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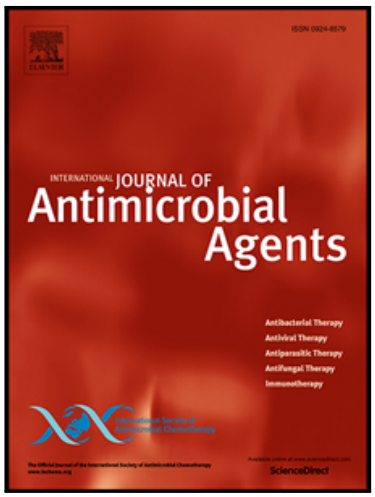

Interregional spread in Spain of linezolid-resistant Enterococcus spp. isolates carrying the optrA and poxtA genes

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HIGLIGHTS

- Emerge of linezolid-resistant *Enterococcus faecalis* carrying *optr*A in Spain.
- High dissemination capability of *optr*A-carrying *E. faecalis* belonging to ST585 and ST480.
- Co-existence of *optrA* and *poxtA* plasmid-born genes in a clinical setting.

Interregional spread in Spain of linezolid-resistant *Enterococcus* spp. isolates carrying the *optrA* and *poxtA* genes

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Running title: *OptrA* and *poxtA* genes in *Enterococcus* spp.

ABSTRACT

The emergence of linezolid-resistant *Enterococcus* spp. (LRE) due to transferable resistance determinants is a matter of concern. To understand the contribution of the plasmid-encoded *optrA* and *poxtA* genes to the emergence of LRE, we analysed clinical isolates from different Spanish hospitals submitted to the Spanish Reference Laboratory from 2015-2018. Resistance mechanisms to linezolid were screened in all isolates by PCR and sequencing. Genetic relatedness of all *Enterococcus* spp. carrying *optrA* and *poxtA* was studied by PFGE and MLST, and antibiotic susceptibility was tested by broth microdilution using EUCAST standards. A total of 97 LRE isolates were studied; in 94 (96.9%) of them, at least one resistance determinant was detected; 84 (86.6%) isolates presented a single resistance mechanism as follows: 45 (53.6%) carried the *optrA* gene, 38 (45.2%) the G2575T mutation, and one (1.2%) the *poxtA* gene; in addition, five (5.1%) carried both the *optrA* gene and the G2575T mutation, and five (5.1%) both *optrA* and *poxtA* genes. The *optrA* gene was more frequent in *E. faecalis* (83.6%) than in *E. faecium* (11.1%) and was mainly associated with community-acquired urinary tract infections. Carriage of the *poxt*A gene was more frequent in *E. faecium* (13.9%) than in *E. faecalis* (1.6%). Among *optrA*-positive *E. faecalis* isolates, two main clusters were detected by PFGE. These two clusters belonged to ST585 and ST480 and were distributed throughout 11 and 6 Spanish provinces, respectively. This is the first description of LRE carrying the *poxtA* gene in Spain, including co-existence of *optrA* and *poxtA* in five isolates.

Keywords: linezolid-resistance; transferable-resistance; optrA; poxtA; Enterococcus

1. Introduction

Enterococcus spp., mainly *Enterococcus faecium* and *Enterococcus faecalis*, are major opportunistic pathogens that cause health-care related infections worldwide [1] as well as community-acquired urinary tract infections [2]. Vancomycin-resistant enterococci (VRE) are of medical and public health importance due to their association with serious multidrug-resistant infections and persistent colonization [3]. In fact, the World Health Organization (WHO) has included vancomycin-resistant *E. faecium* as a "high priority" in the global priority list of antibiotic-resistant bacteria to guide research, discovery and development of new antibiotics [4]. Since its approval in 2000, linezolid has become one of the few therapeutic options available against VRE [5]. Although the global prevalence of linezolid-resistant enterococci (LRE) remains low, the emergence of LRE isolates is concerning [6,7].

Resistance to linezolid in enterococci is often mediated by chromosomal mutations in domain V of the 23S rRNA gene, mainly at position G2576, and/or in the L3, L4, and L22 ribosomal proteins [8]. In addition to mutation-driven resistance, transferable resistance determinants, such as *cfr*-like, *optrA*, and *poxtA* genes, have also been described [9–12]. The *cfr*-like genes encode a methylase that modifies position A2503 of the 23S rRNA gene [9].

