptible enterococci (VSE) and VRE. Our re-assessment also considered that most of the available in vitro studies on dalbavancin efficacy are now more than 10 years old and contained only a few VRE strains, all of which were poorly characterized with respect to species identification and \textit{van}-genotyping [20,21].

For the present study, we compiled a large, comprehensive collection of invasive clinical \textit{E. faecium} isolates that contained both vancomycin-susceptible and vancomycin-resistant isolates of the \textit{vanA}– and \textit{vanB}-types. All isolates originated from submissions to the German National Reference Centre (NRC) for Enterococci between January 2018 and December 2019. The collection was geographically diverse and represented the most common clonal types and \textit{van} genotypes in Germany. Dalbavancin MICs were determined using broth microdilution (BMD) and a commercial gradient strip test. Next Generation Sequencing (NGS) data were available for all of the strains included in this study.

2. Results

2.1. Molecular Typing of the Strain Collection

The strain collection contained a total of 101 \textit{vanB}-positive, 50 \textit{vanA}-positive, and 25 \textit{van}-negative (vancomycin-susceptible) \textit{E. faecium} isolates belonging to 16 MLST and 74 cgMLST types (Supplementary Table S1 and Figure S1). The most frequent MLST types were ST117 (n = 89; 50.6%) and ST80 (n = 36; 20.5%); the most frequent cgMLST types were CT71 (ST117) (n = 39; 22.2%) and CT894 (ST80) (n = 8; 4.5%) (Supplementary Figure S1).

2.2. Determination of Dalbavancin MICs by BMD Testing

Vancomycin-susceptible and \textit{van}-negative \textit{E. faecium} isolates (n = 25) demonstrated dalbavancin MIC\textsubscript{50} and MIC\textsubscript{90} values of 0.064 mg/L and 0.125 mg/L, respectively (Table 1). The ECOFF was 0.5 mg/L (Table 1, Figure 1A). Dalbavancin MICs of \textit{vanB}-positive \textit{E. faecium} isolates (n = 101) tended to be one dilution below from what could be determined for \textit{van}-negative \textit{E. faecium} isolates (MIC\textsubscript{50} = 0.032 mg/L, MIC\textsubscript{90} = 0.064 mg/L) (Table 1). The ECOFF for the \textit{vanB} isolates was 0.25 mg/L (Table 1, Figure 1A). According to the CLSI breakpoint for \textit{E. faecalis} (S ≤ 0.25), all \textit{van}-negative and \textit{vanB}-positive \textit{E. faecium} iso-
lates would be categorized as dalbavancin-susceptible. *E. faecium* possessing *vanA* (*n* = 50) demonstrating a MIC$_{50}$ value of 8 mg/L and a MIC$_{90}$ value of >8 mg/L (Table 1); thus, preventing the definition of an ECOFF. Of note, few vancomycin- and teicoplanin-resistant *vanA*-type VRE demonstrated dalbavancin MICs in the lower range of ≤0.5 mg/L (*n* = 6) (Figure 1A, Supplementary Table S1). Growth deficiencies in MH broth and a low resistance to teicoplanin (8 or 16 mg/L) were observed for almost all of these isolates, as can also be found for some of the now prevalent VRE isolates in Germany (based on experience at the NRC) (Supplementary Table S1). Dalbavancin-resistant and *vanA*-positive isolates generally showed vancomycin and teicoplanin MICs of >16 mg/L (Supplementary Table S1). A single *vanA* isolate that did not reveal growth deficiencies in MH broth was tested vancomycin- and teicoplanin-susceptible (MICs ≤ 1 mg/L) (Supplementary Table S1). This isolate demonstrated dalbavancin MICs of 0.125 and 0.064 mg/L when determined using BMD and MIC test strips, respectively (Supplementary Table S1). Detailed sequence analysis of the corresponding *vanA* gene cluster revealed a 5′ truncated *vanX* gene (data not shown). For isolates that showed deficiencies in growth behavior (*n* = 5), assessment of their *vanA* clusters did not reveal any genetic changes that could potentially be associated with this unusual phenotype. Prolonged incubation for up to 48 h produced dalbavancin MICs in the resistant range for four out of five isolates (Supplementary Table S1).

