

Resistance to linezolid in *Staphylococcus* spp. clinical isolates associated with ribosomal binding site modifications: novel mutation in domain V of 23S rRNA

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SUMMARY

Linezolid is the main representative of the oxazolidinones, introduced in 2000 in clinical practice to treat severe Gram-positive infections. This compound inhibits protein synthesis by binding to the *peptidyl transferase centre* of the 50S bacterial ribosomal subunit. The aim of this study was to characterize 12 clinical strains of linezolid-resistant *Staphylococcus* spp. isolated in Northern Italy. All isolates of *Staphylococcus* spp. studied showed a multi-antibiotic resistance phenotype. In particular, all isolates showed the presence of the *mecA* gene associated with *SSCmec* types IVa, V or I. Mutations in domain V of 23S rRNA were shown to be the most prevalent mechanism of linezolid resistance: among these a new C2551T mutation was found in *S. aureus*, whilst the G2576T mutation was shown to be the most prevalent overall. Moreover, three *S. epidermidis* isolates were shown to have linezolid resistance associated only with alterations in both L3 and L4 ribosomal proteins. No strain was shown to harbor the previously described *cfr* gene. These results have shown how the clinical use of linezolid in Northern Italy has resulted in the selection of multiple antibiotic-resistant clinical isolates of *Staphylococcus* spp., with linezolid resistance in these strains being associated with mutations in 23S rRNA or ribosomal proteins L3 and L4.

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INTRODUCTION

Linezolid is a synthetic drug belonging to the oxazolidinone class of antibacterial agents known to bind to the 50S subunit of the prokaryotic ribosome thereby preventing formation of the initiation complex necessary for protein synthesis. This mode of action differs from that of existing protein synthesis inhibitors, which allow mRNA translation to begin but prevent peptide elongation (Livermore, 2003). This suggests that linezolid has a different ribosomal target site and its activity is therefore unaffected by the rRNA methylases that modify 23S rRNA, resulting in resistance only to macrolides, clindamycin and group B streptogramins. Moreover, linezolid has been demonstrated to possess a good *in vitro* and *in vivo* activity against the majority of Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) (Long *et al.*, 2012). The introduction of linezolid into clinical use in 2000 represented a major advance in the treatment of serious hos-

pital infections caused by multi-drug-resistant Gram-positive cocci, which during the last decade have become an important public health issue (Livermore, 2003). The synthetic nature of this agent determined a low probability of naturally occurring resistance, but after FDA approval, a few case reports described the emergence of linezolid resistance among clinical isolates, particularly following prolonged therapy (>21 days) (Peeters and Sarria, 2005; Mendes *et al.*, 2014). Although this in general remains a rare phenomenon, surveillance data indicate that coagulase-negative *Staphylococcus* spp. (CoNS) currently account for the majority of Gram-positive organisms displaying elevated minimum inhibitory concentrations (MIC) to linezolid (Cojutti *et al.*, 2014; Mendes *et al.*, 2016). Characterization of the linezolid resistance mechanisms in non-susceptible organisms has generally demonstrated distinct nucleotide substitutions in domain V of the 23S rRNA genes and/or alterations in the ribosomal protein L3 and/or L4 (Long *et al.*, 2012). Moreover, a highly effective and transferable form of resistance associated with an RNA methyltransferase (*cfr* gene) was recently described in both MRSA and CoNS isolates in the USA and other geographical areas (Mendes *et al.*, 2014; Russo *et al.*, 2015). The aim of this retrospective study was to investigate the phenotypic and molecular characteristics of linezolid-resistant *Staphylococcus* spp. clinical strains isolated from blood cultures or respiratory samples of patients hospital-

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ized in the North of Italy between 2010 and 2014 for serious infections and who had received linezolid treatment.

MATERIAL AND METHODS

Clinical isolates

Twelve strains of linezolid-resistant (LZD-R) *Staphylococcus* spp. were retrospectively obtained from collections of clinical isolates stored by the Microbiology Laboratories of San Gerardo Hospital in Monza and the Gaslini Institute in Genoa. In particular, these included 9 strains of methicillin-resistant *Staphylococcus epidermidis* (MRSE) and 1 *Staphylococcus hominis* (MRSH), isolated between 2010 and 2011 from blood samples of patients with bloodstream infections (BSIs) admitted to different departments of San Gerardo Hospital in Monza, as well as 2 methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated in 2014 from sputum samples of cystic fibrosis patients hospitalized at the Gaslini Institute in Genoa. All strains were isolated from patients who had received linezolid treatment during the previous 30-days.

