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DNA methylase modifications and other linezolid resistance mutations in coagulase-negative staphylococci in Italy

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Background: Despite 10 years of clinical use, linezolid resistance in *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS) is still a rare phenomenon. This study reports the mechanisms of resistance and strain types seen in clusters of linezolid-resistant CoNS from two different hospitals in Italy during the period 2008–09.

Methods: Genes associated with linezolid resistance were subjected to molecular analysis and isolates were characterized by PFGE macrorestriction analysis using SmaI.

Results: Thirty-three linezolid-resistant isolates of methicillin-resistant CoNS comprising *Staphylococcus epidermidis* (24), *Staphylococcus hominis* (5) and *Staphylococcus simulans* (4) were studied. The isolates showed varying levels of linezolid resistance. Almost all isolates for which linezolid MICs were 64 mg/L possessed point mutations in domain V of 23S rRNA, while isolates for which the MICs were 256 mg/L expressed methylase activity at position A2503 mediated by the *cfr* gene. Overall, the isolates showed reduced susceptibility to vancomycin (MICs 1–2 mg/L) and 11 of the 33 isolates showed no susceptibility to teicoplanin. These strains were also resistant to chloramphenicol (28 of 33), lincomycin (24 of 33), erythromycin (17 of 33) and quinupristin/dalfopristin (13 of 33). *S. epidermidis* isolates, showing mutations or methylase modifications, belonged to different PFGE profiles and to two different sequence types (ST2 and ST23), in which the *cfr* gene was carried on a plasmid of ~50 kb.

Conclusions: Clinical CoNS strains with resistance to linezolid and other second-line antibiotics, as well as reduced susceptibility to glycopeptides, have emerged in Italy.

Keywords: 23S rRNA, G2576T substitution, *cfr* gene

Introduction

Linezolid, the first oxazolidinone to be used clinically, is still effective in the treatment of infections caused by various Gram-positive pathogens, including multidrug-resistant enterococci and methicillin-resistant *Staphylococcus aureus*. It has been used successfully for the treatment of patients with bacteraemia, osteomyelitis, joint infections and tuberculosis; it is often used for treatment of complicated infections, including endocarditis, when other therapies have failed.[1]

Although linezolid resistance in Gram-positive cocci has been encountered in the clinical setting and has also been selected *in vitro*, it is still a rare phenomenon. Epidemiological data show that it occurs in ≤1% of *S. aureus* isolates and ≤0.1% of coagulase-negative staphylococci (CoNS) in the USA[2,3] and 0.4% and 0.3% of *S. aureus* and CoNS, respectively, in Germany.[4]

Resistance to this antibiotic has been, until recently, principally associated with distinct nucleotide substitutions in domain V of the 23S rRNA gene, arising in at least two copies of the rRNA operons, with a stepwise increase due to successive accumulation of single point mutations.[5,6] Post-transcriptional methylation of A2503 in the 23S rRNA by the horizontally transferred *cfr* gene, recently described in clinical isolates and derived from staphylococci of animal origin, added concern about the possible high transmission of linezolid resistance among clinical isolates.[7] In rare cases, mutations in L4 and, recently, L3 proteins, due to their proximity to regions that interact closely with the oxazolidinone binding site, have been associated with resistance.[8–10]

During 2008–09, two hospitals in Italy found CoNS isolates that appeared less susceptible and/or resistant to linezolid. This prompted us to examine these strains and to characterize them at the molecular level.

