

Mechanisms of Linezolid Resistance Among Enterococci of Clinical Origin in Spain—Detection of *optrA*- and *cfr*(D)-Carrying *E. faecalis*

Figure S1. Location of the six Spanish hospitals that took part in this study.



HSJ, Hospital San Jorge; HUG, Hospital Universitario de Getafe; HSP, Hospital San Pedro; HVC, Hospital Verge de la Cinta; HLB, Hospital Lozano Blesa; HRV, Hospital Royo Villanova.

Table S1. Primer pairs used for the molecular typing, and the detection of antimicrobial resistance and virulence genes.

Target gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
Multilocus sequence typing <i>E. faecium</i>			
<i>adk</i>	F: TATGAACCTCATTTTAATGGG	437	[1]
	R: GTTGACTGCCAAACGATTTT		
<i>atpA</i>	F: TTCAAATGGCTCATACGG	556	
	R: AGTTCACGATAAGCAACAGC		
<i>ddl</i>	F: GAGACATTGAATATGCCTTAT	465	
	R: AAAAAGAAATCGCACCG		
<i>gdh</i>	F: GGCGCACTAAAAGATATGGT	530	
	R: CCAAGATTGGGCAACTTCGTCCCA		
<i>pstS</i>	F: TTGAGCCAAGTCGAAGCTGGA	583	
	R: CGTGATCACGTTCTACTTCC		
<i>gyd</i>	F: CAAACTGCTTAGCTCCAAGGC	395	
	R: CATTTCGTTGTCATACCAAGC		
<i>purK</i>	F: GCAGATTGGCACATTGAAAGT	492	
	R:TACATAAATCCCGCCTGTTY		
Multilocus sequence typing <i>E. faecalis</i>			
<i>aroE</i>	F: TGGAAAACTTTACGGAGACAGC	459	[2]
	R: GTCCTGTCCATTGTTCAAAAGC		
<i>gki</i>	F: GATTTTGTGGGAATTGGTATGG	438	
	R: ACCATTAAAGCAAAATGATCGC		
<i>pstS</i>	F: CGGAACAGGACTTTCGC	583	
	R: ATTTACATCACGTTCTACTTGC		

<i>xpt</i>	F: AAAATGATGGCCGTGTATTAGG R: AACGTCACCGTTCCTTCACTTA	456	
<i>yqil</i>	F: CAGCTTAAGTCAAGTAAGTGCCG R: GAATATCCCTTCTGCTTGTGCT	436	
<i>gdh</i>	F: GGCGCACTAAAAGATATGGT R: CCAAGATTGGGCAACTTCGTCCCA	530	
<i>gyd</i>	F: CAAACTGCTTAGCTCCAAGGC R: CATTTTCGTTGTCATACCAAGC	395	
Resistance genes			
<i>optrA</i>	F: AGGTGGTCAGCGAACTAA R: ATCAACTGTTCCCATTCA	1395	[3]
<i>poxA</i>	F: TCAATGCAGAGCAGGAAGCA R: GGTGGATTTACCGACACCGT	791	[4]
<i>cfr</i>	F: TGAAGTATAAAGCAGGTTGGGAGTCA R: ACCATATAATTGACCACAAGCAGC	746	[5]
<i>cfr(B)</i>	F: TGAGCATATACGAGTAACCTCAAGA R: CGCAAGCAGCGTCTATATCA	293	[6]
<i>cfr(D)</i>	F: AGAAGTCGCAACAAGTGAGGA R: GCAACTGCATGAGTCAAAGAA	595	This study ¹
<i>fexA</i>	F: GTACTTGTAGGTGCAATTACGGCTGA R: CGCATCTGAGTAGGACATAGCGTC	1272	[7]
<i>fexB</i>	F: TTCCCACTATTGGTGAAAGGAT R: GCAATTCCCTTTTATGGACGTT	816	[8]

