JCM
Journals.ASM.org

Mechanisms of Linezolid Resistance among Staphylococci in a Tertiary Hospital

Inmaculada Quiles-Melero,[a] Rosa Gómez-Gil,[a] María Pilar Romero-Gómez,[a] Ana María Sánchez-Díaz,[b] Manuela de Pablos,[a] Julio García-Rodriguez,[a] Avelino Gutiérrez,[a] Jesús Mingorance[a]

Servicio de Microbiología, Hospital Universitario La Paz, IdiPAZ, Madrid, Spain[a]; Servicio de Microbiología, Hospital Universitario Ramón y Cajal, IRYCIS, Madrid, Spain[b]

The mechanisms of linezolid resistance among 86 staphylococcal isolates from two intensive care units were investigated. The most frequent was the G2576T mutation in the 23S rRNA (82%). The *cfr* gene was found in 17% of the isolates, seven *S. aureus* and eight *S. epidermidis* isolates. Four of the *S. epidermidis* isolates had the G2576T mutation and the *cfr* gene. In four *S. haemolyticus* isolates, the mechanism could not be identified.

Linezolid is an oxazolidinone with antimicrobial activity against Gram-positive bacteria indicated for the treatment of infections by multiresistant *Staphylococcus aureus* and coagulase-negative staphylococci, as well as penicillin-resistant *Streptococcus pneumoniae* and vancomycin-resistant enterococci (VRE) (1). Activity results from binding to the 23S rRNA in the 50S ribosomal subunit (1, 2), and resistance arises most frequently from a G-to-T mutation at position 2576 of the 23S rRNA (*Escherichia coli* numbering) (1). Other mutations in the 23S rRNA (G2447T, T2500A, and C2534T) have been found in clinical and laboratory-derived staphylococcal isolates (3, 4), as well as mutations in the L3, L4, and L22 ribosomal proteins (5–7). Another mechanism involves the *cfr* gene, which codes for an adenine methyltransferase that modifies adenosine at position 2503 in the 23S rRNA (8).

Linezolid use started in our hospital in 2002, and in 2005–2006 a small outbreak of linezolid-resistant *Enterococcus faecalis* was detected in two intensive care units (9). In subsequent years, linezolid-resistant staphylococci appeared in the same units and later in other hospital areas. This study focused on isolates from two intensive care units, which are called REA and ICU here and are described in reference 9.

Between 2005 and 2009, 256 linezolid-resistant staphylococci were isolated from different hospital units (2.5% of the total number of staphylococcal isolates). Among the resistant isolates, the most abundant were *Staphylococcus epidermidis* (43.4%) and *S. haemolyticus* (35.4%). There were also sporadic isolates of *S. hominis* (7.3%), and *S. aureus* (2.8%) (Table 1). Eighty-six staphylococcal isolates were obtained from the two ICUs (18% of the ICU staphylococcal isolates). Samples included blood, catheters, and cerebrospinal fluids (four patients had spinal catheters). Of these, 66 were obtained from 46 ICU patients and 20 were from 13 REA patients. Only isolates considered clinically significant (grown in at least two out of three culture bottles) were included. One isolate per event was considered, with the exceptions of four pairs of isolates obtained from blood and catheter from the same event. Events were considered independent if they were separated by 1 month or more.

Identification and susceptibility testing were done using the Wider (Francisco Soria Melguizo, S.A., Madrid, Spain) and Vitek 2 (bioMérieux Vitek, Marcy-l'Etoile, France) systems. MICs of linezolid were determined by Etest as concentrations resulting in 90% growth inhibition (bioMérieux Vitek, Marcy-l'Etoile, France) and interpreted according to the CLSI guidelines (10). Isolates with linezolid MICs of >4 μg/ml were considered resistant.

The G2576T mutation in the 23S rRNA was investigated by pyrosequencing (9). Nearby mutations were searched for by Sanger sequencing of domains II and V. A search for the *cfr* gene was done by PCR (11), and mutations in the L3, L4, and L22 ribosomal protein sequences, i.e., the *rplC*, *rplD* and *rplV* genes, were studied by sequencing and comparison with GenBank sequences (12).