The novel plasmid-borne *optrA* and *poxtA* genes, involved in transferable resistance to linezolid, encode related ATP-binding cassette F (ABC-F) proteins, resulting in either resistance to or elevated minimal inhibitory concentrations (MICs) for oxazolidinones and phenicols [10,11]. Additionally, *poxtA* decreases susceptibility to tetracyclines [12].

In 2015, the *optrA* gene was first described in enterococcal isolates from human and

animals originating from China [10]. Since then, it has been increasingly detected, mainly in *E. faecalis*, revealing its great capacity for dissemination [11,13–15]. The *poxtA* gene was detected for the first time in a clinical MRSA strain that was highly resistant to linezolid and also carried the *cfr* gene [12].

Accordingly, the aims of this study were to investigate the presence of the *optrA* and *poxtA* genes in a country-wide collection of LRE submitted to the Spanish National Reference Laboratory for Antibiotic Resistance between 2015 and 2018, and to gain insight into the microbiological features and molecular epidemiology of isolates carrying the *optrA* and *poxtA* genes in Spain.

2. Material and methods

2.1. Study design and bacterial isolates

This study was performed by the unrestricted but non-mandatory national Spanish Antibiotic Resistance Surveillance Program operated by the Public Health Instituto de Salud Carlos III. We included all *E. faecalis* and *E. faecium* isolates resistant to linezolid (MIC > 4 mg/L) submitted to this program between January 2015 and December 2018. Only the first isolate obtained from a given patient was analysed. Identification was initially performed by each of the participating laboratories using different standard methods and further confirmed by 16S ribosomal DNA sequencing.

2.2. Molecular characterisation of resistance genes

The presence of *cfr-like*, *poxtA*, and *optrA* genes was screened in all LRE isolates by PCR and DNA sequencing [9,10,16,17]. Additionally, an internal region of domain V of the 23S rRNA gene was amplified by PCR and subsequently sequenced to detect the

presence of mutated alleles [18].

2.3. Molecular epidemiology

The genetic relationship between the *optrA*-carrying LRE isolates was examined by pulsed-field gel electrophoresis (PFGE) after total chromosomal DNA digestion with *Sma*I [19]. Moreover, multilocus sequence typing (MLST) was performed using the pubMLST schemes for representative *E. faecalis* (https://pubmlst.org/efaecalis/ and *E. faecium* (https://pubmlst.org/efaecium/) isolates of all clusters or single profiles previously detected by PFGE.

2.4. WGS and genetic environment

WGS was performed in four isolates: two *E. faecalis* carrying *optrA* gene and two isolates with both *optrA* and *poxtA* genes, one *E. faecium* and one *E. faecalis*. Genomic DNA paired-end libraries were generated using the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA). These libraries were sequenced using an Illumina HiSeq500 next-generation sequencer with 2×150 bp paired-end reads (Illumina). In order to disclose *optrA* and *poxtA* gene environments, assembled contigs were compared against NCBI nucleotide and CARD 2020 [20] database using BLAST+ [21]

2.5. Antibiotic susceptibility testing

Linezolid minimum inhibitory concentration was determined by Etest (bioMérieux, Marcy-l'Étoile, France) in all isolates included in the study. Additionally, antibiotic susceptibility testing was performed in all *optrA*- and *poxtA*-positive isolates

using broth microdilution (Pos MIC E37; MicroScan, Beckman Coulter**,** CA, USA) and interpreted according to EUCAST breakpoints [22]. Susceptibility to daptomycin and chloramphenicol was determined according CLSI criteria because EUCAST does not list breakpoints for these antibiotics [23]. *Enterococcus faecalis* ATCC 29212 was used as a quality control strain.

2.6. Statistical analyses

Clinical and epidemiological differences between *Enterococcus* spp. resistant to linezolid via presence of the *optrA* gene or the G2575T mutation of the 23S rRNA gene were assessed using Fisher's exact test. The null hypothesis was rejected when a *P* value less than or equal to 0.05 was calculated. Statistical analyses were performed using GraphPad Prism software (version 7.02; GraphPad Software, Inc., San Diego, CA, USA)

3. Results

3.1. Bacterial isolates and mechanisms of linezolid resistance

Between January 2015 and December 2018, 97 unduplicated LRE isolates of which 61 (62.9%) were *E. faecalis* and 36 (37.1%) *E. faecium*, were submitted to the Spanish Reference Laboratory for Antibiotic Resistance. The isolates originated from 24 hospitals located in 17 Spanish provinces (Figure 1).

