Detection of the *optrA* gene among polyclonal linezolid-susceptible isolates of *Enterococcus faecalis* recovered from community patients

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29
30 **Abstract**

31 Dispersion of transferable oxazolidinone-resistance genes among enterococci poses a
32 serious problem to human health. Prompt detection of bacteria carrying these genes is
33 crucial to avoid their spread to multidrug-resistant bacteria. The aim of the study was to
34 describe the presence of *optrA*-positive isolates among enterococci in a Spanish hospital,
35 and to determine their genetic context and location through whole genome sequencing.
36 All enterococci recovered in a Spanish hospital (Hospital El Bierzo; HEB) from February
37 to December 2018 (n=443), with MICs to linezolid (LZD) ≥4 mg/L, were tested by PCR
38 for the presence of *cfr, optrA* and *poxtA* transferable genes. Only four *E. faecalis* isolates
39 (0.9%) had LZD MICs ≥4 mg/L and none of them was positive for *cfr* or *poxtA* genes.
40 However, the *optrA* gene was detected in three isolates collected from urine samples of
41 community patients, whose genomes were sequenced and subjected to bioinformatics
42 analysis. These isolates belonged to different clones: ST7, ST480 and ST585. In these
43 three isolates, the *optrA* gene was located on plasmids, associated with IS*1216* in different
44 arrays. In one isolate, the *optrA* plasmid co-exists with a second plasmid which carried
45 multiple resistance genes for different classes of antibiotics. Detection of *optrA*-positive
46 *E. faecalis* isolates in the community is a matter of concern. Spread of these bacteria into
47 hospital settings, particularly in those, like the HEB, where vancomycin-resistant
48 enterococci are endemic should be avoided, in order to preserve the efficacy of the last-
49 resort oxazolidinones.

50

1. Introduction

The oxazolidinones linezolid (LZD) and tedizolid are effective last-resort antimicrobials for treating infections, caused by multidrug-resistant (MDR) Gram-positive bacteria, including vancomycin-resistant enterococci (VRE).[1] Oxazolidinones inhibit protein synthesis at the initial stage by binding to the peptidyl transferase centre of the bacterial ribosome. Oxazolidinone resistance among Gram-positive bacteria (enterococci and staphylococci) remains low and it is mainly mediated by chromosomal mutations in the V domain of the 23S rRNA gene.[2] However, in the latest years three new oxazolidinone-resistance genes, which can be horizontally disseminated by plasmids: *cfr*-like, *optrA* and *poxtA*, have been discovered.[3–6] The *cfr*-like gene encodes a 23S rRNA methyltransferase which confers resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A (PhLOPS$_A$ phenotype).[3,6] The original variant of the *cfr* gene was first reported in a *Staphylococcus sciuri* isolate and was thereafter found in several Gram-positive and Gram-negative bacteria, being more prevalent in staphylococci.[3,6] New variants of this gene [*cfr(B), cfr(C), cfr(D)* and *cfr(E)*] have been already described, two of them [*cfr(B), cfr(D)*] also in enterococcal isolates.[6,7] On the other hand, both *optrA* and *poxtA* code for ribosomal protection proteins of the ABC-F family, which confer resistance to oxazolidinones, phenicols and also to tetracyclines in the case of *poxtA*.[4,5] The *optrA* gene was first reported in *E. faecalis* and *E. faecium* strains from humans and food-producing animals in China.[5] Since then, isolates carrying this gene have been recovered from animal, human and environmental samples, mainly in China but also in other countries of the Asia-Pacific region, Africa, North America, Latin America, and Europe (including Spain).[5,8–11] Lastly, *poxtA* gene was first reported in a clinical methicillin-resistant *S. aureus* isolate and subsequently in *E. faecalis* and *E. faecium* strains.[4,8] Linezolid-resistant *Enterococcus*, although uncommon, are

76 increasingly being detected in Spain as well as in many other countries worldwide, and
77 *optrA* has become the most prevalent mechanism of oxazolidinone resistance in *E.*
78 *faecalis*.[2,6,8,12,13] The potential spread of these genes between MDR Gram-positive
79 bacteria is a cause of concern, so a prompt detection of bacteria carrying them is required.
80 Accordingly, the aim of the present study was to investigate the presence of transferable
81 oxazolidinone-resistance genes among enterococci isolated from clinical samples from
82 "Hospital El Bierzo" (HEB), a 400-bed teaching hospital in Northern Spain, where
83 vancomycin-resistant enterococci (VRE) are endemic.[14] Additionally, the genomes of
84 isolates positive for such genes were sequenced and subsequently analyzed.