<i>cat</i> _{pC194}	F: CGACTTTTAGTATAACACAGA R: GCCAGTCATTAGGCCTAT	570	
<i>cat</i> _{pC221}	F: ATTTATGCAATTATGGAAGTTG R: TGAAGCATGGTAACCATCAC	434	[9]
<i>cat</i> _{pC223}	F: GAATCAAATGCTAGTTTAACTC R: ACATGGTAACCATCACATAC	283	
<i>catA</i>	F: GGATATGAAATTTATCCCTC R: CAATCATCTACCCTATGAAT	505	[10]
<i>erm</i> (A)	F: TCTAAAAAGCATGTAAAAGAA R: CTTCGATAGTTTATTAATATTAG	645	
<i>erm</i> (B)	F: GAAAAGTACTCAACCAAATA R: AGTAACGGTACTTAAATTGTTTA	639	[11]
<i>erm</i> (C)	F: TCAAAACATAATATAGATAAA R: GCAAATATTGTTTAAATCGTCAAT	642	
<i>msr</i> (C)	F: CTAGATGGGTTGTTGGCTCGT R: AGCTTTGGCAACCAGCTAGAA	361	[12]
<i>lnu</i> (B)	F: CCTACCTATTGTTTGTGGAA R: ATAACGTTACTCTCCTATTC	944	[13]
<i>lsa</i> (B)	F: TGCCGAAGCCATGTACCGTCC R: CGGTTAGACCAACCAGCCGAACG	396	[14]
<i>lsa</i> (E)	F: CGGCTATAGAACCGTTTGTTTT R: AGTTATTGTGGCAACTCAAAATC	1819	[15]
<i>aac</i> (6')-II	F: GCGGTAGCAGCGGTAGACCAAG R: GCATTTGGTAAGACACCTACG	323	[16]

<i>aac(6'')-Ie-aph(2'')-Ia</i>	F: CCAAGAGCAATAAGGGCATA R: CACTATCATAAACCCTACCG	220	[17]
<i>aph(3')-III</i>	F: GCCGATGTGGATTGCGAAAA R: GCTTGATCCCCAGTAAGTCA	282	
<i>ant(6)-Ia</i>	F: ACTGGCTTAATCAATTTGGG R: GCCTTTCCGCCACCTCACCG	597	[18]
<i>str</i>	F: TATTGCTCTCGAGGGTTC R: CTTTCTATATCCATTCATCTC	646	[9]
<i>ant(4')-Ia</i>	F: GCAAGGACCGACAACATTTTC R: TGGCACAGATGGTCATAACC	165	[17]
<i>tet(K)</i>	F: TTAGGTGAAGGGTTAGGTCC R: GCAAACCTCATTCCAGAAGCA	697	[10]
<i>tet(L)</i>	F: CATTTGGTCTTATTGGATCG R: ATTACACTTCCGATTTCGG	456	
<i>tet(M)</i>	F: GTTAAATAGTGTTCTTGGAG R: CTAAGATATGGCTCTAACAA	576	
<i>tet(O)</i>	F: ACGGARAGTTTATTGTATACC R: TGGCGTATCTATAATGTTGAC	171	[19]
<i>dfrA</i>	F: CCTTGGCACTTACCAAATG R: CTGAAGATTCGACTTCCC	374	[9]
<i>dfrD</i>	F: TTCTTTAATTGTTGCGATGG R: TTAACGAATTCTCTCATATATATG	582	
<i>dfrG</i>	F: TCGGAAGAGCCTTACCTGACAGAA R: CCCTTTTTGGGCAAATACCTCATTCCA	323	[14]