Materials and methods

CoNS strains and antibiotic resistance tests

The activity of linezolid and comparator agents was evaluated against 33 CoNS clinical isolates (24 *Staphylococcus epidermidis*, 4 *Staphylococcus simulans* and 5 *Staphylococcus hominis*), obtained from 29 hospitalized patients. Twenty-eight isolates were from blood and five were from central venous catheters. The isolates, recovered during 2008–09, came from three intensive care units and one emergency room of two Italian hospitals, where they were initially detected as strains 'not susceptible' to linezolid by routine laboratory Vitek testing. All isolates were identified by biochemical tests (API-Staph system; bioMérieux SA, Marcy l'Étoile, France) and confirmed by the sequencing of an internal fragment of the 16S rRNA gene, as previously described.[11]

MICs of linezolid (Pfizer Pharma, Milan, Italy), oxacillin, erythromycin, clindamycin, lincomycin, vancomycin, teicoplanin, chloramphenicol, quinupristin/dalfopristin (all obtained as pure powders for analysis from Sigma Aldrich, Milan, Italy) and daptomycin (Novartis Pharma, Basel, Switzerland) were determined using the microdilution method, in accordance with CLSI guidelines (2010).[12] The EUCAST guidelines were also used for comparison.[13]

Genome analyses

PFGE macrorestriction analysis was performed with SmaI (New England Biolabs, Ipswich, MA, USA) following a modified protocol previously reported.[11,14] Similarities between macrorestriction patterns were identified according to established criteria.[15] For long-term evaluation the comparison of similarities between groups was performed using the type strain of each PFGE profile at each time frame as an internal standard. For the multilocus sequence typing (MLST) scheme, PCR conditions and sequencing followed the instructions given at http://sepidermidis.mlst.net. Numbers for alleles and sequence types (STs) were assigned according to the *S. epidermidis* MLST database.

PCR of the resistance determinants

Chromosomal extraction of whole genomic DNA was performed as previously described.[11] All strains were screened for the presence of *mecA* (PBP2A),[16] *cfr*,[17] *erm*(A), *erm*(B), *erm*(C), *vga*, *vgb*, *vat*, *vat*(B) and *cat* genes.[18]

The SCC*mec* cassettes were first determined by a multiplex PCR protocol previously described, and assigned to the corresponding types. Furthermore, the results were confirmed by different multiplex PCR protocols, focusing on the *mec* gene complex and the *ccr* gene complex.[16,19] PCR and multiplex PCR amplifications were performed in a Biometra T3000 Thermal Cycler (M-medical Srl, 20010 Cornaredo, Milan, Italy)

Testing for the presence of linezolid resistance mutations

Domain V of the 23S rRNA gene was amplified by PCR using the primers V1—GCGGTCGCCTCCTAAAAG and V2—ATCCCGGTCCTCTCGTACTA. The 420 bp amplicons were treated with restriction enzyme NheI and analysed by agarose gel electrophoresis to confirm the absence/presence of the most frequent mutation G2576T, which generates a new NheI site.[5] Fragments were also sequenced for confirmation. PCR and sequence analysis were also performed in order to identify possible mutations in *rplD*, *V* and *C* genes, encoding, respectively, L4, L22 and L3 proteins, as described previously.[20]

Results

All 33 linezolid-resistant CoNS isolates were methicillin resistant. Table 1 summarizes the putative mechanisms responsible for linezolid resistance and the presence in these strains of other resistance determinants that can alter regions adjacent to the linezolid target, ultimately able to confer a multidrug resistance phenotype involving different classes of antibiotic, i.e. oxazolidinones, macrolides and chloramphenicol. All strains were also characterized at the molecular level.

Three different mechanisms of resistance were found in the *S. epidermidis* isolates. Seven isolates were found to have the G2576T mutation in all ribosomal alleles of domain V of 23S rRNA. All isolates were susceptible to erythromycin and were lincomycin and chloramphenicol resistant. These strains were clonally related, possessing the same pulsotype (L), belonging to the international *S. epidermidis* clone ST2, and carried *mec*-

Table 1. Molecular characteristics of the linezolid-resistant CoNS isolates and relationship to the level of linezolid resistance