85 2. Materials and methods

86 2.1. Identification of linezolid resistant isolates

87 From February 2018 to December 2018 all enterococci with minimal inhibition
88 concentrations (MICs) to LZD ≥4 mg/L detected at the HEB were recovered. They were
89 obtained from either clinical samples of inpatients or from samples sent to the hospital by
90 primary health centers. Bacterial identification and antimicrobial susceptibility testing
91 (AST) was performed with the MicroScan System (MicroScan; Beckman Coulter, CA,
92 USA). The antimicrobials tested by broth microdilution with the MicroScan System
93 included ampicillin, ciprofloxacin, daptomycin, gentamicin, levofloxacin, linezolid,
94 synercid, teicoplanin, tetracycline and vancomycin. Enterococci with MICs of LZD ≥4
95 mg/L were tested for susceptibility to chloramphenicol by disk-diffusion, and MICs of
96 tedizolid were determined by E-test strips (Liofilchem s.r.l., Roseto degli Abruzzi, Italy).
97 All AST results were interpreted according to the Clinical and Laboratory Standards
98 Institute guidelines.[15] For all isolates, screening of *cfr* (original variant) and *optrA* genes
99 was performed by PCR as previously reported,[16] and PCR detection of *poxtA* was carried

100 out with primers *poxtA*-F (TTCCACCTCTAAGGGAACTTGTG) and *poxtA*-R
101 (TATGCAGAGGAACAGCGGATT).[17]

102 2.2. Whole genome sequencing of isolates positive for transferable oxazolidinone
103 resistance genes and bioinformatics analysis

104 The genomes of three *E. faecalis* isolates positive for a transferable oxazolidinone
105 resistance gene (*Efae*-HEB1, *Efae*-HEB2 and *Efae*-HEB3) were sequenced using the
106 Illumina platform, and bioinformatic analysis was performed. Genomic DNA was
107 extracted with the "GenElute Bacterial Genomic DNA Kit" (Sigma Aldrich), following
108 the manufacturer's instructions. Libraries (ca. 500 bp fragment size) were prepared with
109 the TruSeq PCR-free DNA Sample Preparation Kit at the "Plataforma de Genómica y
110 Bioinformática, Centro de Investigación Biomédica", La Rioja (Spain), and sequenced
111 on a HiSeq 2000 (Illumina) to generate 130-150 nt paired-end reads. Assembly of the
112 reads into contigs was achieved with the VelvetOptimiser.pl script of the Velvet
113 assembler implemented in PLACNETw (https://openebench.bsc.es/tool/placnetw).
114 PLACNETw also distinguishes contigs belonging to the chromosome or to plasmids,
115 hence facilitating plasmid reconstruction.[18] The assembled contigs were annotated by the
116 NCBI Prokaryotic Genome Annotation Pipeline
117 (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/). MultiLocus Sequence
118 Typing (MLST), as well as identification of resistance determinants, comprising
119 chromosomal mutations in the 23S RNA gene and acquired resistance genes, were
120 accomplished *in silico*, using online tools, including MLST 2.0, LRE-Finder 1.0,
121 ResFinder 4.1 (which identifies genes leading to linezolid resistance, including *optrA*,
122 *poxtA* and all variants of *cfr* reported so far) and PlasmidFinder 2.1 from the Center for
123 Genomic Epidemiology (www.genomicepidemiology.org) of the Technical University of
124 Denmark,[19–24] in conjunction with PLACNETw.[18] The genetic context of the

125 oxazolidinone resistance genes was determined by detailed analysis of the annotated
126 contigs using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The complete nucleotide
127 sequence of two resistance plasmids found in one of the isolates (*Efae*-HEB2) was
128 generated by assembling the contigs from each plasmid identified by PLACNET by PCR
129 amplification (using the primers shown in Tables S1 and S2), followed by Sanger
130 sequencing of overlapping amplicons (performed at STAB VIDA, Lisbon, Portugal).

131 2.3. Accession numbers

132 The genomes of *Efae*-HEB1, *Efae*-HEB2 and *Efae*-HEB3, and plasmids p*Efae*-HEB2-1
133 and p*Efae*-HEB2-2, were deposited in the GenBank database under accession numbers
134 JAAOEK000000000, JAAOEL000000000, JAAOEM000000000, OM574792 and
135 OM574793, respectively.

