Resistance to Linezolid Is Mediated by the cfr Gene in the First Report of an Outbreak of Linezolid-Resistant Staphylococcus aureus

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Background. From April through June 2008, we identified 12 patients in the intensive care unit and 3 patients on other wards infected with methicillin-resistant Staphylococcus aureus that was also resistant to linezolid. We investigated the mechanism of resistance—point mutations in domain V of 23S ribosomal RNA (rRNA) or presence of the cfr gene—involved in the outbreak.

Methods. Strains for the study were obtained in the intensive care unit and other wards. Minimal inhibitory concentrations were determined using automated methods, the E-test, or dilution in Mueller-Hinton agar in accordance with Clinical and Laboratory Standards Institute guidelines. Strains were genotyped using pulsed-field gel electrophoresis and were sequenced to determine the presence of point mutations in 23S rRNA. The presence of the cfr gene was determined by specific polymerase chain reaction.

Results. The minimal inhibitory concentrations of linezolid ranged from 16 mg/L to 32 mg/L, and all the strains were susceptible to tigecycline, vancomycin, and daptomycin. Typing of strains sequentially isolated by pulsed-field gel electrophoresis showed that each patient carried only 1 clonal type of linezolid-resistant, methicillin-resistant S. aureus as detected by sequential isolations. The presence of the cfr gene was confirmed in all the isolates. Furthermore, sequencing of domain V of 23S rRNA showed that the most common mechanism of linezolid resistance reported to date, mutation G2576T, was not detected in any of the strains analyzed.

Conclusions. We report the presence of the cfr gene underlying the resistance mechanism involved in a clinical outbreak of linezolid-resistant S. aureus.
fenicol resistance). The cfr gene was initially described in a bovine Staphylococcus scuri isolate [10]. It has been found primarily in plasmids and appears to be capable of horizontal transfer between staphylococci [11]. The product of the cfr gene is a methyltransferase that catalyzes methylation of A2503 in the 23S rRNA gene of the large ribosomal subunit, conferring resistance to chloramphenicol, florfenicol, and clindamycin [12]. In 2007, Toh et al [13] reported the first cfr-mediated, linezolid-resistant clinical isolate of MRSA. Two new cases of cfr-mediated resistance in clinical isolates of Staphylococcus epidermidis and S. aureus were described in 2008 in the United States [14]. In human isolates, the gene was located in the chromosome, unlike the animal isolates, but it was probably part of an integrated plasmid that was potentially capable of excision and mobilization [13]. Therefore, the gene could be transmitted to other pathogenic strains and spread quickly.

We recently described [15] the first outbreak of linezolid-resistant MRSA. The outbreak took place in the intensive care unit (ICU) of a public hospital [15], lasted ∼3 months (April through June 2008), and affected 12 patients (M.S.-G., M.-A.d.L.T., G.M., B.P., M. J. Tolón, S. Domingo, E.J.C., R.A., A.A., N. García, F. Martínez-Sagasti, J.F., and J.J.P., unpublished data). During the same period, 3 more patients were detected with linezolid-resistant MRSA in other hospital wards (neurosurgery, traumatology, and general surgery). Measures to control the outbreak included isolation under barrier precautions and use of linezolid only in documented respiratory tract and complicated skin and soft-tissue infections caused by linezolid-susceptible microorganisms. No new cases have been identified since July 2008. We describe the mechanism of resistance of the linezolid-resistant MRSA strains isolated in the first reported outbreak in the ICU [15] and from other wards of a public hospital during the same period.

METHODS

Bacterial strains. We studied 16 linezolid-resistant clinical isolates, 12 of which were obtained from patients in the ICU, 3 from patients in other hospital wards, and 1 from fomites in the ICU. NCTC 8325 was the reference strain for pulsed-field gel electrophoresis (PFGE) and ATCC 29213 was the reference strain for quality control in determinations of the minimum inhibitory concentration (MIC).

