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ARTICLE

Outbreak of linezolid-resistant *Staphylococcus haemolyticus* in an Italian intensive care unit

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Abstract We report an outbreak of linezolid-resistant *Staphylococcus haemolyticus* strains (MIC 32 mg/L) in patients admitted to the Verona University Hospital Intensive Care Unit. The strains proved to be clonally related at pulsed field gel electrophoresis. All the strains showed the G2576T mutation responsible for linezolid-resistance and retained their resistance even after several passages on antibiotic-free medium. After a decade of linezolid use, multifocal emergence of linezolid resistance in coagulase-negative staphylococci has become an important matter of concern and mandates stricter control over the use of this antibiotic in order to preserve its clinical utility.

Introduction

Staphylococci with acquired multi-drug resistance associated with methicillin resistance are a major problem recently spreading from hospital- to community-acquired infections. The oxazolidinone antimicrobial compound linezolid is normally active against many drug-resistant Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE).

Although even recent reports confirm that resistance to linezolid is minimal among coagulase-negative staphylococci (CoNS) [1, 2], a number of documented cases have occurred in Europe [3–5].

Linezolid binds to the domain V region of 23S rRNA and the effect of binding to 50S is inhibition of 70S formation. Even though the binding sites are similar, the action mechanism of oxazolidinones differs from those of all known protein synthesis inhibitors. Linezolid mainly works by binding the P site, thus inhibiting initiation complex formation and also translocation of peptydil-tRNA from the A site to the P site [6].

Decreased ribosomal affinity for the oxazolidinones due to mutations at 23S rRNA in the central loop of domain V is the main cause of bacterial resistance to these drugs [7–10]. Most mutations defined in various species and associated with linezolid resistance occur by a G to U substitution at position 2576 in the vicinity of the P site [7, 11]. Furthermore, mutation in the conserved region of L4 riboprotein encoded by the *rplD* gene conferred cross-resistance to oxazolidinones, macrolides and chloramphenicol in *Streptococcus pneumoniae* [12]. As a third possibility, a *cfr*-gene- encoded methyltransferase, which alters adenosine at position 2503 in 23S rRNA, has also been reported in clinical strains [13, 14].

We report here on an outbreak of linezolid-resistant *S. haemolyticus* (MIC, 32 mg/L) isolated from patients admitted to the Verona University Hospital ICU.

Materials and methods

Patients and strains

The SHA 200 linezolid-resistant *S. haemolyticus* strain was isolated from a blood culture of a 65-year-old male admitted to the Verona University Hospital ICU on 1 August 2006, after extensive surgery for acute pancreatitis.

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Empirical therapy with teicoplanin (600 mg) was started on admission followed by 200 mg/day after 48 h. On the sixth day of therapy, a strain of *S. haemolyticus* was isolated from the blood culture. The strain proved resistant to teicoplanin (MIC, 16 mg/L), which was immediately suspended and replaced with linezolid (MIC, 0.25 mg/L). After a further 8 days, a new blood culture showed that the same strain had become resistant to linezolid (MIC, 32 mg/L). The strain was investigated with a view to establishing its resistance mechanism.

We also included in the study two linezolid-susceptible strains of *S. haemolyticus* (SHA 203 and SHA 204) isolated from blood cultures of two different patients admitted to the ICU over the same period as the index case. All the strains were methicillin-resistant.

SHA 200 was also compared to ten additional linezolid-resistant strains of *S. haemolyticus* subsequently isolated in the same unit from non-duplicated patients over the following 6 months. Table 1 summarizes the clinical data for the isolates.

Antimicrobial susceptibility testing

The MICs of all antibiotics were determined by means of the E-test and confirmed by microdilution tests according to the latest EUCAST documents [15].

Stability of the resistant phenotype

To study the stability of the resistant phenotype in the absence of antimicrobial selective pressure, a single colony of the linezolid-resistant *S. haemolyticus*, SHA 200, was serially transferred 30 times on antibiotic-free medium (Müller-Hinton agar) incubated overnight at 37°C. The linezolid MICs for all organisms recovered after each of the 30 passages were determined by E-test.

Growth curves

The growth curves of linezolid-susceptible and linezolid-resistant strains were compared by measuring the optical density at 640 nm at one-hour intervals. The initial inocula were obtained by diluting the overnight cultures so as to achieve the same concentrations of the two strains.