Antibiotic susceptibility testing

Automated identification and susceptibility testing of strains was carried out using Vitek II, (bioMérieux, Marcy l'Etoile, France) following bacterial isolation. Antibiotic susceptibility to linezolid, erythromycin, clindamycin, gentamicin, tetracycline, fusidic acid, cefotaxime, vancomycin and ciprofloxacin, (Sigma-Aldrich, Italy) was further confirmed by MIC determination by means of the broth microdilution method according to *Clinical and Laboratory Standards Institute* (CLSI) guidelines (CLSI, 2013). Interpretation of results was based on CLSI and EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines (CLSI, 2013; EUCAST, 2015). *Staphylococcus aureus* ATCC 29213 strain was tested in parallel for quality control.

Molecular characterization

Bacterial DNA was extracted using GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, Italy) and subsequently stored in elution buffer at -2°C. The concentration and purity of extracted DNA was evaluated by spectrophotometric analysis (Nanodrop, Thermo Scientific).

The SCCmec typing method was performed by a multiplex PCR analysis (Mastercycler® EP, Eppendorf) using the previously described primers and protocol (Kondo *et al.*, 2007). Some strains required a second multiplex PCR analysis to differentiate the SCCmec type II/VI and IV subgroup using the method previously described by Zhang *et al.* (Zhang *et al.*, 2005).

Isolates were screened for *cfr*, *ermA-B-C* and *mrsA* genes, as well as for the Pantone-Valentine leukocidin gene in *S. aureus*, by means of PCR analysis as previously described (Lina *et al.*, 1999; Martineau *et al.*, 2000; Kehrenberg *et al.*, 2006). Mutations in domain V of 23S rRNA and for L3, L4, L22 ribosomal proteins were also evaluated by PCR analysis and sequencing of *rplC*, *rplD*, *rplV* and *SER02*, *SER05*, *SER08*, *SER11*, *SER15* genes as described by Pillai *et al.* (Pillai *et al.*, 2002), using ABI Genetic Analyzer 3130 (Thermo Fischer Scientific) and Bioedit software by Ibis Biosciences. All isolates were further characterized by pulsed field gel electrophoresis (PFGE) analysis to investigate clonal type as previously described (Chung *et al.*, 2000). SmaI enzyme was used for restriction analysis.

RESULTS

The studied strains were isolated from clinical samples collected from hospitalized patients who had received linezolid treatment during the previous 30-days. Strains isolated prior to the start of treatment were not available for analysis. Of these, 10 CoNS strains (9 *S. epidermidis* and 1 *S. hominis*) were isolated from blood culture samples of patients in the Intensive Care Unit at San Gerardo Hospital, Monza as well as 2 strains of *Staphylococcus aureus* isolated from respiratory tract samples obtained from cystic fibrosis patients admitted to the Gaslini Institute, Genoa.

The multi-antibiotic resistance profiles of the studied isolates are shown in *Table 1*. All linezolid-resistant *Staphylococcus* spp. strains, however, were shown to be susceptible to tetracycline, fusidic acid and vancomycin. Resistance to linezolid (MIC values ranging from 8 to 64 µg/mL) was always associated with resistance to erythromycin, clindamycin and methicillin.

Results for the presence of the main antibiotic resistance genetic determinants are shown in *Table 2*. Two main genetic clusters were detected among MRSE strains: 3 (3/9; 33%) isolates characterized by SCCmec type IVa and ribosomal proteins mutations (H146Q, V154L, A157R modifications in L3 and ₇₁GGR₇₂ e N158S in L4) and a second cluster of 5 (5/9; 55%) characterized by the co-presence of SCCmec type V and G2576T mutation present in 4 out of the 5 gene copies in domain V of 23S rRNA. Only one MRSE strain (Monz04) was found to have the G2447T mutation in all 5 copies of 23s rRNA.