Susceptibility testing. The MICs of tigecycline, vancomycin, daptomycin, trimethoprim-sulfamethoxazole, gentamicin, erythromycin, ciprofloxacin, clindamycin, and linezolid were determined using VITEK panels (bioMérieux SA) or MicroScan panels (Dade MicroScan) processed using the WIDER system (Francisco Soria Melguizo SA). The MICs of linezolid were further confirmed using the E-test (AB BIODISK). Resistance to linezolid was defined as an MIC >4 mg/L. For some antibiotics (linezolid, chloramphenicol, erythromycin, tigecycline, teicoplanin, clindamycin, and vancomycin), MICs were also confirmed using an agar dilution method following the 2009 guidelines of the Clinical and Laboratory Standards Institute [16].

Molecular typing. Linezolid-resistant MRSA isolates were genotyped by PFGE following the protocol described by Mur- chan et al [17] with minor modifications. Briefly, the linezolid-resistant MRSA suspensions were adjusted to a density equivalent of 5 × 10⁸ cells/mL, mixed with an equal volume of 2% low melting agarose gel, and poured into the block molds. The agarose plugs obtained were treated with proteinase K/lysostaphin and digested with 20 U of SmaI restriction enzyme. The plugs were loaded into 1% agarose gels and electrophoresed using a CHEF-DRIII apparatus (Bio-Rad Laboratories) in 0.5× TBE buffer. Run time was 20 h with an initial switch time of 5 s and a final switch time of 40 s. The ramping factor was linear. Temperature was set at 14°C, voltage at 6 V/cm, and the included angle at 120°. The gels were stained with ethidium bromide, visualized under ultraviolet light, and documented using the Molecular Imager ChemiDoc XRS (Bio-Rad Laboratories). The dendrogram was constructed using the Dice correlation coefficient and the unweighted pair group method with arithmetic mean with a 3% band tolerance. The criteria used to define a cluster were a similarity cutoff of 80% [17] and a difference ≤6 bands, as described by Tenover et al [18].

DNA purification. Total DNA of each isolate was extracted using the Nuclisense Easymag automated system (bioMérieux SA).

Amplification of the cfr gene using polymerase chain reaction (PCR). The presence of cfr was assessed by PCR with use of oligonucleotide primers described elsewhere [19]. PCR conditions were as follows: denaturation for 2 min at 94°C, 30 cycles of denaturation for 10 s at 94°C, annealing for 30 s at 55°C, extension for 30 s at 72°C, and a final extension of 7 min at 72°C.

PCR amplification of individual 23S rRNA genes and domain V. Five specific primer sets [20] were used to amplify each of the 5 copies of the 23S rRNA genes individually. As the amplification products range from 5.5 to 6.5 kb, the Expand Long Template PCR System (Roche Diagnostics GmbH) was used for long-range PCR. The PCR conditions were denaturation for 2 min at 94°C, 30 cycles of denaturation for 10 s at 94°C, annealing for 30 s at 53°C, extension for 4 min (increasing 20 s/cycle from cycle 11 through cycle 30) at 68°C, and a final extension of 7 min at 68°C. PCR products were analyzed using agarose gel electrophoresis to assess their size. Amplified DNAs were diluted 1:1000, and 5 μL was used for nested PCR to amplify the domain V region that contains nucleotide 2576 of the Escherichia coli sequence with use of oligonucleotide primers.
Table 1. Patients With Linezolid-Resistant Methicillin-Resistant *Staphylococcus aureus* Isolates