**Figure 1.** Distribution of dalbavancin MICs. MICs were obtained for 25 vancomycin-susceptible, 50 *vanA*-positive, and 101 *vanB*-positive *E. faecium* isolates using BMD (A) and MIC test strips (B). The number of isolates with corresponding MICs is given in %. Gradient strip MICs were both extrapolated to the next double dilution value equivalent to values for BMD and downsized to a maximum of >8 mg/L. The MIC breakpoint for dalbavancin for *E. faecalis* according to CLSI is ≤0.25 mg/L.
Table 1. Distribution of dalbavancin MICs for *E. faecium* isolates (n = 176). MIC gradient strip values were converted into doubling dilution as found in BMD, and values of 16, 32, and >32 mg/L were downsized to >8 mg/L (maximum measurable value by BMD).

| Genotype/Method | n  | MIC [mg/L] | MIC<sub>90</sub> [mg/L] | MIC<sub>90</sub> [99%] | ECOFF * [% S] | CLSI ** [% S] |
|-----------------|----|------------|------------------------|-----------------------|---------------|---------------|
|                 |    | ≤0.08 0.016 0.032 0.064 0.125 0.25 0.5 1 2 4 8 >8 |                        |                       |               |               |
| vanA, BMD       | 50 | 1 1 3 1 1 21 22 | 8 >8 - 10 |               |               |               |
| vanA, strip     | 50 | 1 1 1 1 1 45 | >8 >8 - 8 |               |               |               |
| vanB, BMD       | 101 | 1 6 64 27 3 | 0.032 0.064 0.25 100 |               |               |               |
| vanB, strip     | 101 | 6 45 44 6 | 0.016 0.032 0.125 100 |               |               |               |
| van-, BMD       | 25 | 3 6 11 5 | 0.064 0.125 0.5 100 |               |               |               |
| van-, strip     | 25 | 6 11 7 1 | 0.032 0.064 0.25 100 |               |               |               |

Legend: BMD, broth microdilution; strip, MIC gradient strip test. The vertical line indicates the CLSI breakpoint for *E. faecalis*. *ECOFFs were identified by the use ECOFFinder (EUCAST). **We applied the susceptibility breakpoint for vancomycin-susceptible *E. faecalis* as provided CLSI3.
2.3. Determination of Dalbavancin MICs by MIC Gradient Strip Testing

For reasons of comparison and the determination of an ECOFF, we converted the MICs of MIC gradient strips into doubling dilution as found in BMD. Furthermore, MIC values of 16, 32 and >32 mg/L were downsized to a maximum of >8 mg/L (Supplementary Table S1). In case of deviations between the BMD and MIC Test Strips, the exact MICs are shown in brackets. Vancomycin-susceptible and van-negative E. faecium isolates (n = 25) demonstrated a MIC$_{50}$ value of 0.032 mg/L and a MIC$_{90}$ of 0.064 mg/L (Table 1). The ECOFF was 0.25 mg/L (Table 1, Figure 1B). Vancomycin-resistant and vanB-positive E. faecium isolates (n = 101) demonstrated a MIC$_{50}$ value of 0.016 mg/L and a MIC$_{90}$ of 0.032 mg/L (Table 1), which was one dilution step below that of van-negative E. faecium isolates. The ECOFF was 0.125 mg/L (Table 1, Figure 1B). All van-negative and vanB-positive E. faecium isolates revealed a gradient strip MICs of ≤0.25 mg/L (Table 1, Figure 1B, Supplementary Table S1). E. faecium possessing vanA (n = 50) showed a MIC$_{50}$ value of >8 mg/L (>32 mg/L) and a MIC$_{90}$ of >8 mg/L (>32 mg/L) (Table 1), thus preventing the definition of an ECOFF. In accordance with results from BMD, few vancomycin- and teicoplanin-resistant vanA isolates demonstrated dalbavancin gradient strip MICs in the lower range of ≤0.5 mg/L (n = 4) (Figure 1B, Supplementary Table S1). Using the macro method (McFarland 2.0, BHI agar, 48 h incubation), only two vanA isolates demonstrated dalbavancin MICs ≤0.5 mg/L (Supplementary Table S1), one of which carried the truncated vanX gene (see above). Isolates showing growth within a defined inhibition zone were categorized as dalbavancin-resistant with a MIC >32 mg/L (Supplementary Figure S2, see discussion).