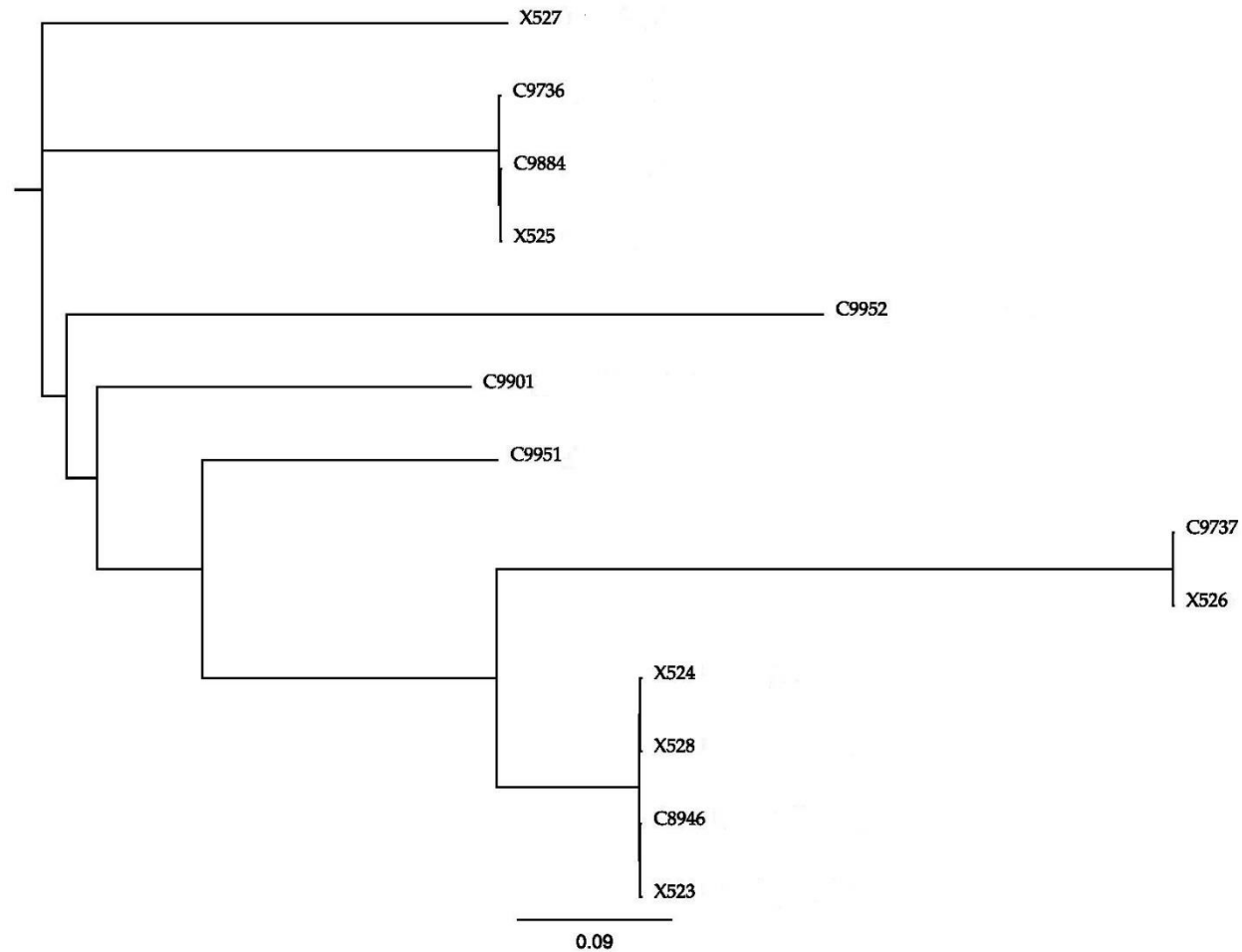
<i>dfrK</i>	F: GAGAATCCCAGAGGATTGGG R: CAAGAAGCTTTTCGCTCATAAA	423	
Mutations			
23S ARN	F: GCGGTCGCCTCCTAAAAG R: ATCCCGGTCCTCTCGTACT	420	[20]
<i>rplC</i> (L3)	F: ATGACCAAAGGAATCTTAGGG R: CACAGCTGATTGATWGTGATT	618	
<i>rplD</i> (L4)	F: GCCGAATGTAGCATTATTCAA R: CAAGCACCTCCTCAATTTGAGT	617	[21]
<i>rplV</i> (L22) (<i>E. faecium</i>)	F: GGACATGCTGCTGACGATA R: ACCATTTAGCATCCCAGTCG	486	
<i>rplV</i> (L22) (<i>E. faecalis</i>)	F: GCCACGTTGCTGACGATAA R: ACCCACTGATTGTCCCTCCT	476	[6]
<i>gyrA</i>	F: CGGGATGAACGAATTGGGTGTGA R: AATTTTACTCATACGTGCTTCGG	240	
<i>parC</i>	F: TTCCCGTGCATTTTCGATCAGTACTTC R: CGTATGACAAAGGATTCGGTAAATC	151	[22]
<i>pbp5</i>	F : CGGGATCTCACAAGAAGAT R : TTATTGATAATTTTGGTT	861	[23]
	F : AAAAATCGAACAGGCGCTTA R : TTGTGAGATCCCGTTTGTC	1140	[24]
Genetic environment of the <i>optrA</i> gene			
<i>optrA-fexA</i>	F: TTAGTTCGCTGACCACCT R: GACGCTATGTCCTACTCAGATGCG	1348	This study ²