<table>
<thead>
<tr>
<th>Patient</th>
<th>Infection</th>
<th>Treatment</th>
<th>ICU outcome</th>
<th>PFGE Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Primary bacteremia</td>
<td>Vancomycin</td>
<td>Survived</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>Primary bacteremia</td>
<td>Vancomycin</td>
<td>Survived</td>
<td>A</td>
</tr>
<tr>
<td>3</td>
<td>Primary bacteremia</td>
<td>Vancomycin</td>
<td>Survived</td>
<td>A</td>
</tr>
<tr>
<td>4</td>
<td>VAP</td>
<td>Tigecycline</td>
<td>Survived</td>
<td>A</td>
</tr>
<tr>
<td>5</td>
<td>VAP</td>
<td>Tigecycline</td>
<td>Died</td>
<td>C</td>
</tr>
<tr>
<td>6</td>
<td>VAP</td>
<td>Tigecycline</td>
<td>Survived</td>
<td>A</td>
</tr>
<tr>
<td>7</td>
<td>Colonization</td>
<td>...</td>
<td>Died</td>
<td>A</td>
</tr>
<tr>
<td>8</td>
<td>Colonization,</td>
<td>Vancomycin-tigecycline</td>
<td>Died</td>
<td>A</td>
</tr>
<tr>
<td>9</td>
<td>VAP</td>
<td>Tigecycline</td>
<td>Died</td>
<td>A</td>
</tr>
<tr>
<td>10</td>
<td>VAP</td>
<td>Vancomycin</td>
<td>Survived</td>
<td>A</td>
</tr>
<tr>
<td>11</td>
<td>Colonization</td>
<td>...</td>
<td>Died</td>
<td>A</td>
</tr>
<tr>
<td>12</td>
<td>VAP</td>
<td>Tigecycline</td>
<td>Survived</td>
<td>A</td>
</tr>
<tr>
<td>13</td>
<td>Colonization</td>
<td>...</td>
<td>Survived</td>
<td>A</td>
</tr>
<tr>
<td>14</td>
<td>SSI</td>
<td>Tigecycline</td>
<td>Survived</td>
<td>D</td>
</tr>
<tr>
<td>15</td>
<td>VAP, CSF infection</td>
<td>Vancomycin</td>
<td>Survived</td>
<td>A</td>
</tr>
</tbody>
</table>

**NOTE.** CSF, cerebrospinal fluid; ICU, intensive care unit; PFGE, pulsed-field gel electrophoresis; SSI, surgical site infection; VAP, ventilator-assisted pneumonia.

* a Positive blood sample drawn through catheter. Simultaneous peripheral blood cultures and catheter tip cultures were negative.

* b Initial therapy with vancomycin; tigecycline rescue treatment added later.

Described elsewhere [20]. The conditions for PCR were as follows: denaturation for 2 min at 94°C, 30 cycles of denaturation for 10 s at 94°C, annealing for 30 s at 55°C, extension for 30 s at 72°C, and a final extension of 7 min at 72°C.

**Sequences.** Amplified DNA was purified using the Ultra-clean PCR Clean-up kit (MOBIO Laboratories). Sequencing was performed in a thermal cycler with use of the dRhodamine Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), the same primers as for the PCR, and 2–4 μL of the purified DNA.

**RESULTS**

**Patients and outbreak.** The outbreak of linezolid-resistant MRSA affected 12 patients in the ICU from April through June 2008. Three more patients with linezolid-resistant MRSA were identified in different hospital wards during the same period. These patients were not considered to be part of the outbreak, because they were detected outside the ICU, although they were included in the study of the resistance mechanism. An environmental sample obtained in the ICU was also positive for linezolid-resistant MRSA. A summary of the infections, treatments, and outcomes of patients as well as the PFGE profile of the strains is shown in the Table 1.

**Antibiotic susceptibility.** All clinical strains were susceptible to tigecycline, vancomycin, teicoplanin, and daptomycin, and 20% of the strains were resistant to trimethoprim/sulfamethoxazole. More than 85% of the strains were resistant to gentamicin, erythromycin, and ciprofloxacin, and all strains were resistant to clindamycin. The MICs of linezolid ranged from 16 mg/L to 32 mg/L.

**Clonal analysis of linezolid-resistant MRSA strains.** PFGE analysis of the outbreak in the ICU (12 patients and 1 environmental sample) (M.S.-G., M.-A.d.L.T., G.M., B.P., M. J. Tolón, S. Domingo, F.J.C., R.A., A.A., N. García, F. Martínez-Sagasti, J.F., and J.J.P., unpublished data) showed that the isolates from 11 patients belonged to clone A. Clones B and C were identified in an environmental sample and in 1 patient, respectively. We also analyzed the isolates obtained from the 3 patients located in non-ICU wards (patients 13–15). PFGE patterns are shown in Figure 1A. The isolates from patients 13 and 15 belonged to the main clone (clone A), whereas the isolate from patient 14 showed a different pattern (clone D).