2.4. Comparing Results of Broth Microdilution and MIC Test Strips

Dalbavancin MICs derived from BMD and MIC gradient strip tests (BMD adapted MIC values) were in good overall concordance (Supplementary Figure S3). For van-negative and vanB-positive isolates, however, the MIC$_{50}$/MIC$_{90}$ values of MIC gradient strips were slightly lower when compared to the BMD results (Table 1). Thus, the ECOFF was one doubling dilution higher when determined using BMD (Table 1). Irrespective of the testing method, all van-negative and all vanB-positive E. faecium isolates demonstrated MICs of ≤0.25 mg/L (CLSI breakpoint for E. faecalis). Dalbavancin MIC values for vanA-positive isolates ranged from ≤0.008 to >8 mg/L in BMD and 0.016 mg/L (0.012 mg/L) to >8 mg/L (>32 mg/L) for the MIC test strips (Supplementary Figure S3C). In contrast to BMD, the majority of the vanA-positive E. faecium isolates exhibited dalbavancin MIC values >8 mg/L (>32 mg/L) when determined using MIC gradient test strips (Supplementary Figure S3C).

3. Discussion

To identify a dalbavancin ECOFF for E. faecium, we determined the dalbavancin MICs for a total of 176 well-characterized, vancomycin-sensitive, vanA- or vanB-positive E. faecium isolates.

To date, available data on the in vitro efficacy of dalbavancin mainly come from complementary clinical studies [22–26]. However, within these studies, enterococci were hardly considered, and many of these studies have been conducted in the USA, where the majority of E. faecium infections is caused by vanA-type VRE. Since dalbavancin has no in vitro activity against vanA-carrying E. faecium, VRE data were not included in most clinical or registration trials. As a result, data on E. faecium are generally scarce, and knowledge about the in vitro activity of dalbavancin against different van genotypes is almost non-existent.

The determination of dalbavancin MICs is challenging for several reasons. First, all lipoglycopeptides show a facile adsorption to plastic surfaces [27]. The strength of the surface adhesion depends on the plastic material and thus on the type of microtiter plates used in the experiment. In order to prevent the depletion of the active substance from the test medium, the administration of polysorbate 80 (Tween 80) is essential. Second, readout of dalbavancin MICs can be challenging for BMD and MIC gradient strip tests because some clinical VRE strains may demonstrate growth deficiencies in standard media.
This effect, in conjunction with an inducible resistance mechanism like VanA, can lead to delayed or weak growth in susceptibility tests. In BMD, this growth behavior is manifested by either very weak or diffuse colony growth when compared to the antibiotic-free growth control. For MIC gradient strip assays, the lack of growth leads to effects reminiscent of “heteroresistance”, which is detected either by microcolony growth in an otherwise clear inhibition zone (Supplementary Figure S2B) or a visible inhibition zone, but overall shaded growth within this zone (Supplementary Figure S2A). Third, dalbavancin is not available for the commercial test panels of automated systems, requiring manual handling, sample preparation, and readout.

