MINIREVIEW

Penicillin Resistance in *Enterococcus faecalis*: Molecular Determinants and Epidemiology

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**Abstract**

*Enterococcus faecalis* plays a significant role in hospital-acquired infections (HAIs), and combination of penicillin with aminoglycoside is important in therapy of invasive HAIs. Penicillin resistance in this organism is due to modification of the drug target, penicillin-binding protein (PBP5), its overproduction and expression of β-lactamase. Although rare, this phenotype is often associated with multi-resistant high-risk enterococcal clonal complexes (HiRECCs), such as CC2 and CC9 which may promote its spread in the near future.

**Key words:** *Enterococcus faecalis*, β-lactamase, HiRECC, PBP, penicillin, resistance

Enterococci are a part of natural intestinal flora of both human and animals. These Gram-positive bacteria are typically harmless commensals, but in particular conditions they can also cause serious infections. So far, several *Enterococcus* species have been identified (Hardie and Whiley, 1997), of which two, *Enterococcus faecalis* and *Enterococcus faecium* have gained clinical importance. Enterococci have emerged as a serious nosocomial pathogens in the 1990s (Edwards, 2000), and their impact is increasing due to growing numbers of patients at-risk (Sydnor and Perl, 2011). Enterococci were reported as the third most common cause of hospital-associated infections in the United States (Hidron et al., 2008). While the majority (around 80%) of enterococcal infections in humans is caused by *E. faecalis* (Jett et al., 1994; European Centre for Disease Prevention and Control, 2010), the prevalence of *E. faecium* is currently on the rise (Top et al., 2007; Lester et al., 2008; Hidron et al., 2008). The most serious forms of infection caused by *E. faecalis* are endocarditis and bloodstream infections; other diseases include urinary tract infections (UTIs) and post-surgery wound infections (Jett et al., 1994); central nervous system and neonatal infections occur with low frequency (Murray, 1998).

Enterococci possess several traits that facilitate dissemination and survival in the hospital settings, and make theirs infections difficult to treat; they are able to grow in the range of 10–45°C, at pH 9.5, in presence of 6.5% NaCl and to survive at 60° for 30 min (Sherman, 1937). Enterococci show an intrinsic lack of susceptibility to various antibiotics, including cephalosporins, monobactams, sulphonamides, low concentrations of aminoglycosides, as well as, in the case of *E. faecalis*, streptogramins and lincosamides. Increasing antibiotic resistance is associated with the capability of these bacteria to acquire and to transfer mobile genetic elements encoding resistance genes (Marothi et al., 2005; Palmer et al., 2010). The resistance to antibiotics can be also developed due to spontaneous mutations. Hospital-associated strains of *E. faecalis* often present acquired resistance to antibiotics of several classes such as tetracyclines, quinolones and high-level aminoglycoside resistance (HLAR), while vancomycin-resistant enterococci (VRE) of this species remain rare (Marothi et al., 2005; Hidron et al., 2008). The HLAR phenotype in *E. faecalis* is of particular concern as the combination of aminoglycoside with penicillin (ampicillin or penicillin G) represents a therapy of choice for enterococcal invasive infections (Arias and Murray, 2008). Combination of these two drugs has a synergistic bactericidal effect on enterococci (Moellering and Weinberg, 1971). Normally, the enterococcal cell-wall is poorly permeable for aminoglycosides but these drugs can penetrate to the intracellular target (ribosome) in the presence of the cell-wall synthesis inhibitors as like β-lactams. In Europe, percentage of HLAR among *E. faecalis* reach relatively high values – from 30% to 50% (European Centre for Disease Prevention and Control, 2010).

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In recent years, we witness an emergence of penicillin-resistant *E. faecalis* isolates in hospital environment. Three mechanisms for penicillin resistance in *E. faecalis* have been reported, and include (i) production of β-lactamase which inactivates the drug, (ii) overproduction of the drug target, penicillin-binding proteins (PBPs) and (iii) their decreased affinity for β-lactams.