In patients with >1 isolate (patients 4, 5, 6, 8, 11, 12, 13, and 15), PFGE analysis was performed on all sequential iso-
lates to study whether >1 clone per patient was present. The restriction pattern obtained for these strains showed that each patient carried only 1 clonal type (Figure 1B). In summary, only patient 5 had clone C (in both isolates) and only patient 14 had clone D, whereas all the other patients had clone A. It is worth noting that all patients except patient 14 had been in the ICU. Patient 5 carried a different clone than clone A.

Identification resistance mechanism to linezolid. PCR targeting the cfr gene was performed to establish the resistance mechanism (Figure 2). All the resistant strains showed an amplification band of the expected size (746 basepairs), which was compatible with the cfr fragment. To confirm the nature of the amplified DNA, 5 PCR products were chosen for sequencing. The selected samples (patients 5, 9, 12, 13, and environmental sample E) included all the patterns obtained by PFGE. All the sequences were identical to the cfr sequence in the National Center for Biotechnology Information gene database (data not shown).

To further confirm the mechanism and rule out mutations in domain V of 23S rRNA, the same isolates underwent PCR with specific primer sets for each of the 5 23S rRNA copies and subsequent nested PCR to amplify the domain V fragment containing nucleotide 2576 (E. coli numbering). None of the sequences showed mutations at this or at other nucleotides in this region (data not shown). Thus, resistance in the isolates of the outbreak in the ICU and other hospital wards was mediated by the presence of the cfr gene in their chromosome.

**DISCUSSION**

Resistance to linezolid is uncommon, and few cases have been reported for *Staphylococcus* species [8, 21, 22]. This is the first report of the mechanism underlying an outbreak of linezolid-resistant MRSA. In <3 months, we detected 15 patients who were infected/colonized with linezolid-resistant MRSA (12 were in the ICU). PFGE analysis of isolates obtained from the patients and 1 environmental sample showed that most, but not all, of the strains were genetically related.

The results of molecular genotyping suggest that resistance disseminated along 2 different routes: transmission of a resistant strain (clonal group A) between patients receiving broad-spectrum antibiotics and horizontal transmission by interclonal spread (resistance to linezolid is also present in different clones). This also points to a plasmid-mediated resistance mechanism such as that described for the cfr gene. In fact, all the isolates in the study carried the cfr gene, as demonstrated by PCR. The presence of the cfr gene in a plasmid has been shown for *Staphylococcus* species of animal origin, including a porcine *S. aureus* strain, but the human cfr-positive *S. aureus* strains studied to date have the cfr gene integrated in the genome. Nevertheless, horizontal transmission of resistance is a serious threat, because the cfr gene can also be transmitted between species, such as from *S. epidermidis*, which although not pathogenic, could become a reservoir for resistance genes. This mode of transmission is more difficult to prevent and stop than the nosocomial spread that is usually controlled with standard measures, such as isolation, barrier precautions, and antibiotic restriction.

Earlier in vitro studies showed a number of mutations in domain V of 23S rRNA associated with linezolid resistance in *Enterococcus faecium* [4], *Enterococcus faecalis* [4], *S. aureus*, and *S. epidermidis*, but only 2 have been found in clinical isolates: G2576T (E. coli numbering) [3] and T2500A (E. coli numbering) [5]. More recently, the presence of cfr has been described as a nonmutational mechanism for linezolid resistance [13]. To date, only 2 cfr-carrying *S. aureus* isolates have been reported [14, 23]. Here, we show that cfr-mediated resistance to linezolid was responsible for the first clinical outbreak of linezolid-resistant MRSA. The occurrence of the outbreak is probably attributable to a combination of factors. Linezolid is a relatively novel agent and is completely synthetic; therefore, this emerging resistance was unexpected. Furthermore, the use of linezolid in ventilator-associated pneumonia is now standardized on the basis of retrospective studies showing that it was more effective and had fewer adverse effects than vancomycin [24, 25]. In addition, the presence in the hospital of coagulase-negative *Staphylococcus*, which may be a reservoir of cfr-mediated resistance, could explain why outbreaks such as this one have not occurred before. Our results raise concerns about the future clinical efficacy of several antimicrobial classes, including the oxazolidinones, and highlight the need for drugs able to overcome ribosome-based linezolid resistance.
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References