136 **3. Results and discussion**

137 3.1. Screening of transferable oxazolidinone-resistance genes in clinical isolates
138 of *Enterococcus faecalis* from community patients

139 During the period of study, a total of 443 enterococci were isolated and assigned to *E.*
140 *faecalis* (n=367) or *E. faecium* (n=76). Only four isolates (0.9%) had LZD MICs ≥4 mg/L,
141 and they were identified as *E. faecalis* resistant to chloramphenicol. Three of them (*Efae*-
142 HEB1, *Efae*-HEB2 and *Efae*-HEB3), proved to be positive for the *optrA* gene, while
143 neither *poxtA* nor any of the reported *cfr* variants were detected (Table 1). The fourth
144 isolate, which did not contain any of the transferable oxazolidinone resistance genes
145 tested, could not be further characterized because it was not kept in the strain collection
146 of the hospital.

147 According to the last results published by the SENTRY Antimicrobial Surveillance
148 Program, the worldwide prevalence of LZD resistance in enterococci is very low, with

149 only 26 *optrA*-positive *E. faecalis* isolates detected among a total of 26,648 enterococci
150 (ca. 0.1%).[8] This frequency is lower than the obtained in the present study (0.7% of the
151 total enterococci), with three *optrA* positive isolates obtained from urine samples of
152 unrelated patients in the community suspected to suffer urinary tract infections (Table 1).
153 The samples were sent to the HEB Microbiology laboratory from primary health centers
154 to confirm the diagnosis and identify the etiological agent. None of the patients had
155 antecedents of recent therapy with oxazolidinones, although two had received other
156 antimicrobials, like quinolones, cefuroxime or azithromycin in the last 3 months. The
157 isolates studied belonged to different clones, revealing a polyclonal spread of *optrA*-
158 positive *E. faecalis* in the community. *Efae*-HEB1 and *Efae*-HEB-2, belonged to clones
159 previously shown to carry the *optrA* gene, i.e. ST480 and ST585, respectively.[6,8–10,13,25–
160 29] The remaining isolate (*Efae*-HEB3) was assigned to the new ST7, which is a single
161 locus variant (affecting the *pstS* gene) of ST317.

162 3.2. The *optrA* genes and encoded proteins

163 After sequencing the genomes of the three isolates, the analysis of the amino acid
164 sequences translated from the *optrA* genes identified two variants of the OptrA proteins
165 (Table 1). The protein of *Efae*-HEB2 coincided with $\text{OptrA}_{\text{E349}}$, considered as wild-type
166 since it is encoded by plasmid pE349, where the *optrA* gene was originally detected.[5] In
167 contrast, the OptrA proteins of *Efae*-HEB1 and *Efae*-HEB3 contain the Tyr176Asp and
168 Thr481Pro substitutions corresponding to the DP variant previously reported in clinical
169 isolates from hospitals in China[12,26,30] and recently found in Spain.[6] Regardless of the
170 variant, the three *optrA* positive *E. faecalis* isolates had the same linezolid MIC of 4 mg/L,
171 which was previously associated with some but not all of the isolates producing either the
172 wild-type protein or the DP variant in China. In fact, it has been already noticed that the
173 number and type of amino acid substitutions reported so far in OptrA has little effect on

the level of linezolid resistance.[31] It is noteworthy that both CLSI and EUCAST consider MICs >4 mg/L as “resistant”, while a MIC equal to 4 mg/L is regarded as ”intermediate” by CLSI and “susceptible” by EUCAST.[15,32] According to this, the dispersal of the *optrA* gene among enterococci may well be underestimated if present in isolates with MIC of 4 mg/L, particularly if EUCAST breakpoints are used. The recent introduction of chloramphenicol in the AST panels for enterococci could aid the detection of transferable oxazolidinone-resistance genes. Expert alert messages can be created in the automated AST systems if a Gram-positive bacterium shows MICs to LZD ≥4 mg/L together with chloramphenicol resistance. Bacteria which fulfill these criteria can be analyzed by multiplex-PCR for simultaneous detection of transferable oxazolidinone-resistance genes.[33]