Clonal analysis by repetitive extrapalindromic sequence (rep)-PCR DNA fingerprinting using DiversiLab (bioMérieux, Marcy-l'Etoile, France) did not find intraspecific variability. Consequently, different approaches were tested for each species to select methods with discriminatory capacity. SmaI pulsed-field gel electrophoresis (PFGE) was used for *S. epidermidis* and *S. haemolyticus*, and ApaI PFGE for *S. hominis*. Randomly amplified polymorphic DNA (RAPD) was used to study *S. haemolyticus* and *S. hominis*, using the primers OPA2 (5′-TGCCGAGCTG-3′), OPI12 (5′-AGAGGGCACA-3′), and OPA18 (5′-AGGTGACCGT-3′). *S. aureus* was analyzed by *spa* typing (13) and multilocus sequence typing (MLST) (14), and *S. epidermidis* was analyzed by PCR of the repeat region of the *sdrF* gene (15) and MLST (16). The staphylococcal chromosomal cassette *mec* (SCC*mec*) types were identified by multiplex PCR (17).

There were seven linezolid-resistant *S. aureus* isolates. All of them were methicillin-resistant nosocomial isolates (HA-MRSA). In addition, they were resistant to fluoroquinolones, erythromycin, and clindamycin. They belonged to sequence type ST125 and carried the *cfr* gene. Their linezolid MICs ranged from 8 to 16 μg/ml. One isolate obtained in 2007 had *spa* type t109 and SCC*mec* type I. Six clustered isolates obtained in 2008 had *spa* type t067 and SCC*mec* type IV. None of them had mutations in the 23S rRNA, although a single methicillin-sensitive *S. aureus* isolate carrying the G2576T mutation was found later in an unrelated hos-

Received 15 June 2012 **Returned for modification** 16 July 2012
Accepted 14 December 2012
Published ahead of print 26 December 2012
Address correspondence to Jesús Mingorance, jesus.mingorance@idipaz.es.

doi:10.1128/JCM.01598-12

TABLE 1 Numbers of linezolid-resistant staphylococci isolated in ICUs during the study period

Yr	*S. aureus*			*S. epidermidis*			*S. haemolyticus*			*S. hominis*		
	No. of Lin^r isolates	%[a]	Total	No. of Lin^r isolates	%[a]	Total	No. of Lin^r isolates	%[a]	Total	No. of Lin^r isolates	%[a]	Total
2005	0		115	6	2.3	258	5	31.3	16	2	3.6	55
2006	1	0.7	139	2	0.7	268	11	42.3	26	0		90
2007	1	0.6	164	11	2.1	521	35	47.9	73	3	1.6	187
2008	6	4.0	150	34	5.6	606	26	49.1	53	6	3.8	158
2009	0		124	46	8.7	530	17	63.0	27	6	4.9	122
Total	8	1.0	692	125	4.0	2,183	102	46.0	195	21	3.0	612

[a] Percentage of the total number of clinically significant blood and catheter isolates of that species.

pital area (18). Linezolid resistance could be transferred by conjugation to a rifampin-resistant *S. aureus* recipient strain (ATCC 29213) that acquired resistance to clindamycin and linezolid.

Among the coagulase-negative staphylococci, the mutation G2576T was detected in 67 of 79 isolates, and the *cfr* gene was detected in eight *S. epidermidis* isolates, four of which also had the G2576T mutation. Most of the isolates were multiresistant, with only two *S. haemolyticus* isolates being susceptible to levofloxacin and three *S. hominis* isolates being susceptible to methicillin. All the isolates were susceptible to vancomycin, daptomycin, teicoplanin, and quinupristin-dalfopristin.

Both PFGE and *sdrF* typing identified a major group of *S. epidermidis* that included 28 SCC*mec* type III isolates that had the G2576T mutation in all the alleles of the 23S rRNA gene. Four isolates from this group also had the *cfr* gene. Besides PFGE, *sdrF* and SCC*mec* typing identified six types, five of them represented by a single isolate each (Table 2). The sixth type included three isolates with pulsotype 2, *sdrF* type IV, and SCC*mec* type IV, two of them having the G2576T mutation in a 3:2 wild-type-to-mutant ratio and the third having the mutation in all the alleles. MLST analysis of selected isolates showed that the major group belongs to ST2 (CC2), while the pulsotype 2 group IV isolates belong to ST23 (CC23). Linezolid resistance has been observed previously in isolates from these sequence types (19–21).