Virulence factors			
<i>esp</i>	F: TTGCTAATGCTAGTCCACGACC	933	[25]
	R: GCGTCAACACTTGCATTGCCGAA		
<i>hyl</i>	F: GAGTAGAGGAATATCTTAGC	661	[26]
	R: AGGCTCCAATTCTGT		
<i>ace</i>	F: GGAATGACCGAGAACGATGGC	616	[27]
	R: GCTTGATGTTGGCCTGCTTCCG		
<i>agg</i>	F: AAGAAAAAGAAGTAGACCAAC	1553	[25]
	R: AAACGGCAAGACAAGTAAATA		
<i>gelE</i>	F: AGTTCATGTCTATTTCTTCAC	402	
	R: CTCATTATTACACGTTTG		

¹The *cfr*(D) PCR was performed using the following conditions: 94°C for 7 min, followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, with a final extension at 72°C for 10 min.

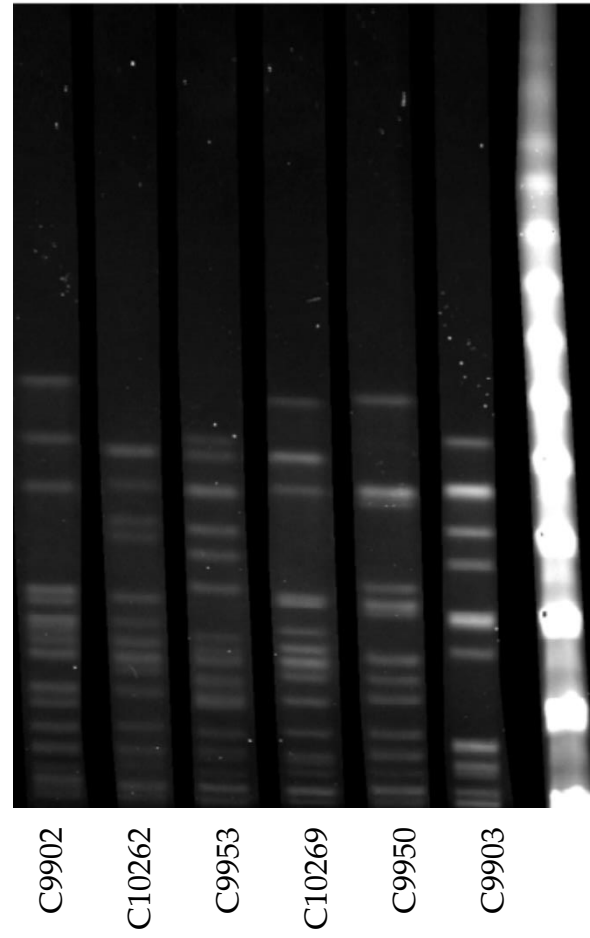
²The *optrA-fexA* PCR was performed using the following conditions: 94°C for 7 min, followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C, with a final extension at 72°C for 10 min.

