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Emergence of *cfr*-Mediated Linezolid Resistance in a Methicillin-Resistant *Staphylococcus aureus* Epidemic Clone Isolated from Patients with Cystic Fibrosis

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Resistance to linezolid (LZD) in methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from patients with cystic fibrosis (CF) is due mainly to ribosomal mutations. We report on four CF patients with LZD-resistant MRSA bronchopulmonary infections by strains carrying the *cfr* gene. Strains from one patient also harbored the G2576U mutation (23S rRNA) and the G139R substitution (L3 protein). All strains belonged to the epidemic clone ST125 MRSA IVc. Our results support the monitoring of LZD resistance emergence in CF and non-CF MRSA isolates.

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections in cystic fibrosis (CF) patients are associated with a more rapid decline in lung function, higher morbidity, and worse survival than in CF patients not colonized with this bacterium (1–4). Linezolid (LZD) is widely used in CF patients for the treatment of MRSA infections, although no specific treatment guidelines are available (5). LZD-resistant MRSA strains (LZD^r MRSA) with ribosomal mutations have been reported in CF patients, often associated with long-term treatments (6, 7–10). Recently, Locke et al. described an LZD^r MRSA strain with the *cfr* gene isolated from a CF patient in 2005 (11). We describe, for the first time, four CF patients with infections by an epidemic LZD^r MRSA clone harboring the *cfr* gene, two of whom had well-documented long-term colonizations.

S. aureus isolates were identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Leipzig, Germany). Antibiotic susceptibility testing was performed by automated microdilution (MicroScan; Beckman-Coulter Inc., Brea, CA) and by the disk diffusion method following EUCAST criteria (12). The MIC of LZD was studied by the agar dilution method and by antibiotic gradient strips (Etest; bioMérieux, Marcy l'Étoile, France) (12). The *cfr* gene was confirmed by PCR (13). Domain V of 23S rRNA and the genes of the ribosomal proteins L3 (*rplC*), L4 (*rplD*), and L22 (*rplV*) were amplified and sequenced for the screening of LZD resistance mutations (14, 15). Genetic relatedness was assessed by pulsed-field gel electrophoresis (PFGE) with SmaI digestion (16). Multilocus sequence typing (MLST) was performed (17), and the results were analyzed using the MLST database (http://www.mlst.net). Staphylococcal cassette chromosome *mec* (SCC*mec*) and *spa* types were also determined (16).

Patient 1 was a 16-year-old female with K710X and R560G CF transmembrane regulator (CFTR) mutations. She was diagnosed with CF at birth in a tertiary-care hospital in Murcia, Spain, as she had a deceased elder sister with the same disease. Her respiratory function was normal until age 10 years (2009), when she experienced frequent coughing with green expectoration and a decrease in median forced expiratory volume in 1 s (FEV_1) (52.5%). Multiresistant *Pseudomonas aeruginosa* and MRSA were isolated from sputum samples and chronically colonized the patient since then. From 2009, she experienced a rapid decline in pulmonary function (FEV_1 = 30%) and frequent exacerbations, whose treatment included 15 courses of oral (600 mg twice daily [BID]) and intravenous (10 mg/kg three times daily [TID]) LZD. In 2013, she was included in a multicenter study in which an LZD^r MRSA was detected (18). The patient received a successful lung transplant in 2014 at age 16 years.

Patient 2 was the elder sister of patient 1. She was chronically colonized by MRSA since age 16 years (2008) and received 13 courses of oral LZD (600 mg BID) from November 2008 to March 2014. LZD^r MRSA isolates were detected in November 2012, January 2013, and December 2013. She died in July 2014 from a pulmonary embolism at age 21 years.

Three MRSA isolates from patient 1 and three from her sister were available for the study (Fig. 1). All LZD^r MRSA isolates harbored the *cfr* gene, whereas the sequences of the *rplC*, *rplD*, and *rplV* genes and of domain V of 23S rRNA showed no LZD resistance mutations (Fig. 1). Interestingly, the isolate obtained from patient 1 after lung transplant was susceptible to LZD, and neither resistance mutations nor the *cfr* gene was detected (Fig. 1). All of these isolates had identical PFGE patterns and corresponded to the ST125 strain, with *spa* and SCC*mec* types t067 and IVc, respectively (Fig. 1). This may indicate a cross-transmission event be-