Studied *S. hominis* and *S. aureus* isolates were shown to carry SCCmec type I as well as mutations in domain V of 23S rRNA: G2576T in *S. hominis* and in one *S. aureus* isolate whilst the second *S. aureus* isolate showed a C2551T mutation, which to our knowledge has not been previously described.

None of the studied isolates showed evidence of modifications in L22 ribosomal proteins or of the acquisition of the more recently described plasmid encoded *cfr* gene.

The concomitant presence of macrolide resistance genetic determinants was demonstrated in 10/12 (83%) of the linezolid resistant isolates studied, as shown in *Table 2*, with a heterogeneous profile. Both *S. aureus* isolates were found to be negative for the Pantone-Valentine leukocidin gene.

Finally, PFGE analysis demonstrated different clonal types among the studied isolates (data not shown). In particular, the profiles obtained by PFGE analysis showed a clonal cluster only for the 5 strains of *S. epidermidis* characterized by possessing the G2576T mutation in domain V of 23S rRNA. Neither *S. epidermidis* strains, characterized by mutations at L3 and L4 level, nor the *S. aureus* strains were found to be clonally related.

DISCUSSION

Infections caused by multi-antibiotic-resistant Gram-positive cocci become serious threat, particularly to vulnerable hospitalized patients, and currently represent an important public health issue (Livermore, 2003). The dramatic emergence and worldwide dissemination of antibiotic resistance in these pathogens has resulted in an increased clinical use of the few most recently approved antimicrobial agents, such as linezolid.

Table 1 - Characteristics and antimicrobial susceptibility profiles (MIC values, expressed in mg/mL) of linezolid-resistant *Staphylococcus* spp.

Microbial strain	Strain ID	Department of origin	Source	LZD	ERY	DA	CTX	GM	TET	FUS	OXA	CIP	VA
<i>S. epidermidis</i>	Monz01	Intensive care unit (Monza)	Blood culture	R (64)	I (4)	R (1)	R (>128)	S (4)	S (2)	S (<0,5)	R (4)	R (128)	S (2)
<i>S. epidermidis</i>	Monz02	Intensive care unit (Monza)	Blood culture	R (32)	R (8)	R (2)	R (>128)	R (16)	S (1)	S (<0,5)	R (4)	R (64)	S (2)
<i>S. epidermidis</i>	Monz03	Intensive care unit (Monza)	Blood culture	R (64)	I (4)	R (4)	R (>128)	R (16)	S (1)	S (<0,5)	R (4)	R (64)	S (1)
<i>S. epidermidis</i>	Monz04	Intensive care unit (Monza)	Blood culture	R (64)	R (8)	R (0,5)	R (>128)	R (16)	S (4)	S (<0,5)	R (4)	R (64)	S (2)
<i>S. epidermidis</i>	Monz05	Surgery (Monza)	Blood culture	R (32)	R (8)	R (2)	R (>128)	R (16)	S (2)	S (<0,5)	R (4)	R (128)	S (2)
<i>S. epidermidis</i>	Monz06	Intensive care unit (Monza)	Blood culture	R (32)	R (8)	R (1)	R (>128)	R (16)	S (0,5)	S (<0,5)	R (4)	R (128)	S (1)
<i>S. epidermidis</i>	Monz07	Infectious diseases (Monza)	Blood culture	R (32)	R (8)	R (0,5)	R (>128)	R (16)	S (2)	S (<0,5)	R (4)	R (128)	S (1)
<i>S. epidermidis</i>	Monz08	Intensive care unit (Monza)	Blood culture	R (32)	R (8)	R (1)	R (>128)	R (16)	S (2)	S (<0,5)	R (4)	R (128)	S (2)
<i>S. epidermidis</i>	Monz09	Intensive care unit (Monza)	Blood culture	R (32)	R (8)	R (1)	R (>128)	R (16)	S (2)	S (<0,5)	R (4)	R (128)	S (4)
<i>S. hominis</i>	Monz10	Intensive care unit (Monza)	Blood culture	R (32)	R (8)	R (1)	R (>128)	I (8)	S (2)	S (<0,5)	R (4)	R (64)	S (2)
<i>S. aureus</i>	Gasl01	Cystic fibrosis unit (Genoa)	Sputum	R (8)	R (8)	R (1)	R (>128)		S (2)	S (<0,5)	R (4)	R (128)	S (2)
<i>S. aureus</i>	Gasl02	Cystic fibrosis unit (Genoa)	Sputum	R (32)	R (8)	R (1)	R (>128)		S (2)	S (<0,5)	R (4)	R (16)	S (2)