3.3. Genetic context of the *optrA* genes

The *optrA* genes of the three isolates were located on plasmids, named p*Efae*-HEB1, p*Efae*-HEB2-1 and p*Efae*-HEB3. In the three plasmids, the *fexA* gene, encoding a chloramphenicol/florfenicol efflux protein of the major facilitator superfamily (MFS), was located upstream of *optrA* (Figure 1). In p*Efae*-HEB1 and p*Efae*-HEB3, the *optrA* region was flanked by IS*1216* elements placed in the same orientation, although in the latter plasmid the second copy was truncated. The DNA comprised between the two copies of the IS differed by the *erm*(A)-like gene, deleted and intact in p*Efae*-HEB1 and p*Efae*-HEB3, respectively, and by the *ubiE* gene (for ubiquinone/menaquinone C-methyltransferase), present in p*Efae*-HEB3 but not in p*Efae*-HEB1. The organization of the p*Efae*-HEB3 *optrA* region roughly coincides with that reported for p10-2-2, a plasmid carried by a pig isolate of *E. faecalis* ST59 from China in which two entire copies of IS*1216* are nevertheless present (Figure 1).[25] In contrast, the structure of the p*Efae*-HEB1 *optrA* region does not exactly match that of any other published so far. Finally, in the case

199 of p*Efae*-HEB2-1, a single copy of IS*1216* was detected downstream of the *optrA* gene,
200 but not at the opposite end, and neither *erm*(A) nor *ubiE* were found. The entire nucleotide
201 sequence of p*Efae*-HEB2-1, which coexists with p*Efae*-HEB2-2 (see below) in the same
202 isolate, was determined (Figure 1). p*Efae*-HEB2-1 consists of ca. 53.3 kb with a GC
203 content of 35%, slightly lower than that of the *E. faecalis* chromosome (37.4%). It appears
204 to be a sex pheromone responsive plasmid closely related to pEF10748 (Figure 1), a
205 conjugative *optrA* plasmid with a high transfer frequency.[27] PlasmidFinder failed to
206 identify the p*Efae*-HEB2-1 replicon, but a BLASTn search of its *repA* gene yielded 100%
207 identity (with 100% coverage) with the *repA* gene of pEF10748 previously assigned to
208 rep_9.[27] Like the former plasmid, p*Efae*-HEB2-1 contains multiple sex pheromone
209 response genes, including *traB*, *prgA*, *prgB*, *prgC*, *prgU*, *prgR*, *prgS* and *prgT*. Apart
210 from pEF10748, p*Efae*-HEB2-1 has a high percentage of identity (more than 99.5%) with
211 plasmids of *E. faecalis* collected from raw-frozen dog food in Portugal (pAPT110;
212 accession number MW012677)[34] and from a human urine sample in the United Kingdom
213 (strain TM6249; contig 000003; accession number OD940433). Taken together, the
214 results obtained are consistent with the previously observed variation in the genetic
215 context of the *optrA* gene,[25,27] and with the involvement of IS*1216* in the evolution of
216 antimicrobial drug resistance/generation of resistance regions in Gram-positive
217 bacteria.[35]

218 3.4. Sequence analysis of p*Efae*-HEB2-2, a second plasmid of *Efae*-HEB2
219 carrying multiple antibiotic resistance genes

220 Apart from *optrA* and *fexA*, additional genes conferring resistance to aminoglycosides,
221 trimethoprim, fenicols (other than *optrA* and *fexA*), and antibiotics of the MLS_B
222 (macrolides-lincosamides-streptogramin B), LS_AP (lincosamides, streptogramin A and
223 pleuromutilins) and L (lincosamide) groups, were identified on plasmids or the