Linezolid-resistant strains (*n*)	Mechanism of linezolid resistance	Linezolid MIC mg/L (*n*)	Associated genes (*n*)	Associated resistance (*n*)	PFGE profiles/ST	*mec* complex class	*ccr* types
S. epidermidis (7)	G2576T	64 (6)–128 (1)	*vga* (7) *cat* (7)	lincomycin (7) chloramphenicol (7)	L/ST2	A	A_3B_3, A_4B_4, *C*
S. epidermidis (8)	unknown	16	*erm*(A) (7) *vga* (8) *cat* (3)	erythromycin (8) lincomycin (8) chloramphenicol (7)	A1, A2, A3/ST23	B	A_2B_2
S. epidermidis (9)	*cfr*	64 (2)–256 (7)	*erm*(A) (9) *erm*(C) (2) *vga* (9) *cat* (2)	erythromycin (9) lincomycin (9) Q/D (1) chloramphenicol (9)	A1, A3/ST23	B	A_2B_2
S. hominis (5)	G2576T	>128	*vga* (5)	lincomycin (5) chloramphenicol (1)	H1, H2/ND	*mecA*	A_1B_1
S. simulans (4)	G2576T	64	*vga* (2)	lincomycin (4) chloramphenicol (4)	S1, S2/ND	A	A_3B_3, A_4B_4

Q/D, quinupristin/dalfopristin; ND, not defined.

complex A and $ccrA_4B_4$, $ccrA_3B_3$ and *ccrC* recombinase genes, in agreement with previously published data on the *mec* structure of *S. epidermidis*.[21]

Eight *S. epidermidis* strains showed mutations neither in the gene encoding domain V of the 23S rRNA nor the recently described *cfr* gene. To look for the possible mechanism of resistance, mutations in the genes responsible for L4, L22 or L3 proteins were investigated. While the sequence of *rplD* showed 100% nucleotide identity with the corresponding gene of the susceptible strain ATCC 12228, three mutations were found in the *rplV* gene encoding the L22 protein. These three mutations were not considered significant because they were not able to alter the protein itself. In an attempt to find new mutations in L3, a recently described protein involved in resistance to pleuromutilins, one mutation (C363G) was found that was different from the ATCC 12228 strain. Despite their susceptibility to quinupristin/dalfopristin, these strains carried the *vga* gene together with *erm*(A) and *cat*. These genes conferred resistance to erythromycin, lincomycin and chloramphenicol. All isolates were genotypically related (PFGE profiles A1, A2, A3) and belonged to the international ST23 clone.

Nine isolates of *S. epidermidis* showed varying degrees of resistance to linezolid, the MICs ranging from 64 (2/9) to 256 mg/L (7/9), by virtue of the methylation of A2503; this methylase is codified by the *cfr* gene. These strains were also resistant to erythromycin, lincomycin and chloramphenicol, with one isolate additionally resistant to quinupristin/dalfopristin. All isolates carried *erm*(A) and *vga* genes, while only two isolates carried *erm*(C) and *cat* genes. These strains belonged to the ST23 clone. Preliminary results performed in our laboratory, showed that the *cfr* gene is located on a plasmid of ~50 kb in size (data not shown).

In all *S. epidermidis* isolates shown to belong to ST23, methicillin resistance was associated with the *mec*-complex B and $ccrA_2B_2$ recombinase genes.

The linezolid-resistant isolates also included two other CoNS species, *S. hominis* and *S. simulans*. In both cases the G2576T mutation was the only mutation found in all alleles and in these strains, the *vga* gene was responsible for resistance to lincomycin and chloramphenicol.

All *S. hominis* strains belonged to closely related PFGE subtypes (H1–H2), carrying only the *mecA* gene and $ccrA_1B_1$ recombinase genes. All *S. simulans* strains belonged to similar PFGE subtypes (S1–S2) and carried *mec*-complex A and $ccrA_4B_4$ and $ccrA_3B_3$ recombinase genes (see Table 1).