Legend: LZD: linezolid, ERY: erythromycin, CIP: ciprofloxacin, GM: gentamicin, TET: tetracycline, FUS: fusidic acid, OXA: oxacillin, CTX: cefotaxime, DA: clindamycin, VA: vancomycin, R: resistant, I: intermediate, S: susceptible.

Linezolid was introduced in clinical practice in 2000 with the claim that, in view of its synthetic origin, resistance would be rare and difficult for bacteria to develop (Fines *et al.*, 2000). However, already in 2001 Tsiodras *et al.* reported the first staphylococcal clinical isolate with resistance to linezolid recovered from a patient receiving oral linezolid for the treatment of peritoneal dialysis-associated peritonitis (Tsiodras *et al.*, 2001). Since then there have been several reports worldwide describing the emergence of linezolid resistance in *Staphylococcus* spp., also associated with multi-antibiotic resistance, particularly during the course of linezolid treatment, emphasizing the leading role of linezolid in selecting resistant strains. Surveillance data indicate that CoNS isolates, mainly *Staphylococcus epidermidis*, currently account for the majority of Gram-positive organisms displaying reduced susceptibility to linezolid (Mendes *et al.*, 2014). This is of particular concern as CoNS, which commonly inhabit human skin, are now recognized as important opportunistic pathogens responsible for nosocomial bloodstream infections and

in-dwelling catheter-related bacteraemia. Furthermore, small colony variant (SCV) methicillin-resistant *Staphylococcus aureus*, such as those typically isolated from cystic fibrosis patients who often undergo long-term antibiotic therapy, are exposed to increasing antibiotic selection pressure favoring the emergence of linezolid-resistant isolates (Morelli *et al.*, 2015).

Linezolid resistance has been characterized by several different modifications of the linezolid binding site on the ribosome, initially associated with mutations in domain V of 23S rRNA and more recently also to L3 and L4 ribosomal proteins modifications (Long *et al.*, 2012). Moreover, the number of rRNA genes mutated has been shown to influence the level of linezolid resistance and to depend on the duration of linezolid exposure and dosage (Pillai *et al.*, 2002). The only reported horizontally transferable plasmid gene conferring cross resistance to linezolid, chloramphenicol, clindamycin and streptogramin A is the *cf*r gene encoding for a 23S rRNA methyltransferase (Schwarz *et al.*, 2000; Zhang *et al.*, 2005).

Linezolid-resistant CoNS have been reported in Italy since 2008 (Campanile *et al.*, 2013). Molecular characterization of 50 strains isolated between 2010 and 2011 from different Italian geographical areas showed the *cfr* gene, alone or in combination with ribosomal modifications, to be the major cause of linezolid resistance in *Staphylococcus* spp. (22/50; 44%) (Campanile *et al.*, 2013). However, in a more recent Italian multicenter clinical study on linezolid-resistant staphylococcal bacteraemia, the majority of strains, isolated between 2012 and 2013, showed the G2576T mutation in domain V of 23S rRNA (22/28; 78.6%) to be the principal mechanism of resistance and only one isolate (3.6%) demonstrated the *cfr* gene (Russo *et al.*, 2015). This clinical study also demonstrated that linezolid-resistant staphylococcal bloodstream infections are associated with a more unfavorable outcome in a significant proportion of patients.

Linezolid resistance in clinical strains of *Staphylococcus* spp. isolated in geographical areas near Monza or Genoa has not, however, to our knowledge been previously reported.

The present retrospective study determined the mechanisms responsible for linezolid resistance in 10 CoNS and 2 methicillin-resistant *S. aureus*, isolated from hospitalized patients in 2 cities of the North of Italy.