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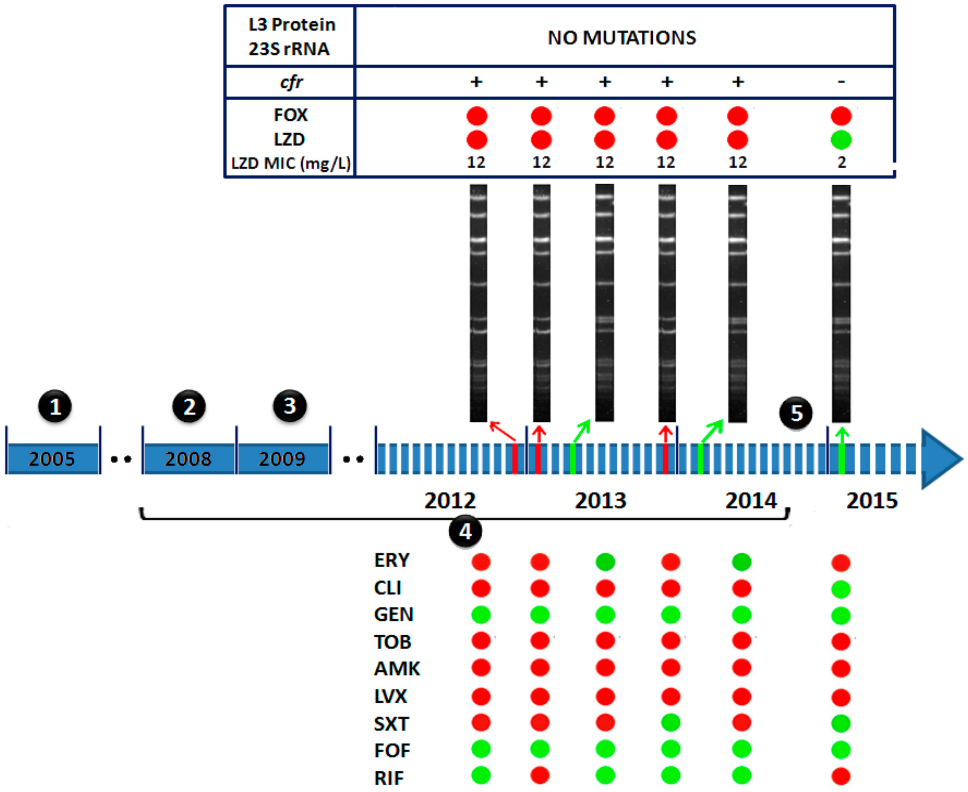

FIG 1 Characteristics of the MRSA isolates obtained from patients 1 and 2. (1) Patient 2 spent 5 months in the lung transplant reference center of Valencia, but she was excluded from the transplant list due to an improvement in her clinical status. (2) First isolation of MRSA in patient 2. (3) First isolation of MRSA in patient 1. (4) LZD treatment period. (5) Patient 1 received a successful lung transplant. Green arrows, LZD^r MRSA isolates from patient 1; red arrows, LZD^r MRSA isolates from patient 2; red dots, resistant; green dots, susceptible; FOX, cefoxitin (for the detection of MRSA isolates); LZD, linezolid; ERY, erythromycin; CLI, clindamycin; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; LVX, levofloxacin; SXT, cotrimoxazole; FOF, fosfomycin; RIF, rifampin. No resistance to glycopeptides was observed (data not shown). All three isolates had the same PFGE type.

tween the siblings, as MRSA can be transmitted within households with CF patients (19).

Patient 3 was an 18-year-old female with F508del and N1303K CFTR mutations who was diagnosed with CF in our center (Madrid, Spain) at age 7 years (July 2003). The patient experienced a rapid decrease in pulmonary function from 2003 (FEV_1 = 84%) to 2008 (FEV_1 = 52%), with repeated findings of *P. aeruginosa*, *Mycobacterium chelonae*, *Aspergillus fumigatus*, and methicillin-susceptible *S. aureus* (MSSA) isolates. The patient moved from our institution to a lung transplant reference center in Valencia from 2008 to 2009. Her clinical status improved from January 2009, and she was excluded from the lung transplant list and returned to our institution in Madrid in 2010. LZD was administered twice in 2008 (10 mg/kg TID intravenously) and at least 15 days per month from January 2009 to February 2011 (600 mg BID orally), as part of treatment regimens against *M. chelonae*. An LZD^r MRSA strain was first isolated in September 2010, and from then on, all of the isolates were LZD^r MRSA with only one exception (strain 19) (Fig. 2).