Pulsed field gel electrophoresis (PFGE)

PFGE was carried out using standard procedures. The DNA preparation was digested with 30 U of *SmaI* for 3 h. The fragments were separated with a linear ramped pulse time of 6.8–63.8 over a period of 23 h at 14°C [16].

Detection of the G2576T mutation

Both the linezolid-susceptible and the linezolid-resistant strains were analyzed for the presence of the G2576T mutation (according to the *Escherichia coli* numbering system) in the V domain of the 23S rRNA gene. The primers used were 23Sfw and 23Srev [7] with the following cycling conditions: 30 cycles consisting of 94°C for 1 min, 50°C for 30 sec, and 72°C for 1 min.

The PCR fragments (596 pb) were purified and sequenced.

We also used a polymerase chain reaction (PCR) to check for the other known mechanisms of linezolid resistance, namely, the *rplD* (L4 ribosomal protein) and *cfr* (chloramphenicol florfenicol resistance) genes. All the oligonucleotide primers applied had been described previously [13, 17]. In the case of the *rplD* gene, the cycling parameters were the following: 30 cycles consisting of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Amplification of the *cfr* gene was performed according to PCR parameters published previously [17]. The rplCfw

Table 1 Clinical data of the *S. haemolyticus* strains included in the study

Strain	Patient's age	Ward	Source	Collection date	Previous therapy with	Outcome of hospitalization
SHA200	65	ICU	Blood	24 August 2006	LIN 8 days	Discharge
SHA205	56	ICU	Blood	4 November 2006	ND	Discharge
SHA206	70	ICU	Blood	11 November 2006	ND	Discharge
SHA214	69	ICU NCH	Blood	27 November 2006	ND	Death
SHA219	56	ICU	Blood	18 December 2006	LIN 4 days	Discharge
SHA226	33	ICU	Blood	03 January 2007	LIN 8 days	Discharge
SHA228	30	ICU NCH	Blood	12 January 2007	ND	Discharge
SHA233	74	ICU	Blood	29 January 2007	LIN 11 days	Death
SHA234	74	ICU	Blood	19 February 2007	LIN 10 days	Discharge
SHA239	74	ICU	Blood	10 March 2007	ND	Discharge
		ICU	CVC	05 March 2007	ND	Discharge
SHA240	55	CARDIAC SURGERY	Blood	6 March 2007	LIN 10 days	Discharge

primer was earlier published by Locke et al. [18], and the rplCrev primer (CGT AAT GAC GCA CGT TGT AAG: corresponding to 1861260–1861280 nucleotide bases of *S. epidermidis* RP62A, GenBank accession no. NC_002976) was designed on the MWG Operon homepage. For the amplification of the *rplC* gene, 33 cycles were applied with 94°C denaturation (1 min), 53°C annealing (1 min) and 72°C temperature (1 min).

Positive PCR products were purified (Qiagen) and sequenced (MWG Operon) on both strands. The DNA sequences obtained were aligned with the corresponding nucleotide sequences of the *E. coli* reference strain (GenBank accession no V00331) in NCBI Blast. The *rplC* and *rplD* gene sequence results were analysed on amino-acid level aligned with reference strain S. *haemolyticus* JCSC1435 DNA (L3 riboprotein ID: BAE04111.1 and L4 riboprotein ID: BAE04112.1).

Determination of number of alleles mutated

Five µl of PCR product were digested with 10 U of Nhe I restriction enzyme at 37°C for 1 h. The complete digestion of the PCR products indicated that all the alleles were mutated.

Results

Table 2 reports the MIC values of linezolid, vancomycin, teicoplanin and other recently released anti-staphylococcal compounds for SHA 200 (linezolid-resistant) and SHA 203 (linezolid-susceptible). The MIC values for all the additional linezolid-resistant isolates of *S. haemolyticus* were exactly the same as for SHA 200, and those for SHA 203 were exactly the same as for SHA 204.

After 30 passages on antibiotic-free medium, the resistance of strain SHA 200 was still unvaried at 32 mg/L.

Table 2 Minimum inhibitory concentrations (MICs) of linezolid-resistant and linezolid-susceptible strains for different anti-staphylococcal compounds

Antimicrobials	MIC (mg/L)	
	SHA 200	SHA203
Linezolid	32	0.5
Daptomycin	0.125	0.125
Quinopristin/dalfopristin	0.5	0.25
Telavancin	0.12	0.06
Tygecycline	1	1
Teicoplanin	16	4
Vancomycin	4	2

Compared with the linezolid-susceptible strain, all the linezolid-resistant strains showed a significant difference in their in-vitro growth characteristics. Their growth rates were significantly slower, although at 24 h the OD_{640} nm was the same as for the susceptible strain.