Figure S2. Phylogenetic relatedness of the 13 *E. faecalis* isolates based on single nucleotide polymorphism (SNP) analysis performed using CSI Phylogeny.



The scale bar indicates the number of substitutions per site. The reference *E. faecalis* ATCC® 29212 was not included in final phylogeny.

Figure S3. Pulsed field gel electrophoresis (PFGE) of whole-cell DNA of *E. faecium* isolates after digestion with the enzyme *Sma*I.



References

1. Homan, W.L.; Tribe, D.; Poznanski, S.; Li, M.; Hogg, G.; Spalburg, E.; Van Embden, J.D.; Willems, R.J. Multilocus sequence typing scheme for *Enterococcus faecium*. *J. Clin. Microbiol.* **2002**, *40*, 1963-1971. <https://doi.org/10.1128/jcm.40.6.1963-1971.2002>.
2. Ruiz-Garbajosa, P.; Bonten, M.J.; Robinson, D.A.; Top, J.; Nallapareddy, S.R.; Torres, C.; Coque, T.M.; Cantón, R.; Baquero, F.; Murray, B.E.; et al. Multilocus sequence typing scheme for *Enterococcus faecalis* reveals hospital-adapted genetic complexes in a background of high rates of recombination. *J. Clin. Microbiol.* **2006**, *44*, 2220-2228. <https://doi.org/10.1128/JCM.02596-05>.
3. Wang, Y.; Lv, Y.; Cai, J.; Schwarz, S.; Cui, L.; Hu, Z.; Zhang, R.; Li, J.; Zhao, Q.; He, T.; et al. A novel gene, *optrA*, that confers transferable resistance to oxazolidinones and phenicols and its presence in *Enterococcus faecalis* and *Enterococcus faecium* of human and animal origin. *J. Antimicrob. Chemother.* **2015**, *70*, 2182-2190. <https://doi.org/10.1093/jac/dkv116>.
4. Ruiz-Ripa, L.; Feßler, A.T.; Hanke, D.; Sanz, S.; Olarte, C.; Mama, O.M.; Eichhorn, I.; Schwarz, S.; Torres, C. Coagulase-negative staphylococci carrying *cfr* and PVL genes, and MRSA/MSSA-CC398 in the swine farm environment. *Vet. Microbiol.* **2020**, *243*, 108631. <https://doi.org/10.1016/j.vetmic.2020.108631>.
5. Kehrenberg, C.; Schwarz, S. Distribution of florfenicol resistance genes *fexA* and *cfr* among chloramphenicol-resistant *Staphylococcus* isolates. *Antimicrob. Agents Chemother.* **2006**, *50*, 1156-1163. <https://doi.org/10.1128/AAC.50.4.1156-1163.2006>.