Similarly, PFGE and RAPD analyses of *S. haemolyticus* isolates showed a major group divided into two subgroups by their SCC*mec* type: 26 had SCC*mec* type I and nine had SCC*mec* type V. Among them, 31 isolates had the G2576T mutation in all five alleles and four had neither the G2576T mutation nor the *cfr* gene (Table 2). PFGE, RAPD, and SCC*mec* typing identified six additional types, all of them represented by a single isolate each. Four of these had the G2576T mutation in all the alleles, while in the other two a mechanism for linezolid resistance could not be identified. The four isolates with no known resistance mechanism were

TABLE 2 Numbers of linezolid-resistant staphylococci isolated in ICUs during the study period, according to molecular typing and linezolid resistance mechanisms

Species	PFGE pulsotype	*sdrF* type	RAPD pattern	*spa* type	SCC*mec* type	No. of isolates with linezolid resistance mechanism			
						G2576T	*cfr*	G2576T + *cfr*	NT[a]
S. aureus				t109	I		1		
				t067	IV		6		
S. epidermidis	1	I			III	24		4	
	1	II			III	1			
	1	III			I		1		
	1	V			NT		1		
	2	II			III		1		
	2	IV			IV	3			
	3	III			I		1		
S. haemolyticus	1		1		I	21			2
	1		1		V	6			
	1		3		V				1
	1		4		I	1			
	2		1		I	1			
	3		1		I				1
	4		1		I	1			
	4		4		V	1			
			2		V	1			
S. hominis	1		1		NT	5			
	1		1			3			

[a] NT, nontypeable.

further investigated by sequencing domains II and V of the 23S rRNA genes and the L3, L4, or L22 ribosomal protein gene, but no mutations were found.

All of the eight *S. hominis* isolates were identical by both PFGE and RAPD; three of them were methicillin sensitive, and five were resistant, but their SSC could not be typed. Six of the isolates had the G2576T mutation in a 1:4 ratio of wild type to mutant, while the other two had the mutation in all the alleles.

The MICs of these isolates span the full range of the standard assays, from 8 to >256 μg/ml for *S. epidermidis* and 8 to 64 for *S. haemolyticus* and *S. hominis*. No relation between the MIC and the number of mutant alleles was observed, and even among the group of *S. epidermidis* isolates having the G2576T mutation in all five alleles of the 23S rRNA gene, the MICs span the full range of values. Four *S. epidermidis* isolates from group I with MICs of >256 were further investigated by sequencing, but no additional mutations in the 23S rRNA gene or the L3, L4, and L22 genes were found. A correlation between MICs and numbers of mutant alleles has been found after selection of isogenic lines under laboratory conditions (22, 23), but the relation is less clear when independent clinical isolates are analyzed (24). The heterogeneity of MICs even among isolates with the same number of mutant alleles suggests that there might be additional unidentified factors affecting linezolid susceptibility.

The *cfr* methyltransferase gene was associated with different *S. aureus* and *S. epidermidis* clones and was shown to be transferable at least among *S. aureus*, suggesting that *cfr* might have spread by transfer between different clones. It is known that resistance plasmids can be transferred among *S. aureus* and *S. epidermidis* in the context of hospital outbreaks (25), and the spread of the *cfr* gene from *S. aureus* to *S. epidermidis* in a patient was recently reported (26).

The diversity of species and clones found suggests that linezolid resistance among Gram-positive cocci in our hospital resulted from several independent selection events followed by the expansion of a few clones. This suggests that both antibiotic selection pressure and cross transmission have played a role in the local emergence and spread of the resistant clones. After reaching a maximum in 2009, the number of resistant isolates decreased in 2010 and 2011. The reasons for this rapid decrease are unclear, but improved linezolid use might have been an important factor (27). Global linezolid consumption in the hospital has stayed stable at around 6,000 defined daily doses (DDD) per year, but there might be significant differences among different wards (27). Other factors, like the reinforcement in infection control measures, increased awareness of the problem among clinicians, and the development of a program to prevent catheter-related infections in ICUs, might have contributed.