Of the 61 *E. faecalis*, 52 (85.2%) presented a single resistance mechanism: 45 (86.5%) carried the *optrA* gene, and 7 (13.5%) had G2575T mutation; six (9.8%) additional isolates had double resistance mechanisms: 5 (83.3%) *optrA* gene plus G2575T mutations, and 1 (16.7%) carried both *optrA* and *poxtA* genes. Of the 36 *E.*

faecium, 32 (88.9%) presented a single resistance mechanism: 31 (96.9%) G2575T mutations and 1 (3.1%) the *poxtA* gene; 4 (11.1%) additional isolates carried both *optrA* and *poxtA* genes. No *cfr-like* genes were detected, and in three linezolid-resistant *E. faecalis* none of the tested mechanisms were identified.

Overall, the *optrA* gene was more frequently found in *E. faecalis* (83.6%) than in *E. faecium* (11.1%) ($P < 0.0001$), whereas the G2575T mutation was more frequently detected in *E. faecium* (86.1%) than in *E. faecalis* (19.7%) ($P < 0.0001$).

3.2. Statistical clinical and epidemiological differences between Enterococcus spp. resistant to linezolid either producing the optrA gene or the G2575T mutation.

The presence of the *optrA* gene was statistically more frequent than the G2575T mutation in community-acquired urinary tract infections from females ($P < 0.03$), whereas the G2575T mutation occurred more frequently in nosocomial infections in males over 65 years of age (Table 1).

3.3. OptrA- and poxtA-mediated mechanisms of linezolid resistance in Enterococcus spp.

Isolates carrying the *optrA* gene came from 17 different hospitals located in 13 Spanish provinces (Figure 1). The distribution of *optrA* per year was 9 cases in 2015, 11 cases in 2016, 17 cases in 2017 and 18 cases in 2018 (Table S1). The isolates were collected from genitourinary samples (65.5%), wounds/abscesses (21.8%), blood, ascitic- and synovial fluids (10.9%) and from rectal samples (1.8%).

Five isolates carrying both the *poxtA* and *optrA* genes were also found in four different hospitals from three different provinces throughout the study period.

Remarkably, four of these five isolates caused invasive nosocomial infections: abdominal abscess, joint infection, ascitic fluid infection and bacteremia (Table S1).

3.4. Molecular epidemiology of Enterococcus spp. carrying the optrA and poxtA genes

In *E. faecalis* carrying the *optrA* gene, PFGE analysis revealed a total of 32 different PFGE profiles among the 51 isolates analysed. Considering a genetic linkage greater than or equal to 85%, seven well-defined PFGE clusters (C1–C7), containing two or more isolates, were detected. Two main clusters, C1 and C2, included 20 and 10 isolates, respectively, whereas clusters C3–C7 had two isolates each. In addition, 11 isolates with single PFGE profiles were detected (Figure 2).

By MLST typing, the 51 *E. faecalis* isolates carrying the *optrA* gene belonged to seven sequence types (STs; ST585, ST480, ST16, ST324, ST6, ST25, and ST476). The two most predominant STs were ST585 and ST480, encompassing 62.7% of the 51 isolates studied (Figure 2). All the 20 isolates belonging to C1 by PFGE were ST585, spanning 11 different provinces, whereas 12 isolates submitted from 6 provinces were ST480, 10 of them belonging to PFGE C2 and 2 belonging to PFGE C7 (Figure 1). It is worth noting that one of the ST480/C2 isolates also carried the *poxtA* gene (Figure 2).

All four *E. faecium* isolates carrying the *optrA* gene that also carried the *poxtA* gene, belonged to ST25 (n = 1), ST323 (n = 1) and ST17 (n = 2); they came from different hospitals and were unrelated by PFGE (Table S1). The single *E. faecium* isolate carrying the *poxtA* but not the *optrA* gene was ST117.