None of the linezolid-resistant strains analyzed were found to be carrying the *cfr* gene or modifications in the L22 ribosomal proteins. Linezolid resistance mechanisms clustered the 9 *S. epidermidis* isolates in two different

groups: one cluster included 5 (55%) *S. epidermidis* strains characterized by the SCCmec type IVa and the G2576T mutation in 4 out of 5 copies of 23S rRNA, whilst 3 (33%) strains showed SCCmec type V associated with ribosomal protein modifications in L3 and L4. Interestingly, one of the *S. epidermidis* studied strains showed a G2447T mutation to be present in all the 23S rRNA gene copies (Mendes *et al.*, 2014; Wong *et al.*, 2010).

The *S. aureus* isolates studied were both found to harbor SCCmec type I; one strain was found to have G2576T mutation of 23S rRNA gene (linezolid MIC =8 µg/mL), whilst the second isolate was characterized by a new C2551T mutation in domain V of 23S rRNA (currently under investigation), associated with linezolid MIC values equal to 32 µg/mL, which to our knowledge has not been previously described in *S. aureus* (Mendes *et al.*, 2014). Interestingly, the new C2551T mutated position in domain V of 23S rRNA was previously associated with ribosomal stability and peptide elongation (Sirum-Connolly *et al.*, 1995; Green *et al.*, 1997).

The molecular basis for linezolid resistance in the *Staphylococcus* spp. strains analyzed in this study is comparable to what has recently been described in other areas of Italy (Russo *et al.*, 2015; Mendes *et al.*, 2014), suggesting the accumulation of independent mutational events in both CoNS and *S. aureus* strains, possibly resulting from antibiotic selective pressure.

In conclusion, although overall linezolid resistance in *Staphylococcus* spp. remains uncommon, these findings

Table 2 - Genetic resistance markers of linezolid resistance in *Staphylococcus* spp. Strains.

Microbial strain	Strain ID	<i>mecA</i> gene presence	SCCmec Type	rRNA 23S mutations (mutated copies/total copies)	Ribosome-protein mutation			<i>cfr</i> gene presence	Macrolide resistance genes (<i>ermA</i> , <i>ermB</i> , <i>ermC</i> , <i>msrA</i>)	Panton-Valentine leukocidin
					L3	L4	L22			
<i>S. epidermidis</i>	Monz01	Positive	IVa	WT	H146Q, V154L, A157R	71GGR72, N158S	WT	negative	-	n.d.
<i>S. epidermidis</i>	Monz02	Positive	IVa	WT	H146Q, V154L, A157R	71GGR72, N158S	WT	negative	<i>ermC</i>	n.d.
<i>S. epidermidis</i>	Monz03	Positive	IVa	WT	H146Q, V154L, A157R	71GGR72, N158S	WT	negative	-	n.d.
<i>S. epidermidis</i>	Monz04	Positive	V	G2447T (5/5)	WT	WT	WT	negative	<i>ermC-msrA</i>	n.d.
<i>S. epidermidis</i>	Monz05	Positive	V	G2576T (4/5)	WT	WT	WT	negative	<i>ermC-msrA</i>	n.d.
<i>S. epidermidis</i>	Monz06	Positive	V	G2576T (4/5)	WT	WT	WT	negative	<i>msrA</i>	n.d.
<i>S. epidermidis</i>	Monz07	Positive	V	G2576T (4/5)	WT	WT	WT	negative	<i>msrA</i>	n.d.
<i>S. epidermidis</i>	Monz08	Positive	V	G2576T (4/5)	WT	WT	WT	negative	<i>msrA</i>	n.d.
<i>S. epidermidis</i>	Monz09	Positive	V	G2576T (4/5)	WT	WT	WT	negative	<i>ermC-msrA</i>	n.d.
<i>S. hominis</i>	Monz10	Positive	I	G2576T	n.d.	n.d.	n.d.	negative	<i>ermC</i>	n.d.
<i>S. aureus</i>	Gasl01	Positive	I	G2576T	WT	WT	WT	negative	<i>ermA</i>	negative
<i>S. aureus</i>	Gasl02	Positive	I	C2551T (new)	n.d.	n.d.	WT	negative	<i>ermA</i>	negative

Legend: WT: wild type, n.d.: not determined.

emphasize the need for continued surveillance and for improved infection control measures in order to prevent the emergence of linezolid resistance in Gram-positive cocci.

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