**Determination and interpretation criteria of penicillin susceptibility in *E. faecalis***

According to the guidelines of Clinical and Laboratory Standard Institute (CLSI) determination of *E. faecalis* susceptibility to penicillin and ampicillin can be carried out with the use of either disc diffusion method or by establishing the minimal inhibitory concentration (MIC) values by an agar or broth dilution methods (Clinical and Laboratory Standard Institute, 2011). Following the CLSI interpretation criteria, strains of *E. faecalis* can be defined as a susceptible (S), or resistant (R) to penicillin, without an intermediate (I) category (Table I). For the detection of β-lactamase-producing strains, the nitrocefin test is recommended (Clinical and Laboratory Standard Institute, 2011). The European Committee on Antimicrobial Susceptibility Testing (EUCAST) provides interpretations of MICs and zone diameters only for ampicillin (Table I) (European Committee on Antimicrobial EUCAST Testing, 2011). Criteria used by CLSI and EUCAST are different, MIC values equal to 8 µg/ml for ampicillin denote susceptible strain according to CLSI but intermediate following the EUCAST criteria.

In addition, the EUCAST provides so-called epidemiological cut-off values (ECOFFs), which separate the wild-type (WT) strains from the strains that exhibit acquired and mutual resistance mechanisms to the drug in question. In the case of *E. faecalis*, the ECOFF value for penicillin is 16 µg/ml, and for ampicillin is 4 µg/ml, *i.e.* the WT strains show MICs lower than or equal to this values. According to MIC distributions determined by the EUCAST, for most of the *E. faecalis* strains MIC of penicillin ranges between 2 and 4 µg/ml and MIC of ampicillin ranges between 1 and 2 µg/ml (www.eucast.org/mic_distributions/; 16th November 2011, date last accessed).

**Resistance due to the target modification**

Penicillin-binding proteins (PBPs) are produced by almost all bacteria. These membrane proteins are involved in the final stages of peptidoglycan synthesis. Binding β-lactam antibiotic to PBPs inhibits enzymatic activity of protein and leads to cell growth inhibition or cell death (Lleó et al., 1987). However, certain PBPs show lower affinity to penicillin and because of that, they are responsible for reduced susceptibility to penicillin in a number of Gram-negative and Gram-positive bacteria, including enterococci (Fontana et al., 1983; Canepari et al., 1986; 1987). Low-affinity PBPs replace other PBPs, inhibited by drugs and take over their transpeptidase function (Fontana et al., 1983; 1985).

*E. faecalis* produces five PBPs, including four high-molecular weight PBPs and one low-molecular weight PBP (Williamson et al., 1983, Duez et al., 2001; Ono et al., 2005). One of these PBPs, designed PBP5, and sometimes named PBP4, is a low-affinity PBP, and its changes and/or overproduction can be related to penicillin resistance. The *E. faecalis* PBP5 encoded by the genome of V583 strain (Paulsen et al., 2003) is a high-molecular weight protein of about 75 kDa (680aa) and is organized in three distinct domains. The N-terminal hydrophobic domain, about 30aa long, is responsible for PBP5 anchoring to the cell membrane (Ghuysen et al., 1996; Signoretto and Canepari, 2000). The penicillin-binding domain, with typical penicillin-binding motifs, with binding motifs, _SXXK_, _SXXS_, _SXXG_ and _SXXG_ is localized at the C-terminus of the protein (Zapun et al., 2008). These two parts are connected by a central non-penicillin-binding domain of presumable transglycosylase activity proposed by analogy with high-molecular weight *Escherichia coli* PBPs (Ghuysen et al., 1996; Signoretto and Canepari, 2000). Non-penicillin-binding domain seems to be essential for the proper folding, stability and the biochemical activity of the penicillin-binding domain, like in *E. hirae* (Mollerach et al., 1996). This specific structure classifies enterococcal PBP5 in the class B multimodular PBPs (Ghuysen et al., 1996).