The majority of the vanA-positive E. faecium isolates showed dalbavancin MICs of >8 mg/L (BMD) (Figure 1A, Supplementary Table S1). In contrast to vanB-type resistance, the regulatory system of vanA-positive VRE are sensitive to induction by teicoplanin and dalbavancin, resulting in the synthesis of modified peptidoglycan precursors and thus a resistance against all (lipo-) glycopeptides [28]. A few vanA-type VRE also revealed dalbavancin MICs in the lower range (≤0.5 mg/L) (Figure 1A, Supplementary Table S1). Similarly, data from a study by Zhanel et al. indicated a wide range of dalbavancin MICs for vanA-type E. faecium isolates reaching from 0.03 mg/L to >32 mg/L [17]. However, the authors did not address this aspect and did not provide any explanation as to whether and why dalbavancin might be active against some vanA-type isolates. Our research showed that five out of six vanA isolates with a dalbavancin MIC of ≤0.5 mg/L revealed weakened growth in MH broth. As such, it could be argued that more general growth deficiencies were responsible for the comparably low dalbavancin MICs. In case of a weakened growth, prolonged incubation of up to 48 h facilitated BMD MIC determination and prevented the detection of false-negative results (Supplementary Table S1). Similarly, a supplementation with 10% rich media such as BHI in combination with an increased inoculum (McFarland 2.0) and 48 h incubation period improved experimental outcomes when using MIC test strips (Supplementary Table S1). Both variations could be considered as a deviation from susceptibility testing guidelines and standards. Interestingly, one vanA isolate showed dalbavancin MICs in the susceptible range regardless of the method used, but no growth defect was observed (Supplementary Table S1). Subsequent in silico analysis revealed a trunked vanX gene, most probably leading to a non-functional VanX protein. VanX is essential for glycopeptide resistance because it degrades the D-Ala-D-Ala dipeptide into its individual components and thus prevents the formation of glycopeptide susceptible cell wall precursors.

The majority of published studies that have evaluated the in vitro activity of dalbavancin have focused either only on E. faecalis or did not distinguish between E. faecalis and E. faecium and/or the different van genotypes [21,29,30]. Therefore, these studies do not allow for a comparison with our data. With the beginning of the 2010s, however, several US medical centers presented results on the in vitro activity of dalbavancin for some E. faecium isolates, including VRE. A study conducted by Sader et al. included 30 E. faecium isolates, 18 of which carried the vanA gene. Of these, all but one isolate demonstrated a dalbavancin MIC of >4 mg/L [21]. Similarly, Jones and colleagues published a study on the potency profiles for dalbavancin and found that all included vanA-type enterococci (19 E. faecium, 6 E. faecalis) had dalbavancin MICs of ≥4 mg/L [20].

Due to the general prevalence of vanA-type resistance in clinical E. faecium and E. faecalis isolates for many years, comprehensive data on dalbavancin resistance in vanB-type enterococci are generally scarce [17,20,21,30]. A recently performed and yet unpublished study determined the dalbavancin MICs of clinical VRE isolates (including vanA- and vanB-type E. faecium) (Kresken et al., unpublished). Similar to our study, the MIC50 and MIC90 values for vanB isolates were 0.06 mg/L and 0.125 mg/L, respectively. For the vanA-positive isolates, the MIC50 was 8 mg/L, and the MIC90 was >8 mg/L. All of the vanB strains possessed dalbavancin MICs of ≤0.25 mg/L, and all vanA isolates revealed dalbavancin MICs of ≥4 mg/L.
In conclusion, we determined dalbavancin MICs for the largest and best-described strain collection of clinical \textit{E. faecium} isolates, comprising both vancomycin-susceptible and vancomycin-resistant isolates of the \textit{vanA}– and \textit{vanB}-types. Results are reliable and consistent across different methodologies when dalbavancin susceptibility testing is performed according to the recommended guidelines. Based on the here provided and recently published data, we suggest a tentative dalbavancin ECOFF of 0.25 mg/L or a breakpoint of \(S \leq 0.25 \text{ mg/L}\) for \textit{E. faecium}. Using this ECOFF or breakpoint, \textit{van}-negative and \textit{vanB}-positive \textit{E. faecium} isolates would be considered dalbavancin-susceptible, while most \textit{vanA}-type VRE, with the exception of a few, would be categorized as dalbavancin-resistant.

4. Materials and Methods

\textit{Strain collection.} Within the present study, we included 176 clinical \textit{E. faecium} isolates collected between January 2018 and December 2019 (Supplementary Table S1). All of the isolates derived from cases of bacteremia/blood stream infections and were submitted from clinical diagnostic laboratories to the NRC.