ACKNOWLEDGMENTS

We thank Francisco Moreno Ramos (Division of Hospital Pharmacy, Hospital La Paz) for providing linezolid consumption data, Patricia Gómez-Sánchez for technical support, and Rosa del Campo and Rafael Cantón (Servicio de Microbiología, Hospital Ramón y Cajal) for kindly providing material and technical assistance with PFGE.

J.M. was the recipient of a Ramón y Cajal fellowship financed by the European Social Fund and the Ministerio de Ciencia e Innovación. This work was supported in part by an Investigator Initiated Research Grant from Pfizer Inc. to J.M.

REFERENCES

1. **Livermore DM.** 2003. Linezolid in vitro: mechanism and antibacterial spectrum. J. Antimicrob. Chemother. **51**(Suppl 2):ii9–ii16.
2. **Long KS, Vester B.** 2012. Resistance to linezolid caused by modifications at its binding site on the ribosome. Antimicrob. Agents Chemother. **56**: 603–612.
3. **Meka VG, Pillai SK, Sakoulas G, Wennersten C, Venkataraman L, DeGirolami PC, Eliopoulos GM, Moellering RC, Gold HS.** 2004. Linezolid resistance in sequential *Staphylococcus aureus* isolates associated with a T2500A mutation in the 23S rRNA gene and loss of a single copy of rRNA. J. Infect. Dis. **190**:311–317.
4. **Miller K, O'Neill AJ, Wilcox MH, Ingham E, Chopra I.** 2008. Delayed development of linezolid resistance in *Staphylococcus aureus* following exposure to low levels of antimicrobial agents. Antimicrob. Agents Chemother. **52**:1940–1944.
5. **Bonilla H, Huband MD, Seidel J, Schmidt H, Lescoe M, McCurdy SP, Lemmon MM, Brennan LA, Tait-Kamradt A, Puzniak L, Quinn JP.** 2010. Multicity outbreak of linezolid-resistant *Staphylococcus epidermidis* associated with clonal spread of a *cfr*-containing strain. Clin. Infect. Dis. **51**:796–800.
6. **Farrell D, Morrissey I.** 2004. In vitro activities of telithromycin, linezolid, and quinupristin-dalfopristin against *Streptococcus pneumoniae* with macrolide resistance due to ribosomal mutations. Antimicrob. Agents Chemother. **48**:3169–3171.
7. **Locke JB, Hilgers M, Shaw KJ.** 2009. Mutations in ribosomal protein L3 are associated with oxazolidinone resistance in staphylococci of clinical origin. Antimicrob. Agents Chemother. **53**:5275–5278.
8. **Arias CA, Vallejo M, Reyes J, Panesso D, Moreno J, Castañeda E, Villegas MV, Murray BE, Quinn JP.** 2008. Clinical and microbiological aspects of linezolid resistance mediated by the *cfr* gene encoding a 23S rRNA methyltransferase. J. Clin. Microbiol. **46**:892–896.
9. **Gómez-Gil R, Romero-Gómez MP, García-Arias A, Ubeda MG, Busselo MS, Cisterna R, Gutiérrez-Altés A, Mingorance J.** 2009. Nosocomial outbreak of linezolid-resistant *Enterococcus faecalis* infection in a tertiary care hospital. Diagn. Microbiol. Infect. Dis. **65**:175–179.
10. **National Committee for Clinical Laboratory Standards.** 2006. Performance standards for antimicrobial susceptibility testing. Document M100-S16. NCCLS, Wayne, PA.
11. **Kehrenberg C, Schwarz S.** 2006. Distribution of florfenicol resistance genes *fexA* and *cfr* among chloramphenicol-resistant *Staphylococcus* isolates. Antimicrob. Agents Chemother. **50**:1156–1163.
12. **Wolter N, Smith AM, Farrell DJ, Schaffner W, Moore M, Whitney CG, Jorgensen JH, Klugman KP.** 2005. Novel mechanism of resistance to oxazolidinones, macrolides, and chloramphenicol in ribosomal protein L4 of the pneumococcus. Antimicrob. Agents Chemother. **49**:3554–3557.
13. **Shopsin B, Gómez M, Montgomery SO, Smith DH, Waddington M, Dodge DE, Bost DA, Riehman M, Naidich S, Kreiswirth BN, Arbeit R, Archer G, Biddle J, Byrne S, Goering R, Hancock G, He GA, Hill B, Hollis R, Jarvis WR, Eisner W, Maslow J, Mcdougal LK, Miller JM, Mulligan M.** 1999. Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. J. Clin. Microbiol. **37**:3556–3563.
14. **Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG.** 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J. Clin. Microbiol. **38**:1008–1015.
15. **Ohlin A, Bäckman A, Söderquist B, Wingren S, Björkqvist M.** 2010. Rapid typing of neonatal *Staphylococcus epidermidis* isolates using polymerase chain reaction for repeat regions in surface protein genes. Eur. J. Clin. Microbiol. Infect. Dis. **29**:699–704.
16. **Thomas JC, Vargas MR, Miragaia M, Peacock SJ, Archer GL, Enright MC.** 2007. Improved multilocus sequence typing scheme for *Staphylococcus epidermidis*. J. Clin. Microbiol. **45**:616–619.
17. **Boye K, Bartels MD, Andersen IS, Møller JA, Westh H.** 2007. A new multiplex PCR for easy screening of methicillin-resistant *Staphylococcus aureus* SCCmec types I-V. Clin. Microbiol. Infect. **13**:725–727.
18. **Quiles-Melero I, García-Perea A, de Pablos M, Gómez-Gil R, Mingorance J.** 2012. Resistance to linezolid in a methicillin-susceptible *Staphylococcus aureus* clinical isolate without previous exposure to oxazolidinones. Int. J. Med. Microbiol. **302**:145–147.
19. **Bongiorno D, Campanile F, Mongelli G, Baldi MT, Provenzani R, Reali S, Lo Russo C, Santagati M, Stefani S.** 2010. DNA methylase modifica-