PBP5 of *E. faecalis* is closely related to the other enterococcal low-affinity PBPs described previously (Zorzi et al., 1996). Importance of alterations in PBP5 caused by point mutations for β-lactam resistance is well-described in *E. faecium* (Ligozzi et al., 1996; Zorzi

**Table I**

<table>
<thead>
<tr>
<th>CLSI</th>
<th>MIC breakpoint (µg/ml)</th>
<th>Zone diameter breakpoint (mm)– disc content 10 µg</th>
<th>EUCAST</th>
<th>MIC breakpoint (µg/ml)</th>
<th>Zone diameter breakpoint (mm)– disc content 2 µg</th>
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<tr>
<td>Penicillin</td>
<td>8</td>
<td>16</td>
<td>Ampicillin</td>
<td>4</td>
<td>8</td>
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<tr>
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<td>16</td>
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Due to the changes of the transglycosylase activity of PBP5, the resistant strains show lower affinity to penicillin and because of that, they are responsible for reduced susceptibility to penicillin in a number of Gram-negative and Gram-positive bacteria, including enterococci (Fontana et al., 1983; Canepari et al., 1986; 1987). Low-affinity PBPs replace other PBPs, inhibited by drugs and take over their transpeptidase function (Fontana et al., 1983; 1985).

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et al., 1996; Rybkine et al., 1998). Amino acid substitutions in the region between motifs SDN and KTG of the penicillin-binding domain are involved in the resistance to penicillin and ampicillin by decreasing affinity to these drugs (Ligozzi et al., 1993; Fontana et al., 1996; Ligozzi et al., 1996). Similarly, mutations within the PBP5 penicillin-binding domain in \textit{E. faecalis} result in the decrease of affinity and susceptibility to \beta-lactam antibiotics. Such isolates were first detected in Japan in 1998–2002 (Ono et al. 2005). Four clinical isolates with ampicillin MIC values 8–16 \mu g/ml harbored amino acid alterations of PBP5 at the amino acid positions 520 and 605 (P520S and Y605H), \textit{i.e.} located in the region between the SDN and KTG motifs of the penicillin-binding domain (Ono et al., 2005). These isolates also showed elevated MICs for imipenem; susceptibility to penicillin was not reported in this study. Mutations in the same amino acid positions were detected in UTI clinical isolates with reduced faropenem susceptibility, also found in Japan (Hiraga et al., 2008). Different kind of modifications was observed in the laboratory mutant strain 56R with high penicillin and ampicillin MIC values equal 128 \mu g/ml and 64 \mu g/ml, respectively (Signoretto et al., 1994; Signoretto and Canepari, 2000). Comparison of the deduced amino acid sequence of PBP5 from 56R with its counterpart in the JH2-2 susceptible strain revealed that the resistant strain harbors a small frameshift mutation in the non-penicillin-binding domain (the peptide \texttt{AAQLIGYTGG\textsubscript{281}} is replaced by \texttt{ACAINRVYG\textsubscript{280}} resulting in a protein shorter by a single amino acid residue), as well as the T/I mutation, immediately adjacent to the K residue in the SXXK motif (Duez et al., 2001). As the corresponding sequence of the parental susceptible strain 56 was not available, it was not possible to draw reliable conclusion about these changes (Duez et al., 2001). The role of PBP5 for decreased susceptibility to \beta-lactams in \textit{E. faecalis} is further supported by the fact that inactivation of the \textit{pbp5} gene in the 56 strain by Tn916 mutagenesis resulted in 8-fold decrease in the penicillin MIC value of the PBP5-deficient strain (Signoretto and Canepari, 2000).