Molecular investigation of these linezolid-resistant isolates showed that all ST2 *S. epidermidis* were referred from a single outbreak involving six patients in one hospital, while linezolid-resistant ST23 *S. epidermidis* carrying or not carrying the *cfr* gene were repeatedly isolated in the second hospital, over the course of 1 year, and involved 14 different patients. The other CoNS isolates were single isolates from different patients from the second hospital and comprised five *S. hominis* and four *S. simulans*.

Table 2 shows the distribution of MICs of vancomycin, teicoplanin and daptomycin for all linezolid-resistant strains, with 27 of the 33 strains showing MICs of 1–2 mg/L of vancomycin and an even distribution of MICs of teicoplanin. Daptomycin MICs ranged between 0.125 and 1 mg/L. Considering the EUCAST breakpoints, 15 of the 33 strains were resistant to vancomycin and 11 of the 33 strains resistant to teicoplanin; almost all strains are considered susceptible if CLSI breakpoints are used. All linezolid-resistant strains were strongly inhibited by daptomycin.

Table 2. MIC distribution of vancomycin, teicoplanin and daptomycin for linezolid-resistant CoNS strains

	Number of strains at the following concentrations (mg/L)								
Antibiotics	0.125	0.25	0.5	1	2	4	8	16	32
Vancomycin	0	0	1	17	10	4	1	0	0
Teicoplanin	0	5	1	4	3	9	9	2	0
Daptomycin	10	11	4	8	0	0	0	0	0

Discussion

The first report of a clinical linezolid-resistant staphylococcal strain came only 1 year after the introduction of linezolid[22] and since that time, resistant enterococci and staphylococci have been isolated all around the world, even if only sporadically.[23,24] Linezolid resistance, as described in numerous reports, has been mediated by mutations in 23S rRNA or other ribosomal protein genes, implying the slow dissemination of resistance by these mechanisms. However, the detection of a plasmid-borne *cfr*-mediated linezolid resistance gene in staphylococci, recently recovered from human specimens, adds a new dimension to the threat to the clinical use of several antimicrobial classes, including the oxazolidinones.

This article documents, for the first time to the best of our knowledge, the isolation of linezolid-resistant CoNS in Italy. Our results demonstrate different linezolid resistance mechanisms in multiple CoNS strains, with mutations and methylase modifications being found in different lineages of *S. epidermidis*, *S. hominis* and *S. simulans*. The linezolid-resistant *S. epidermidis* isolates showed different PFGE subtype profiles, and belonged to two different STs, namely ST2 and ST23, already described in this species.[25] The population structure of *S. epidermidis* was initially described in a group of strains isolated from different parts of the world,[26] but the expansion of the MLST database has changed this first description of the founder of the assignment and the composition of the CC2 structure.[27] Further studies of the population structure of this microorganism are clearly needed; however, even if we do not know much concerning the origins of these species, ST2 and ST23 are the most widely disseminated clones[25,26] both among linezolid-susceptible and linezolid-resistant strains.

With regard to the other CoNS species, G2576T mutations were found for the first time in *S. hominis* and *S. simulans*. All these strains variably possessed other resistance determinants conferring resistance to members of the macrolide, lincosamide, streptogramin, ketolide and oxazolidinone (MLSKO) group.

We describe here the methylase modification mechanism mediated by the *cfr* gene, which was first discovered in animal isolates and subsequently found in clinical staphylococcal strains. The presence of *cfr* conferred resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramins A (known as $PhPLOS_aA$), but not to erythromycin, and thus differs from rRNA methyltransferases. Resistance due to *cfr* was first

discovered in 2000 during a surveillance study among staphylococci from animals.[28] Since that time, five different human staphylococcal isolates have been found, and at least three different genetic organizations carrying the gene have been described.[7] In our *cfr*-positive strains, a plasmid of ~50 kb in size carries the gene and further studies are ongoing to completely characterize this genetic element in *S. epidermidis*. The number of isolates will surely increase: a recent outbreak of *cfr*-positive strains was recently described in Spain.[29] Interestingly, all the isolates reported here showed reduced susceptibility to vancomycin and in almost half of the cases resistance to both glycopeptides was observed; however, daptomycin retained full activity against these strains.