6. Lee, S.M.; Huh, H.J.; Song, D.J.; Shim, H.J.; Park, K.S.; Kang, C.I.; Ki, C.S.; Lee, N.Y. Resistance mechanisms of linezolid-nonsusceptible enterococci in Korea: low rate of 23S rRNA mutations in *Enterococcus faecium*. *J. Med. Microbiol.* **2017**, *66*, 1730-1735. <https://doi.org/10.1099/jmm.0.000637>.
7. Kehrenberg, C.; Schwarz, S. Florfenicol-chloramphenicol exporter gene *fexA* is part of the novel transposon Tn558. *Antimicrob. Agents Chemother.* **2005**, *49*, 813-815. <https://doi.org/10.1128/AAC.49.2.813-815.2005>.
8. Liu, H.; Wang, Y.; Wu, C.; Schwarz, S.; Shen, Z.; Jeon, B.; Ding, S.; Zhang, Q.; Shen, J.A. A novel phenicol exporter gene, *fexB*, found in enterococci of animal origin. *J. Antimicrob. Chemother.* **2012**, *67*, 322-325. <https://doi.org/10.1093/jac/dkr481>.
9. Schnellmann, C.; Gerber, V.; Rossano, A.; Jaquier, V.; Panchaud, Y.; Doherr, M.G.; Thomann, A.; Straub, R.; Perreten, V. Presence of new *mecA* and *mph(C)* variants conferring antibiotic resistance in *Staphylococcus* spp. isolated from the skin of horses before and after clinic admission. *J. Clin. Microbiol.* **2006**, *44*, 4444-4454. <https://doi.org/10.1128/JCM.00868-06>.
10. Aarestrup, F.M.; Agerso, Y.; Gerner-Smidt, P.; Madsen, M.; Jensen, L.B. Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. *Diagn. Microbiol. Infect. Dis.* **2000**, *37*, 127-137. [https://doi.org/10.1016/s0732-8893\(00\)00130-9](https://doi.org/10.1016/s0732-8893(00)00130-9).
11. Sutcliffe, J.; Grebe, T.; Tait-Kamradt, A.; Wondrack, L. Detection of erythromycin-resistant determinants by PCR. *Antimicrob. Agents Chemother.* **1996**, *40*, 2562-2566.
12. Ruiz-Ripa, L.; Feßler, A.T.; Hanke, D.; Sanz, S.; Olarte, C.; Eichhorn, I.; Schwarz, S.; Torres, C. Detection of *poxA*- and *optrA*-carrying *E. faecium* isolates in air samples of a Spanish swine farm. *J. Glob. Antimicrob. Resist.* **2019**, pii, S2213-7165(19)30326-1. <https://doi.org/10.1016/j.jgar.2019.12.012>.
13. Bozdogan, B.; Berrezouga, L.; Kuo, M.S.; Yurek, D.A.; Farley, K.A.; Stockman, B.J.; Leclercq, R. A new resistance gene, *linB*, conferring resistance to lincosamides by nucleotidylation in *Enterococcus faecium* HM1025. *Antimicrob. Agents Chemother.* **1999**, *43*, 925-929.