### Resistance mediated by the PBP overproduction

The overproduction of the drug target appears to be another important mechanism of penicillin resistance in clinical isolates and laboratory mutants of \textit{E. faecalis}, and was also observed in other enterococcal species such as \textit{E. faecium}, \textit{Enterococcus avium} and \textit{Enterococcus durans} (Fontana et al., 1994; al-Obeid et al., 1990). The exposure of susceptible \textit{E. faecalis} strains to penicillin led to selection of penicillin-resistant mutants with hyperproduction of PBP5 and this type of resistance was developing both through serial passages on plates containing increasing concentrations of penicillin (al-Obeid et al., 1990; Hodges et al., 1992; Duez et al., 2001) and under continuous penicillin exposure (Hodges et al., 1992). A detailed analysis of the JH2-2r strain with penicillin MIC value of 75 \mu g/ml, overproducing PBP5, and its parental susceptible strain JH2-2 confirmed that both strains harbor identical \textit{pbp5} gene (Duez et al., 2001). Apparently increased amount of PBP5 were also observed in \textit{E. faecalis} from hospital settings in Spain (Cercenado et al., 1996). These two clinical isolates showed MIC values of 64 \mu g/ml and 32–64 \mu g/ml for penicillin and ampicillin, respectively, and were isolated independently from two patients with UTI, treated with ampicillin (Cercenado et al., 1996).

The molecular basis of PBP5 overproduction in \textit{E. faecalis} has not yet been clarified. In \textit{E. hirae}, synthesis of PBP5 is under the control of the \textit{psr} gene (PBP5 synthesis repressor), located upstream \textit{pbp5}, and alteration of \textit{psr} by a point mutation or deletion results in the elevated expression of PBP5 (Ligozzi et al., 1993). In \textit{E. faecalis}, however, no such gene is present in the proximity of to \textit{pbp5}, and \textit{psr}-like gene located elsewhere in the genome showed exactly the same sequence in the penicillin-resistant mutant JH2-2r, described above, in comparison to the JH2-2 parental strain. Moreover, the \textit{pbp5} promoter region in both strains was identical (Duez et al., 2001). Therefore, the pathway leading to PBP5 overexpression in \textit{E. faecalis} remains to be elucidated.

### Resistance due to \beta-lactamase acquisition

\beta-Lactamase production is another mechanism of resistance penicillin, so far restricted almost exclusively to \textit{E. faecalis}. The first \beta-lactamase producing (Bla+) enterococcal isolate, HH22, was detected in Houston, Texas, in 1981 (Murray and Mederski-Samaraj, 1983). MIC values for penicillin and ampicillin of HH22 were above 1000 \mu g/ml, when the high inoculum of \textit{10^7} CFU/ml was used (Murray et al., 1986a). Since then, Bla+ enterococci became more prevalent and were isolated from severe infections and nosocomial outbreaks (Murray et al., 1991; 1992; Patterson et al., 1988a; Rhinehart et al., 1990; Wells et al., 1992; Mazzulli et al., 1992). \beta-Lactamase from \textit{E. faecalis} is a typical penicillinase, able to hydrolyze penicillin, ampicillin and ureidopenicillins (\textit{e.g.} piperacillin), and sensitive to inhibitors such as clavulanic acid (Murray et al., 1986b).

Most likely, enterococci acquired the \beta-Lactamase gene from staphylococci (Murray et al., 1986b). The \textit{E. faecalis} HH22 carries the \textit{blaZ} gene, whose sequence...
is identical to the staphylococcal blaZ genes from the pC1 and pS1 plasmids (Zscheck and Murray, 1991), and from staphylococcal Tn552 transposon (Tomayko et al., 1996). In contrast to the inducible production of large amounts of β-Lactamase by Staphylococcus aureus, E. faecalis produces the enzyme constitutively on relatively low level (Murray et al., 1986a; Okamoto et al., 1996). Particular enterococcal clones may differ by the presence of the regulatory genes blaR1 (encoding antirepressor) and blaI (encoding repressor) from the bla gene cluster of S. aureus. For example, the HH22 strain possesses only a part of blaR1 and lacks blaI (Zscheck and Murray, 1993). Even if these genes are present, the β-lactamase is still produced constitutively. It is possible that blaR1 and blaI contain mutations which make them non-functional or that these regulatory proteins are not active in the enterococcal cell (Zscheck and Murray, 1991; Okamoto et al., 1996; Tomayko et al., 1996).