Twenty-four *S. aureus* isolates (7 MSSA and 17 MRSA) were recovered from patient 3 between 2004 and 2014 (Fig. 2). From 2004 to 2007, all of the isolates were LZD-susceptible MSSA (LZD^S MSSA) strains with different PFGE types. LZD^r MRSA isolates obtained from 2010 onward harbored multiple mechanisms of LZD resistance. All strains had the G2576U mutation (23S rRNA) and the G139R substitution (L3 protein) (6), and eight isolates carried the *cfr* gene. This triple mechanism of resistance was common (5/6 isolates) during the period of LZD treatment (2010 to 2011) (Fig. 2) and conferred high MICs to this antibiotic (>2,048 mg/liter). When LZD was suspended (from March 2011), the strains harboring the *cfr* gene were less common (3/11), but all maintained ribosomal mutations without reverting to a wild-type phenotype, as described previously in some studies (9, 20, 21). LZD MICs of these strains were also very high, indicating a high number of mutated rRNA copies (22). The first and last LZD^r MRSA isolates corresponded to ST393, and all were *spa* type t067 and SCC*mec* type IVa. We hypothesize that patient 3 acquired a *cfr*-positive MRSA during her stay in the transplant center (Valencia), and then, due to her prolonged LZD treatment for nontuberculous mycobacteria (NTM) (nearly 2 years), mutations causing resistance to this antibiotic were selected. A long-term administration of macrolides (azithromycin and clarithromycin) included in the NTM treatment regimen may have helped this process (23).

Patient 4 was a female CF patient with an LZD^r MRSA infection who was identified in Murcia by the time of this study. She had received multiple courses of oral LZD (600 mg BID) since her first positive MRSA culture in 2005. The first LZD^r MRSA isolate was detected in July 2015 and was positive for the *cfr* gene and negative for ribosomal mutations (LZD MIC = 12 mg/liter). The isolate also belonged to the ST125 MRSA IVc *spa* t067 clone.