tions and other linezolid resistance mutations in coagulase-negative staphylococci in Italy. J. Antimicrob. Chemother. **65**:2336–2340.
20. **Lozano C, Ruiz-García M, Gómez-Sanz E, López-García P, Royo-García G, Zarazaga M, Torres C.** 2012. Characterization of a *cfr*-positive methicillin-resistant *Staphylococcus epidermidis* strain of the lineage ST22 implicated in a life-threatening human infection. Diagn. Microbiol. Infect. Dis. **73**:380–382.
21. **Mendes RE, Deshpande LM, Farrell DJ, Spanu T, Fadda G, Jones RN.** 2010. Assessment of linezolid resistance mechanisms among *Staphylococcus epidermidis* causing bacteraemia in Rome, Italy. J. Antimicrob. Chemother. **65**:2329–2335.
22. **Besier S, Ludwig A, Zander J, Brade V, Wichelhaus TA.** 2008. Linezolid resistance in *Staphylococcus aureus*: gene dosage effect, stability, fitness costs, and cross-resistances. Antimicrob. Agents Chemother. **52**:1570–1572.
23. **Marshall S.** 2002. Gene dosage and linezolid resistance in *Enterococcus faecium* and *Enterococcus faecalis*. Antimicrob. Agents Chemother. **46**: 3334–3336.
24. **Ikeda-Dantsuji Y, Hanaki H, Sakai F, Tomono K, Takesue Y, Honda J, Nonomiya Y, Suwabe A, Nagura O, Yanagihara K, Mikamo H, Fukuchi K, Kaku M, Kohno S, Yanagisawa C, Nakae T, Yoshida K, Niki Y.** 2011. Linezolid-resistant *Staphylococcus aureus* isolated from 2006 through 2008 at six hospitals in Japan. J. Infect. Chemother. **17**:45–51.
25. **Forbes BA, Schaberg DR.** 1983. Transfer of resistance plasmids from *Staphylococcus epidermidis* to *Staphylococcus aureus*: evidence for conjugative exchange of resistance. J. Bacteriol. **153**:627–634.
26. **Pérez-Jorge C, Isea-Peña M-C, Heili S, Esteban J.** 2012. Spread of *cfr* gene among staphylococci conferring resistance to linezolid in a patient under treatment. J. Antibiot. **65**:151–152.
27. **Ramírez E, Gómez-Gil R, Borobia AM, Moreno F, Zegarra C, Muñoz R, Reutero Z, de Montreuil C, González D, Hernández S, Herrero A, Gutiérrez A, Frías J.** 2013. Improving linezolid use decreases the incidence of resistance among Gram-positive microorganisms. Int. J. Antimicrob. Agents. **41**:174–178.