3.4. Genetic environment of optrA and poxtA genes

In all sequenced strains *oprtA* and *poxt*A genes were located in plasmids, and ISs of the type IS1216 were found upstream and downstream of both genes, as previously described [12,24]. In two *E. faecalis*, *fexA* resistance gene was located downstream of *optrA* gene, similar to plasmid pE161 described in an *E. faecalis* strain hosted in a human from China [24]. In the sequenced *E. faecium* isolate, no *fexA* gene was detected and *ermA1* gene was located downstream of *optrA*, similar to plasmid pFX13 [24], but two additional resistance genes (*sat*4 and *aph*(3')-IIIa) were located upstream to *optrA* gene.

In *E. faecalis* isolate harbouring *poxtA* gene, it was only constructed the *IS1216E-poxtA-IS1216E* segment because the nucleotide sequence amplicon was not large enough to identify additional resistance genes. In *E. faecium* isolate, upstream of *IS1216E-poxtA-IS1216E* segment, two additional insertion sequences (IS1216) and the resistance genes *tet*(M) and *tet*(L) were detected, as described previously in *S. aureus* AOUC-0915 (MF095097) [25].

3.5. Antibiotic susceptibility testing

Higher linezolid MIC values were observed among isolates expressing the G2576U mutation than in those containing the *optr*A gene (Table 2).

The 51 *optrA*-positive *E. faecalis* isolates had a linezolid MIC range of 8–256 mg/L, with MIC_{50} and MIC_{90} values of 16 and 32 mg/L, respectively. All isolates were susceptible to ampicillin, glycopeptides, and tigecycline, and resistant to chloramphenicol. Nine (17.6%) isolates presented an intermediate susceptibility to daptomycin, and 31 (60.8%) had high resistance levels to gentamicin.

The four *E. faecium* isolates, which were positive for both the *optrA* and *poxtA* genes, and the *E. faecium* isolate, which was positive for the *poxtA* gene, were susceptible to glycopeptides, daptomycin and tigecycline, and resistant to ampicillin, chloramphenicol and had high resistance levels to gentamicin and streptomycin.

4. Discussion

Our multicentre study demonstrates that in Spain the emergence and spread of linezolid-resistant *E. faecalis* in clinical samples is mainly due to the transferable resistance genes, *optrA* and *poxtA*. Prior to this study, the *optrA* gene had only been described in Spain in six *E. faecalis* isolates from a single teaching hospital [15]. Moreover, we here describe the largest collection of isolates carrying the *optrA* gene in Europe; similar studies have also been conducted at the National Reference Centres in France [17] and Germany [14].

An association between distinct linezolid-resistance mechanisms and bacterial species has been previously described [14,26,27]; in fact, resistance to linezolid was almost exclusively limited to *E. faecium* until the emergence of the *optrA* gene. In the present study, we detected a higher frequency of the *optrA* gene than 23S rRNA mutations, probably due to its easy transference by plasmids [28].

Although infrequent in our study compared to the *optrA* gene, the presence of *poxtA* in some isolates is concerning [29]. The majority of *poxtA*-positive isolates detected were *E. faecium* carrying *optrA* (66.7%). Previously, a porcine enterococcal strain was described as co-harbouring both *optrA* and *poxtA* genes in the same plasmid [28]. To the best of our knowledge, the presence of both genes has not been reported previously in a clinical setting, and the implications of the spread of these transferable

resistance genes should be monitored in the future [28,30].

Additionally, secondary mechanisms such as biofilm formation and cell wall thickening, can lead to low-level of linezolide resistance, especially in *E. faecalis* [31]. This fact might be postulated as an alternative cause of resistance for the 3 *E faecalis* with no mechanism detected. However further research is warranted.

Regarding the population structure, ST585 and ST480 were the two main STs of *E. faecalis* associated with *optrA* in our study which is in accordance with previous reports [14,15,17,32]. In addition, we detected different geographical origins for these two STs, even within the same PFGE cluster, suggesting interregional spread. However, our results also reveal some genetic diversity among *optrA*-positive *E. faecalis* circulating in Spain, as widely documented in several studies [27,33].