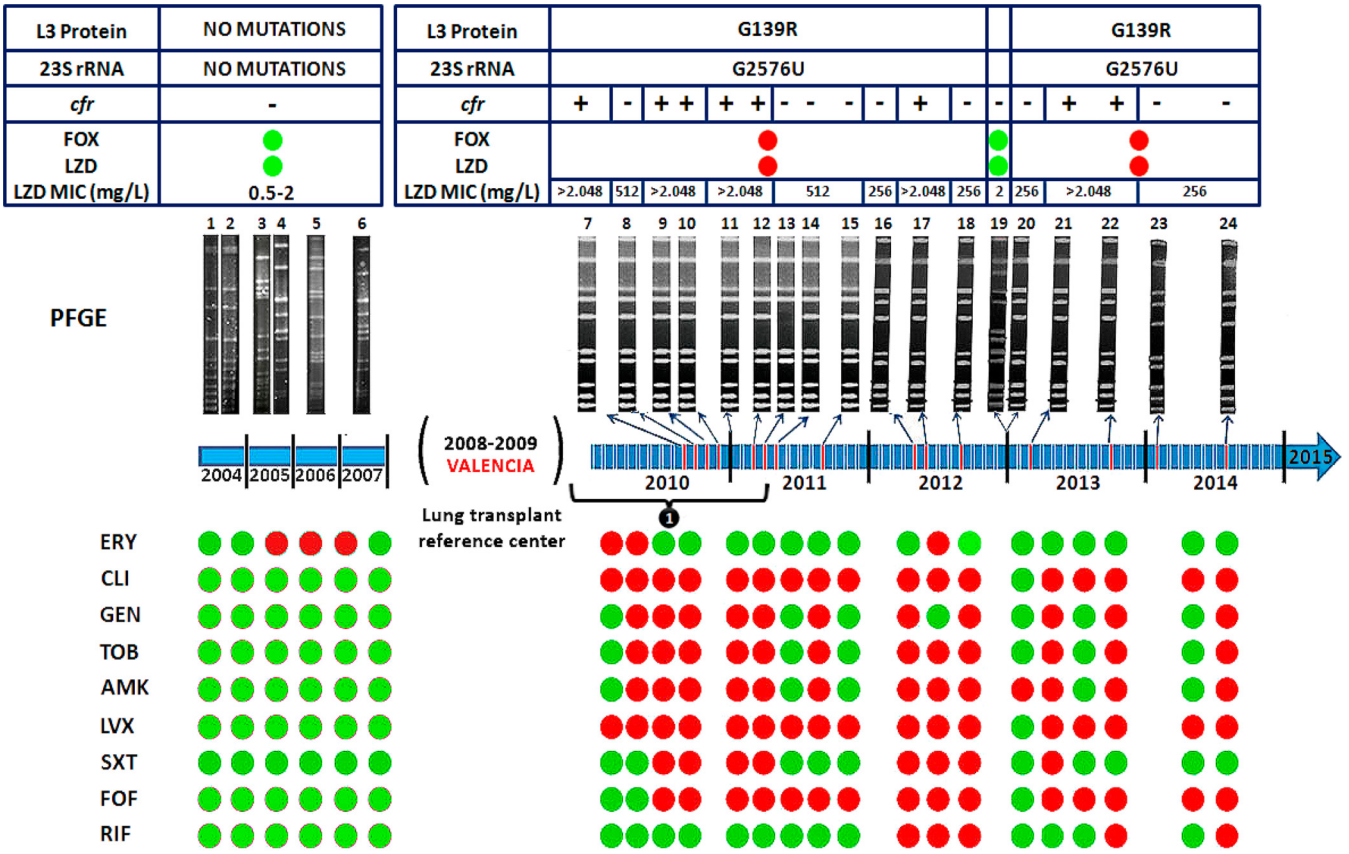

FIG 2 Characteristics of the MRSA and MSSA isolates obtained from patient 2. (1) LZD treatment period. Red dots, resistant; green dots, susceptible; FOX, cefoxitin (for the detection of MRSA isolates); LZD, linezolid; ERY, erythromycin; CLI, clindamycin; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; LVX, levofloxacin; SXT, cotrimoxazole; FOF, fosfomycin; RIF, rifampin. No resistance to glycopeptides was observed (data not shown). All 17 MRSA and 1 MSSA (strain no. 19) isolates had the same or a highly related PFGE type.

Patients 1, 2, and 4 harbored the Spanish epidemic clone ST125 MRSA IVc *spa* t067. LZD^r MRSA strains from patient 3 probably belonged to the same clone, even though they had a different MLST, due to their highly related PFGE (Fig. 3) and their same *spa* type. In fact, ST393 is a single locus variant of ST125, with only a C377T transition in the *aroE* gene. Such variations in MLST profiles due to genetic changes have been demonstrated in *S. aureus* and *P. aeruginosa* isolates as a result of their adaptation to the CF lung (24, 25). ST125 MRSA is a predominant clone in Spain and is responsible for more than half of the nosocomial MRSA infections (16, 26). Another proof of the dissemination of the *cfr* gene among the predominant MRSA clones in Spain is the recent finding in another Spanish hospital of two chronic obstructive pulmonary disease (COPD) patients infected by the ST125 MRSA IVc clone harboring the *cfr* gene (27). The PFGE pattern of this LZD^r MRSA isolate showed that it was very similar to the isolates from our patients (Fig. 3). The presence of a transferable mechanism of LZD resistance in such a successful clone is worrisome, as it can spread to other pathogenic bacteria and confers resistance to 5 classes of antibiotics (22). Further epidemiological studies are required to elucidate the extension of the spread of this LZD^r MRSA strain.

In summary, we describe four CF patients infected with *cfr*-positive LZD^r MRSA strains, one of whom (patient 3) also harbored two stable mutational resistance mechanisms affecting the

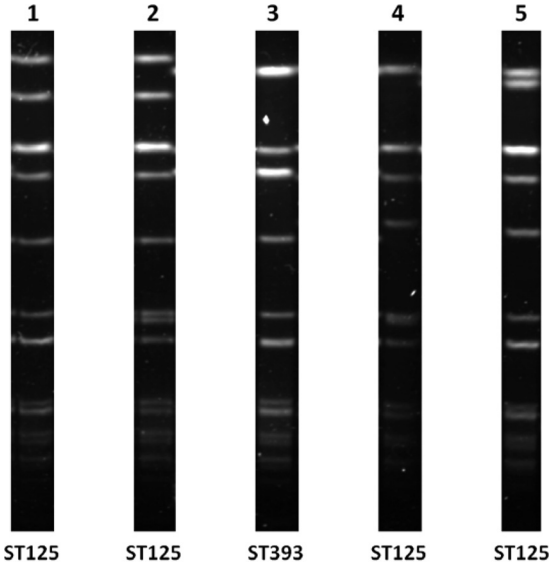

FIG 3 PFGE types observed for the *cfr*-positive LZD^r MRSA isolates studied. Lanes: 1, strain from patient 1; 2, strain from patient 2; 3, strain from patient 3; 4, strain from patient 4; 5, strain isolated from the two COPD patients mentioned in reference 27.

activity of LZD. Moreover, we draw attention to the potential spread of an epidemic clone with these traits in CF patients. The evolution of resistance to LZD should be closely monitored among the MRSA clones circulating in Spain, as should the emergence of LZD resistance in patients with long-term treatments with this antibiotic. Although LZD is one of the most used antibiotics for MRSA infections in CF patients (28), its role should be revised due to its potential effect on the selection of resistant strains.

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We declare that we have no conflicts of interests.

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