In E. faecalis, blaZ genes reside either on conjugative plasmids (Murray et al., 1986a, b, Patterson et al., 1988b; 1990; Markowitz et al., 1991), or on bacterial chromosome (Rice et al., 1991; Chow et al., 1993). The blaZ gene in the first described Bla+ isolate HH22 was shown to be present on the ~70 kb plasmome-responsive conjugal plasmid pBEM10 that was transferred with a high frequency of 10−5 (Murray et al., 1988). Efficient conjugative transfer of plasmome-responsive plasmids, specific for enterococci, is due to the unique mechanism of response to so-called sex pheromones, secreted by recipient cells lacking a particular type of plasmid. Stimulated donor cells respond by production of proteins necessary for cell-to-cell contact and subsequent plasmid transmission (Dunny, 1990; Palmer et al., 2010). The pBEM10 plasmid responds to the cAD1 pheromone and uses the same pheromone system as pAD1 (Murray et al., 1988) with which it shares extensive homology (Galli and Wirth, 1991).

The chromosome-located blaZ gene, detected in isolates from Boston, was transferable at low frequency (below 10−4), together with determinants of resistance to a number of other antimicrobials such as erythromycin, gentamicin, mercuric chloride, streptomycin, and tetracycline (Rice et al., 1991). Later on it was shown that blaZ is associated with a composite ~60 kb transposon Tn5385 which structure was a subject of several studies. Tn5385 comprises sequences of transposons, such as (1) Tn5381 that carries the tetM gene (Rice et al., 1992), (2) Tn5384, that itself contains Tn4001 with the aacA-aphD gene (responsible for gentamicin resistance) and a derivative of Tn917 with the ermB gene (erythromycin resistance determinant) (Rice et al., 1995; Bonafede et al., 1997) and (3) Tn552-like staphylococcal transposon with the blaZ gene and a part of blaR1 (Rice et al., 1996; Bonafede et al., 1997). Additionally, Tn5385 possesses two replication genes of pAMβ1, a broad-host range plasmid originally described in E. faecalis, a gene homologous to the putative relaxase gene from small, mobilizable staphylococcal plasmid pSI94 and three types if insertions sequences (IS256, IS257, and IS1216), indicating a complex series of co-integration events (Rice and Carias, 1998; Bonafede et al., 1997).

Almost all Bla+ E. faecalis isolates identified so far belong to two clonal complexes, BVE (Bla+ Van+) endocarditis) and ACB (Argentina-Connecticut-Bla+), suggesting a limited number of transfer events of staphylococcal blaZ gene to E. faecalis (Nallapareddy et al., 2005). The BVE clonal complex includes the first Bla+ isolate HH22 (Nallapareddy et al., 2005) and the members of BVE were found spreading in five states (Delaware, Texas, Pennsylvania, Florida, and Virginia) of North America (Murray et al., 1991), including a seven-year outbreak in a Virginia hospital where they were named the mid-Atlantic clone (Seetulsingh et al., 1996). BVE was also responsible for bloodstream infections in a North Carolina hospital (Murdoch et al., 2002). Representatives of this clone are also often associated with glycopeptide resistance and possess a pathogenicity island (Nallapareddy et al., 2005), thus demonstrating a high pathogenic potential and adaptation to the hospital environment. The ACB clonal complex occurred in hospital outbreaks in Connecticut (Patterson et al., 1991) and Argentina (Murray et al., 1992). Application of multilocus sequence analysis (MLST) to BVE and ACB isolates clustered them into hospital-associated clonal complexes, CC2 and CC9, respectively (Ruiz-Garbajosa et al., 2006). The described above isolates from Boston carrying the Tn5385 transposon also belonged to CC9 (McBride et al. 2007). Both CC2 and CC9 are considered high-risk enterococcal clonal complexes, or HiRECCs (Leavis et al., 2006). Only a single isolate Bla+ from Beirut, Lebanon, did not belong to any of these two HiRECCs (Nallapareddy et al., 2005; Ruiz-Garbajosa et al., 2006). Diversity of blaZ sequence and variable presence of regulatory genes blaR1 and blal within the mid-Atlantic/BVE clone suggests that the bla gene cluster has originated from more than one source or the gene cluster has diverged significantly after acquisition (Tomayko et al., 1996).