224 chromosome of the analyzed isolates (Table 1). In *Efae*-HEB2, many resistance genes
225 were carried by plasmid p*Efae*-HEB2-2, whose entire nucleotide sequence was
226 determined (Figure 2). This plasmid comprises 75.4 kb with a GC content of 34.6%,
227 similar to that of p*Efae*-HEB2-1. According to PlasmidFinder it contains three different
228 replicons, rep_{7a}, rep_{9a} and rep_{US43}, showing 100%, 95.3% and 99.9% identity with genes
229 of the prototype plasmids for each of these *rep* families (pS194, pAD1 and pDOP1
230 plasmids, respectively). Most of the resistance genes carried by p*Efae*-HEB2-2 (Table 1)
231 were distributed into two clusters (Figure 2). The largest cluster, located between rep_{9a}
232 and rep_{7a}, contains genes conferring resistance to aminoglycosides [*aacA*-*aphD*, *aadE*
233 (three copies), *apt*, *spw*, *sat4* and *aphA3*], trimethoprim (*dfrG*), or involved in the MLS
234 [three copies of *erm*(B)], LS_AP [*lsa*(E)] and L [*lnu*(B)] phenotypes. This entire complex
235 resistance region was only detected in unpublished plasmids of *E. faecalis*, which are
236 identical: contig 000002 of strain TM6294 (accession number OD940432) or nearly
237 identical: p508-2 and pEFS17 (MK465702 and CP085290) to p*Efae*-HEB2-2, as well as
238 in plasmid p505-1 of *E. faecium* (MK465703). However, an internal sub-cluster
239 comprising *lsa*(E) and *lnu*(B), together with a number of additional resistance genes, is
240 widely distributed in Gram-positive bacteria.[36] For instance, it was found in the
241 chromosome of *Erysipelothrix rhusiopathiae* Ery-11 (accession numbe. KP339868), as
242 well as in plasmids of pEf418, pXD4 and pV7037 (AF408195, KF421157 and JX560992)
243 of *E. faecalis*, *E. faecium* and *S. aureus*, respectively (Figure 2). The *lsa*(E) and *lnu*(B)
244 genes encode an ABC-F type ribosomal protection protein and a lincosamide
245 nucleotidyltransferase, responsible for the LS_AP and L phenotypes, respectively. In some
246 cases, the *lsa*(E)-*lnu*(B) cluster was flanked by one (pXD4 and pV7037) or two (pEf418)
247 copies of IS*1216*. In p*Efae*-HEB2-2, a single copy of this insertion sequence was found
248 downstream of *dfrG*, outside the cluster. Apart from the above mentioned resistance

249 genes, the *tet*(M) and *tet*(L) genes, which encode a ribosome protective protein and a
250 efflux protein of the major facilitator superfamily conferring resistance to tetracycline,
251 and the *cat* gene encoding a chloramphenicol acetyltransferase, were also carried by
252 p*Efae*-HEB2-2. In Gram positive bacteria, *tet*(M) is often mobilized by conjugative
253 transposons of the Tn*916* family, which can also harbor additional antimicrobial
254 resistance genes.[37] For instance, *tet*(L) and *cat* have been shown to co-reside with *tet*(M)
255 in Tn*6248* (25,963 bp; accession no. KP834592), which shares a large part of its sequence
256 (18,032 pb) with Tn*916*. In p*Efae*-HEB2-2 these genes are carried by a truncated version
257 of Tn*6248* (18,727 bp) that lacks the genes for insertion and excision of the transposon.
258 As indicated above, p*Efae*-HEB2-2 is closely related to *E. faecalis* plasmids pE508-2
259 carried by a ST256 isolate from a fecal swine sample in China,[30] pEFS17 of unknown
260 origin, and a plasmid (contig 000002) found in strain TM6294, which also harbors an
261 *optrA* plasmid related to p*Efae*-HEB2-1 (contig 000003; accession number OD940433).
262 Interestingly, both Efae-HEB2 and TM6294, for which the ST is not available, derived
263 from urine samples. These results provide evidence that the newly detected plasmid
264 carrying multiple resistance genes has already been established in different *E. faecalis*
265 clones (at least ST585 and ST256), from different environments (isolates derived from
266 swine and human samples), and geographical regions (Europe and China).

267 3.5. Conclusions

268 *E. faecalis*, including *optrA*-positive isolates, are usually susceptible to beta-lactams,
269 which are the first line antimicrobials to treat the infections they cause. In fact, this is the
270 case of the isolates reported in the present study. However, these isolates could serve as
271 a reservoir of the *optrA* gene, which is mainly located in plasmids and could be
272 horizontally disseminated, not only between members of this species but also to other
273 MDR pathogens such as *E. faecium*, including vancomycin resistant isolates. This would

mimic the transfer of the *cfr* gene from coagulase-negative staphylococci into methicillin-resistant *S. aureus*.[8,38] Prompt detection and surveillance of transferable oxazolidinone-resistance genes among enterococci are crucial to avoid their spread in hospital settings, in order to preserve the efficacy of these last-resort antibiotics. This particularly true in the context of MDR as well as in hospitals, like the HEB, where VRE are endemic.

Supplementary information accompanies this paper: Table S1, Table S2.

Disclaimer

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