\textit{Susceptibility testing.} We assessed susceptibilities to vancomycin and teicoplanin using BMD. Testing was performed according to EUCAST guidelines. EUCAST v8 and v9 standards were applied to categorize the MIC data into susceptible (S) and resistant (R). The MIC of dalbavancin (Sigma-Aldrich, St. Louis, USA) was determined by BMD using cation-adjusted Mueller–Hinton broth (Becton-Dickinson, Heidelberg, Germany) as described recently [18]. Deviating from this procedure, microdilution panels contained serial 2-fold dilutions of dalbavancin ranging from 8 to 0.008 mg/L. In order to alleviate adherence of the dalbavancin to the plastic surfaces of microtiter plates (Greiner Bio-One, Frickenhausen, Germany), the solution contained a final concentration of 0.002% polysorbate 80 (Twee 80; Sigma-Aldrich/Merck, Taufkirchen, Germany). In addition to BMD testing, the dalbavancin MICs were determined through a MIC gradient strip test on MH agar (Becton-Dickinson) according to the manufacturer’s instructions (Liofilchem, Roseto degli Abruzzi, Italy [http://www.liofilchem.net/login.area.mic/technical_sheets/MTS38.pdf, accessed on 22 June 2021]). For reasons of comparison, we also applied the macro method using a higher inoculum of McFarland 2.0, Brain Heart Infusion (BHI) agar and a prolonged incubation time of up to 48 h before readout. Since EUCAST does not provide a clinical breakpoint or an ECOFF for dalbavancin, we compared our MIC data with the CLSI breakpoint provided for \textit{E. faecalis} (\(S \leq 0.25 \text{ mg/L}\)). Reference isolates were \textit{S. aureus} ATCC29213 and \textit{E. faecalis} ATCC29212.

\textit{Determination of ECOFFs.} ECOFFs were determined through the use of ECOFFinder [31]. A threshold of 99% was chosen (MIC value that captures 99% of the modelled wild-type population) to increase the specificity for the wild-type population.

\textit{Determination of \textit{van} genotypes.} As part of the daily routine, the \textit{van} genotypes were assessed by multiplex PCR as described recently [32] and verified through in vitro susceptibility testing to vancomycin and teicoplanin. Additionally, \textit{van}-genotypes were derived from de novo assembled read-data (see next paragraph).

\textit{NGS-based analysis.} At the NRC for Enterococci, all isolates from bacteremia and sepsis are routinely sequenced using NGS. Whole genome sequencing was conducted in paired-end mode using the NextSeq 550 workflow with a read length of 2 × 150 bp (Illumina, San Diego, CA, USA). The quality of the raw sequence data was checked using FastQC v0.11.5 [33]. Taxonomic read classification was verified by Kraken v0.10.6 [34]. For the purpose of genome reconstruction, Illumina reads were de novo assembled using SPAdes with default parameters [35]. BWA-MEM was used for assembly-remapping and -polishing [36]. MLST and cgMLST were performed with de novo assembled contigs and Ridom SeqSphere* v6.0.0 (Ridom; Münster, Germany). De novo assembled contigs were screened for \textit{vanA} or \textit{vanB} using BLAST [37]. Corresponding contigs were annotated using a customized database of relevant sequences (\textit{vanR}, \textit{vanS}, \textit{vanH}, \textit{vanA} and \textit{vanX}) and aligned through the use of MAFFT and Geneious Prime v2020.2.3 [38].
Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/antibiotics10080915/s1, Figure S1: Distribution of clonal lineages according to MLST for van-negative, vanB- and vanA-positive E. faecium isolates, Figure S2: Exemplary illustration of dalbavancin MIC determination by the use of MIC Test Strips, Figure S3: Concordance of dalbavancin MICs obtained by BMD and MIC test strips, Table S1: Details on the strain collection.

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Conflicts of Interest: M.K. is a partner and CEO of Antiinfectives Intelligence GmbH, a research organization providing services to pharmaceutical companies. All other authors have no conflicts of interest to declare.

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