Consistent with previous studies, the genetic context of *optrA* and *poxtA* revealed that these genes appear to be flanked by IS*6* family elements contributing to its transferability among enterococci and other species [12,24,28]. Moreover, the fact of finding two additional insertion sequences in the context of *E. faecium poxtA* gene might remark the important role of these IS sequences in the dissemination of these genes. The detection of two additional resistance genes, such as *sat*4 and *aph*(3')-IIIa, located upstream of *optrA* gene reveals the great diversity of these plasmid-borne structures. In *E. faecium* isolate that was sequenced, the contig size was not long enough to confirm that *oprtA* and *poxtA* genes were in the same plasmid, but the environment detected in *optrA* gene was described in plasmids that also harbour *poxtA* gene [25] suggesting that both genes could be located in the same composite transposon.

Hospital consumption of linezolid (WHO code J01XX08) increased in Spain from 0.0249 DID (defined daily dose per 1000 inhabitants per day) in 2012 to 0.0345 DIDs in 2017 (38.5% increase) [34]. The emergence of LRE has been associated with previous linezolid administration in admitted patients[6,35], mainly due to chromosomal mutations in the 23S rRNA gene [33,36]. In this study, we observed different epidemiological contexts in linezolid-resistance due to *optrA* gene acquisition and mutation-driven linezolid resistance (Table 1). The high frequency of *optrA* in strains isolated from females with community-acquired urinary tract infections, together with its clonal distribution among different geographical areas, seems to indicate that it did not originate from a nosocomial site. Recent studies have suggested that the *optrA* and *poxtA* genes arose due to the use of the phenicol antibiotic, florfenicol, in animals [12,37,38], as previously observed in other resistance-acquisition events related to ribosome-targeting agents [39]. This finding could also explain their clonal dissemination throughout different geographical regions.

Although we did not detect co-resistance between glycopeptides and linezolid in isolates carrying the *optrA* and *poxtA* genes, four isolates with mutations in the 23S rRNA gene also carried the *vanA* gene (data not shown). The emergence of enterococci strains resistant to both linezolid and vancomycin represents a great threat to current treatment regimens [40,41].

In conclusion, linezolid-resistant *E. faecalis* harbouring transferable *optrA* and *poxtA* genes is spreading in Spain mainly due to the dissemination of sequence types ST585 and ST480. Our data reveal several findings that require active surveillance: (i) identification of different epidemiological contexts between acquired linezolid resistance due to the presence of the *optrA* gene and mutation-driven linezolid

resistance; (ii) high dissemination capability in different geographic regions of *optrA*-carrying *E. faecalis*; and (iii) co-existence of *optrA* and *poxtA* genes in *E. faecium* isolates belonging to different clones.