Different mechanisms of linezolid resistance were found in three CoNS species, i.e. *S. epidermidis*, *S. hominis* and *S. simulans*. Together with the G2576T mutations, A2503 methylation mediated by the *cfr* gene was found in different strains, and preliminary results demonstrate that *cfr* is localized on a plasmid of ~50 kb. However, we were unable to detect any known mechanisms of linezolid resistance in a group of *S. epidermidis* isolates.

In conclusion, even if the mutations documented here still appear to be sporadic, the recent acquisition of a linezolid resistance mechanism based on a modification of A2503 and mediated by the *cfr* gene localized on a transferable element, indicates a potential to disseminate among Gram-positive pathogenic strains. This gene, originally found in animal strains, is now present clinically and therefore attention should be paid to the fact that these strains might also be selected under treatment with phenicols or macrolides; this could be due to co-selection and might multiply the risk of development of linezolid-resistant strains. Continuous judicious use of linezolid and surveillance of resistance in staphylococci are needed to preserve the therapeutic efficacy of this important antimicrobial.

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Transparency declarations

None to declare.

References

1 Ford C, Hamel J, Stapert D *et al*. Oxazolidinones: a new class of antimicrobials. *Infect Med* 1999; **16**: 435.

2 Jones RN, Ross JE, Castanheira M *et al*. United States resistance surveillance results for linezolid (LEADER Program for 2007). *Diagn Microbiol Infect Dis* 2008; **62**: 416–26.

3 Potoski BA, Adams J, Clarke L *et al*. Epidemiological profile of linezolid-resistant coagulase-negative staphylococci. *Clin Infect Dis* 2006; **43**: 165–71.

4 Brauers J, Kresken M, Hafner D *et al*. Surveillance of linezolid resistance in Germany, 2001–2002. *Clin Microbiol Infect* 2005; **11**: 39–46.

5 Meka VG, Pillai SK, Sakoulas G *et al*. 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* 2004; **190**: 311–7.

6 Shaw KJ, Poppe S, Schaadt R *et al*. *In vitro* activity of TR-700, the antibacterial moiety of the prodrug TR-701, against linezolid-resistant strains. *Antimicrob Agents Chemother* 2008; **52**: 4442–7.

7 Stefani S, Bongiorno D, Mongelli G, Campanile F. Linezolid resistance in staphylococci. *Pharmaceuticals* 2010; **3**: 1.

8 Prystowsky J, Siddiqui F, Chosay J *et al*. Resistance to linezolid: characterization of mutations in rRNA and comparison of their occurrences in vancomycin-resistant enterococci. *Antimicrob Agents Chemother* 2001; **45**: 2154–6.

9 Ippolito JA, Kanyo ZF, Wang D *et al*. Crystal structure of the oxazolidinone antibiotic linezolid bound to the 50S ribosomal subunit. *J Med Chem* 2008; **51**: 3353–6.

10 Locke JB, Hilgers M, Shaw KJ. Novel ribosomal mutations in *Staphylococcus aureus* strains identified through selection with the oxazolidinones linezolid and torezolid (TR-700). *Antimicrob Agents Chemother* 2009; **53**: 5265–74.

11 Campanile F, Bongiorno D, Borbone S *et al*. Characterization of a variant of the SCC*mec* element in a bloodstream isolate of *Staphylococcus intermedius*. *Microb Drug Resist* 2007; **13**: 7–10.

12 Clinical and Laboratory Standard Institutes. *Performance Standards for Antimicrobial Susceptibility Testing: Approved Standard M100-S20*. CLSI, Wayne, PA, USA, 2010.