Figure 1 shows the PFGE results for the linezolid-susceptible strain, the linezolid-resistant strain SHA 200 and an additional 5 linezolid-resistant strains isolated between December 2006 and February 2007 in the Verona University Hospital ICU. All the resistant strains had the same pattern, which is clearly different from the susceptible strain SHA 203, thus proving the clonal relationship between the linezolid-resistant strains.

After genetic analysis, all the linezolid-resistant strains showed the G2576T mutation in domain V of 23S rRNA. The linezolid-susceptible strains did not present this mutation, which has been shown to be responsible for the emergence of linezolid resistance [7].

The *cfr* gene was not detected in any of the isolates with the previously published primer pairs [13, 17]. After amplification of *rplC* and *rplD* genes, the nucleic acid and amino acid sequences of amplicons showed the wild type of L3 and L4 riboprotein without any mutation as compared with the *S. haemolyticus* susceptible strain.

The complete digestion with the NheI of the PCR products compared with undigested PCR indicates that all the alleles were mutated.

Conclusions

After almost ten years of linezolid use, and although the overall prevalence of linezolid resistance is still low [1], multifocal emergence of linezolid resistance in CoNS in

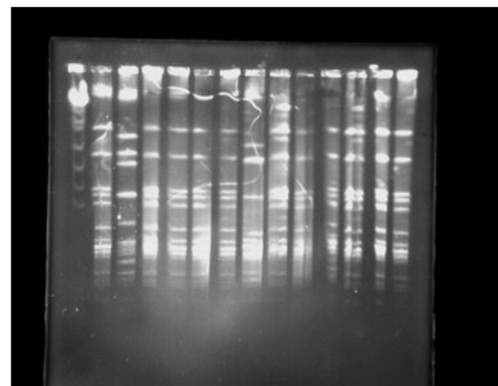

Fig. 1 Pulsed field gel electrophoresis (PFGE) of clinical strains involved in the outbreak. *Line 1* molecular weight; *line 2* SHA200 index strain; *line 3* SHA203 susceptible strain; *line 4* SHA205; *line 5* SHA206; *line 6* SHA214; *line 7* SHA219; *line 8* SHA224 susceptible strain; *line 9* SHA 226; *line 10* SHA228; *line 11* SHA233; *line 12* SHA234; *line 13* SHA239; *line 14* SHA240

different geographical areas has become an important matter of concern and involves species other than *S. epidermidis*.

We reported an outbreak of linezolid-resistant *S. haemolyticus* in the Verona University Hospital, ICU with the index strain isolated from a blood culture of a 65-year-old male after empirical therapy with teicoplanin, and discovered the mechanism of resistance to be the classic mutation G2576T in domain V of 23S rRNA.

This finding confirms the emergence in Italy of CoNS clinical strains with resistance to linezolid and other second-line antibiotics, as well as with reduced susceptibility to glycopeptides. The outbreak described in this paper occurred in a different geographical area and is antecedent to those described in a recent report, which documented for the first time the isolation of linezolid-resistant CoNS in Italy and demonstrated different linezolid resistance mechanisms in multiple CoNS species, namely, *S. epidermidis*, *S. hominis* and *S. simulans*, but not *S. haemolyticus* [4]. Thus, it broadens the picture of linezolid-resistance in CoNS in terms of time of onset, geographical spread and species concerned. Whilst no link with antibiotic usage was observed in the report by Bongiorno et al. (2010), the present outbreak seems to confirm the close relationship between linezolid use and resistance.

S. haemolyticus, a frequent coloniser of human skin which is second in frequency only to *S. epidermidis* among clinical isolates of CoNS [19], has been regarded since the early studies as an important nosocomial pathogen with a tendency to develop multiple resistance [20]. Frequent insertion sequences in its chromosome probably account for frequent genomic rearrangements [21]. Indeed, it was the first Gram-positive pathogen to acquire glycopeptide resistance, and it has been suggested as being more active than other CoNS in generating clones with increased glycopeptide (especially teicoplanin) MICs [22].

The finding of linezolid-resistant strains in this species represents a disquieting addition to the landscape of antimicrobial resistance in Italy, and mandates stricter control over the use of linezolid to preserve its clinical utility.

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