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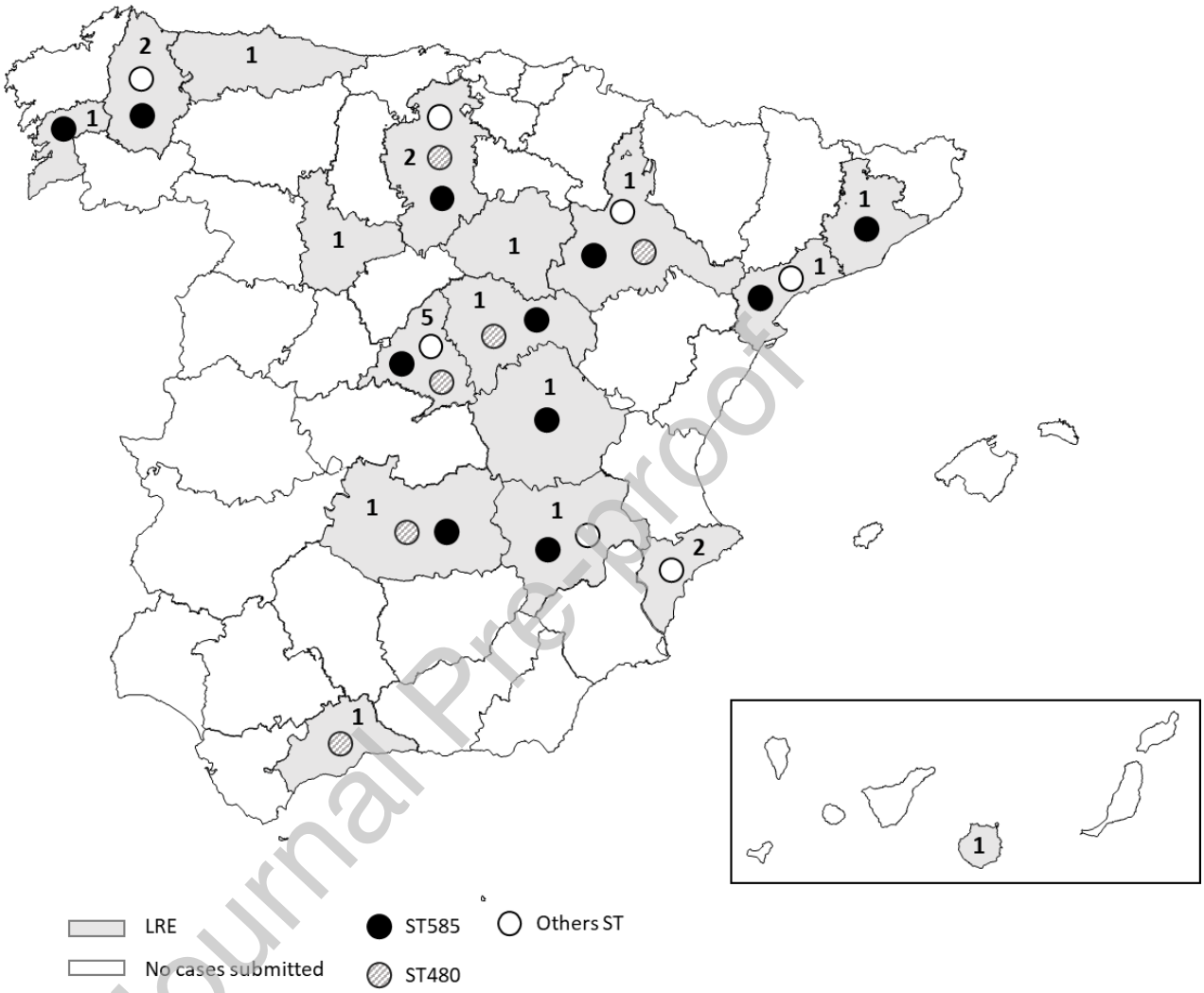
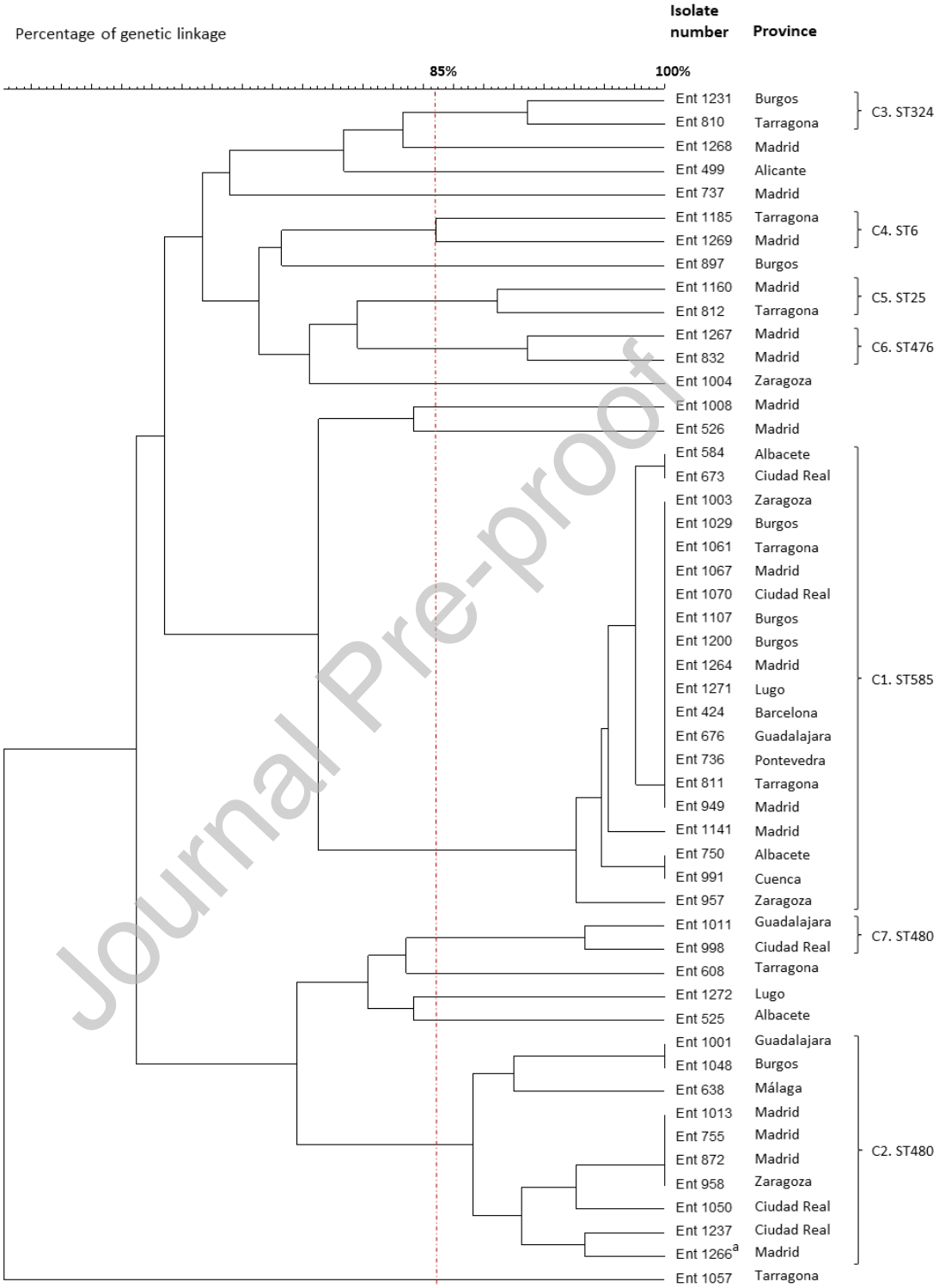
Figure 1. Geographic distribution by province of the linezolid-resistant *Enterococcus* spp. submitted to the Spanish Antibiotic Resistance Surveillance Program, and distribution of the sequence types (STs) of the 51 *optrA*-positive *E. faecalis.*