13 EUCAST. *Clinical Breakpoints*. 2010. http://www.eucast.org/clinical_breakpoints/ (4 August 2010, date last accessed).

14 Mato R, Campanile F, Stefani S *et al*. Clonal types and multidrug resistance patterns of methicillin-resistant *Staphylococcus aureus* (MRSA) recovered in Italy during the 1990s. *Microb Drug Resist* 2004; **10**: 106–13.

15 Tenover FC, Arbeit RD, Goering RV *et al*. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995; **33**: 2233–9.

16 Stefani S, Bongiorno D, Cafiso V *et al*. Pathotype and susceptibility profile of a community-acquired methicillin-resistant *Staphylococcus aureus* strain responsible for a case of severe pneumonia. *Diagn Microbiol Infect Dis* 2009; **63**: 100–4.

17 Kehrenberg C, Schwarz S, Jacobsen L *et al*. A new mechanism for chloramphenicol, florfenicol and clindamycin resistance: methylation of 23S ribosomal RNA at A2503. *Mol Microbiol* 2005; **57**: 1064–73.

18 Lina G, Quaglia A, Reverdy ME *et al*. Distribution of genes encoding resistance to macrolides, lincosamides, and streptogramins among staphylococci. *Antimicrob Agents Chemother* 1999; **43**: 1062–6.

19 Campanile F, Bongiorno D, Borbone S *et al*. Hospital-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) in Italy. *Ann Clin Microbiol Antimicrob* 2009; **8**: 22.

20 Locke JB, Hilgers M, Shaw KJ. Mutations in ribosomal protein L3 are associated with oxazolidinone resistance in staphylococci of clinical origin. *Antimicrob Agents Chemother* 2009; **53**: 5275–8.

21 Hanssen AM, Kjeldsen G, Sollid JU. Local variants of staphylococcal cassette chromosome *mec* in sporadic methicillin-resistant *Staphylococcus aureus* and methicillin-resistant coagulase-negative staphylococci: evidence of horizontal gene transfer? *Antimicrob Agents Chemother* 2004; **48**: 285–96.

22 Tsiodras S, Gold HS, Sakoulas G *et al*. Linezolid resistance in a clinical isolate of *Staphylococcus aureus*. *Lancet* 2001; **358**: 207–8.
23 Jones RN, Ross JE, Bell JM *et al*. Zyvox Annual Appraisal of Potency and Spectrum program: linezolid surveillance program results for 2008. *Diagn Microbiol Infect Dis* 2009; **65**: 404–13.
24 Bonora MG, Solbiati M, Stepan E *et al*. Emergence of linezolid resistance in the vancomycin-resistant *Enterococcus faecium* multilocus sequence typing C1 epidemic lineage. *J Clin Microbiol* 2006; **44**: 1153–5.
25 Wong A, Reddy SP, Smyth DS *et al*. Polyphyletic emergence of linezolid-resistant staphylococci in the United States. *Antimicrob Agents Chemother* 2010; **54**: 742–8.
26 Miragaia M, Thomas JC, Couto I *et al*. Inferring a population structure for *Staphylococcus epidermidis* from multilocus sequence typing data. *J Bacteriol* 2007; **189**: 2540–52.
27 Miragaia M, Carrico JA, Thomas JC *et al*. Comparison of molecular typing methods for characterization of *Staphylococcus epidermidis*: proposal for clone definition. *J Clin Microbiol* 2008; **46**: 118–29.
28 Schwarz S, Werckenthin C, Kehrenberg C. Identification of a plasmid-borne chloramphenicol-florfenicol resistance gene in *Staphylococcus sciuri*. *Antimicrob Agents Chemother* 2000; **44**: 2530–3.
29 Morales G, Picazo JJ, Baos E *et al*. Resistance to linezolid is mediated by the *cfr* gene in the first report of an outbreak of linezolid-resistant *Staphylococcus aureus*. *Clin Infect Dis* 2010; **50**: 821–5.