14. Gómez-Sanz, E.; Torres, C.; Lozano, C.; Fernández-Pérez, R.; Aspiroz, C.; Ruiz-Larrea, F.; Zarazaga, M. Detection, molecular characterization, and clonal diversity of methicillin-resistant *Staphylococcus aureus* CC398 and CC97 in Spanish slaughter pigs of different age groups. *Foodborne Pathog. Dis.* **2010**, *27*, 1269-1277. <https://doi.org/10.1089/fpd.2010.0610>.
15. Wendlandt, S.; Lozano, C.; Kadlec, K.; Gómez-Sanz, E.; Zarazaga, M.; Torres, C.; Schwarz, S. The enterococcal ABC transporter gene *lsa(E)* confers combined resistance to lincosamides, pleuromutilins and streptogramin A antibiotics in methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*. *J. Antimicrob. Chemother.* **2013**, *68*, 473-475. <https://doi.org/10.1093/jac/dks398>.
16. Costa, Y.; Galimand, M.; Leclercq, R.; Duval, J.; Courvalin, P. Characterization of the chromosomal *aac(6')-II* gene specific for *Enterococcus faecium*. *Antimicrob. Agents Chemother.* **1993**, *37*, 1896-1903. <https://doi.org/10.1128/aac.37.9.1896>.
17. van de Klundert, J.A.M.; Vliegthart, J.S. PCR detection of genes coding for aminoglycoside-modifying enzymes In *Diagnostic Molecular Microbiology. Principles and applications*; Persing, D.H., Smith, T.F., Tenover, F.C., White, T.J. Eds.; ASM, Washington, USA, 1993.
18. Clark, N.C.; Olsvik, O.; Swenson, J.M.; Spiegel, C.A.; Tenover, F.C. Detection of a streptomycin/spectinomycin adenyltransferase gene (*aadA*) in *Enterococcus faecalis*. *Antimicrob. Agents Chemother.* **1999**, *43*, 157-160.
19. Aminov, R.I.; Garrigues-Jeanjean, N.; Mackie, R.I. Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *Appl. Environ. Microbiol.* **2001**, *67*, 22-32. <https://doi.org/10.1128/AEM.67.1.22-32.2001>.
20. Dibo, I.; Pillai, S.K.; Gold, H.S.; Baer, M.R.; Wetzler, M.; Slack, J.L.; Hazamy, P.A.; Ball, D.; Hsiao, C.B.; McCarthy, P.L.; et al. Linezolid-resistant *Enterococcus faecalis* isolated from a cord blood transplant recipient. *J. Clin. Microbiol.* **2004**, *42*, 1843-1845. <https://doi.org/10.1128/jcm.42.4.1843-1845.2004>.
21. Diaz, L.; Kiratisin, P.; Mendes, R.E.; Panesso, D.; Singh, K.V.; Arias, C.A. Transferable plasmid-mediated resistance to linezolid due to *cfr* in a human clinical isolate of *Enterococcus faecalis*. *Antimicrob. Agents Chemother.* **2012**, *56*, 3917-3922. <https://doi.org/10.1128/AAC.00419-12>.
22. Leavis, H.L.; Willems, R.J.; Top, J.; Bonten, M.J. High-level ciprofloxacin resistance from point mutations in *gyrA* and *parC* confined to global hospital-adapted clonal lineage CC17 of *Enterococcus faecium*. *J. Clin. Microbiol.* **2006**, *44*, 1059-1064. <https://doi.org/10.1128/JCM.44.3.1059-1064.2006>.
23. Hsieh, S.; Hsu, L.; Hsu, W.; Chen, C.; Chen, H.; Liao, C. Importance of amino acid alterations and expression of penicillin-binding protein 5 to ampicillin resistance of *Enterococcus faecium* in Taiwan. *Int. J. Antimicrob. Agents.* **2006**, *28*, 514-519. <https://doi.org/10.1016/j.ijantimicag.2006.07.027>.
24. Jureen, R.; Top, J.; Mohn, S.C.; Harthug, S.; Langeland, N.; Willems, R.J.L. Molecular characterization of ampicillin-resistant *Enterococcus faecium* isolates from hospitalized patients in Norway. *J. Clin. Microbiol.* **2003**, *41*, 2330-2336. <https://doi.org/10.1128/jcm.41.6.2330-2336.2003>.
25. Eaton, T.J.; Gasson, M.J. Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. *Appl. Environ. Microbiol.* **2001**, *67*, 1628-1635. <https://doi.org/10.1128/AEM.67.4.1628-1635.2001>.
26. Klare, I.; Konstabel, C.; Mueller-Bertling, S.; Werner, G.; Strommenger, B.; Kettlitz, C.; Borgmann, S.; Schulte, B.; Jonas, D.; Serr, A.; et al. Spread of ampicillin/vancomycin-resistant *Enterococcus faecium* of the epidemic-virulent clonal complex-17 carrying the genes *esp* and *hyl* in German hospitals. *Eur. J. Clin. Microbiol. Infect. Dis.* **2005**, *24*, 815-825. <https://doi.org/10.1007/s10096-005-0056-0>.
27. Creti, R.; Imperi, M.; Bertuccini, L.; Fabretti, F.; Orefici, G.; Di Rosa, R.; Baldassarri, L. Survey for virulence determinants among *Enterococcus faecalis* isolated from different sources. *J. Med. Microbiol.* **2004**, *53*, 13-20. <https://doi.org/10.1099/jmm.0.05353-0>.