The figures on the map represent the number of hospitals per province that contributed with linezolid-resistant *Enterococcus* spp. isolates in this study.

Figure 2. Dendrogram of PFGE profiles obtained from 51 *Enterococcus faecalis* isolates carrying the *optrA* gene.



[a] Ent 1266 carried both *optrA* and *poxtA* genes

Table 1. Comparison of some clinical and epidemiological features of *Enterococcus* spp. resistant to linezolid due to the presence of the *optrA* gene or the G2575T mutation of the 23S rRNA gene.[a]

Variable	***optrA* gene (n = 45)**	**G2575T Mutation (n = 38)**	**OR (95% CI)**	***P* Value**
E. faecium (%)	0	31 (81.6)	0 (0-0.02)	< 0.0001
E. faecalis (%)	45 (100)	7 (18.4)	0 (0-0.02)	< 0.0001
Infections (%)	45 (100)	32 (84.2)	∞ (1.76-∞)	0.007
Bacteraemia (%)	2(4.4)	3 (7.9)	0.54 (0.09-2.80)	0.66
Urinary tract infections (%)	33 (73.3)	9 (23.7)	8.86 (3.11-24.60)	< 0.0001
Community acquisition (%)	24 (48)	4 (10.5)	7.85 (2.49–22.53)	0.0002
Female (%)	24 (45)	10 (26.3)	3.20 (1.24-7.62)	0.015
> 65 years of age (%)	24 (48)	25 (65.8)	0.48 (0.21–1.13)	0.130

OR: Odds ratio; 95% CI: 95% confidence interval. Data are reported as absolute numbers and percentage (%) of isolates.

[a] Isolates with double resistance mechanisms are excluded.

Table 2. Susceptibility to linezolid according to the patterns of linezolid resistance mechanisms found in *Enterococcus faecalis and Enterococcus faecium*.

		Linezolid	
Resistance mechanisms detected	**Range**	**MIC50**	**MIC90**
optrA (n=45)	8->256	16	32
G2575T mutation (n=38)	8->256	32	>256
poxtA (n= 1)	16	NA	NA
optrA+ G2575T mutation (n= 5)	8-32	32	32
optrA + *poxtA* (n= 5)	8-64	16	64
No mechanism detected (n=3)	8 - 32	32	32

Range and MIC (minimum inhibitory concentration) expressed in mg/L.
*NA: not applicable