**Current epidemiology of penicillin resistance in E. faecalis**

The resistance to penicillin in clinical isolates of E. faecalis remains a relatively rare phenomenon. A large TEST study collecting isolates in 266 centers in North and Latin America, Asia and Europe in 2004–2006 reported 100% susceptibility to penicillin and ampicillin among 2701 isolates of E. faecalis (Reinert et al.,
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Analysis of the Surveillance Network databases, collecting susceptibility data of predominant ICU pathogens during 2000–2002 from 650 hospitals in Europe (France, Germany and Italy), Canada, and the US revealed ampicillin resistance ranging from 0.2% (France) to 4.7% (Italy) among 7865 isolates of *E. faecalis* (Jones et al., 2004). Some of national studies recently report much higher ratios of penicillin resistance (Table II). In general, little is known about clonal relationships of current penicillin-resistant *E. faecalis*. Recently, twenty *E. faecalis* isolates from bloodstream infections from seven hospitals in Denmark collected in 2007 displaying reduced susceptibility to penicillin (MIC > 16 µg/ml by the Etest method and 4–8 µg/ml by the broth microdilution method) and full ampicillin susceptibility were reported. Most of these isolates (17) belonged to the same sequence type ST6 of CC2. The mechanism underlying this phenotype was not studied (Guardabassi et al., 2010). Pulsed-field gel electrophoresis (PFGE) analysis of 90 penicillin-resistant but ampicillin-susceptible isolates from Greece revealed that the majority (52) of them belonged to a single genotype while the remaining 38 isolates were grouped in five genotypes (Metzidie et al., 2006). None of these isolates produced β-lactamase (Table II).

### Conclusions and perspectives

Penicillin and ampicillin play an important role in the treatment of serious infections caused by *E. faecalis* and the development of resistance to these drugs implies serious clinical problems, especially as it excludes a synergistic bactericidal effect of β-lactams in combination with aminoglycosides. The first cases of ampicillin-resistant *E. faecium* (AREfm) were identified in the early 1980s. AREfm isolates have emerged as a causative agent of nosocomial infections and outbreaks (Coudron et al., 1984). Generally, ampicillin resistance has become a typical trait for hospital-associated strains of *E. faecium*, in particular of CC17, a HiRECC found in hospitals all over the world. One of the first steps of hospital-adaptation of CC17 was acquisition of ampicillin resistance (Leavis et al., 2006; Galloway-Peña et al., 2009). Although still rare, penicillin resistance can be also acquired by *E. faecalis* via various mechanisms and is often associated with HiRECCs circulating in hospitals. Moreover, the determinants of the resistance, such as β-lactamase genes, have a potential to be transferred to susceptible strains due to their localization on pheromone-responsive plasmids and within conjugative transposons. These elements...
contribute to the resistance spread among different species of bacteria, such as acquisition of β-lactamase gene from staphylococci by E. faecalis (Murray et al., 1986b) and subsequent transmission of the trait to E. faecium (Coudron et al., 1992). Also chromosomal determinant, ppb5, may disseminate in the population of E. faecalis due to frequent recombination in this species (Ruiz-Garbajosa et al., 2006). It is necessary to observe phenomenon of penicillin-resistance among E. faecalis.

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Literatura


