

Clinical and Microbiological Aspects of Linezolid Resistance Mediated by the *cfr* Gene Encoding a 23S rRNA Methyltransferase[∇]

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The *cfr* (chloramphenicol-florfenicol resistance) gene encodes a 23S rRNA methyltransferase that confers resistance to linezolid. Detection of linezolid resistance was evaluated in the first *cfr*-carrying human hospital isolate of linezolid and methicillin-resistant *Staphylococcus aureus* (designated MRSA CM-05) by dilution and diffusion methods (including Etest). The presence of *cfr* was investigated in isolates of staphylococci colonizing the patient's household contacts and clinical isolates recovered from patients in the same unit where MRSA CM-05 was isolated. Additionally, 68 chloramphenicol-resistant Colombian MRSA isolates recovered from hospitals between 2001 and 2004 were screened for the presence of the *cfr* gene. In addition to *erm*(B), the *erm*(A) gene was also detected in CM-05. The isolate belonged to sequence type 5 and carried staphylococcal chromosomal cassette *mec* type I. We were unable to detect the *cfr* gene in any of the human staphylococci screened (either clinical or colonizing isolates). Agar and broth dilution methods detected linezolid resistance in CM-05. However, the Etest and disk diffusion methods failed to detect resistance after 24 h of incubation. Oxazolidinone resistance mediated by the *cfr* gene is rare, and acquisition by a human isolate appears to be a recent event in Colombia. The detection of *cfr*-mediated linezolid resistance might be compromised by the use of the disk diffusion or Etest method.

Linezolid is an oxazolidinone antibiotic that binds to the 50S subunit of bacterial ribosomes and inhibits protein synthesis (25). Linezolid is currently approved for the treatment of infections caused by methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* (MSSA and MRSA, respectively) strains and vancomycin-resistant enterococci. Resistance has developed sporadically during therapy in both enterococci (8) and *S. aureus* (29). The most common mechanism of linezolid resistance involves mutations in the central loop of domain V of the 23S rRNA. The most frequent mutation associated with linezolid resistance in both staphylococci and enterococcal clinical strains is G2576T (*Escherichia coli* 23S rRNA gene numbering) (8, 25, 29). Another mutation (T2500A) was characterized in a single patient bloodstream isolate of MRSA (17). Some additional mutations that have been found solely in vitro include the following: G2447T in *S. aureus* and G2505A, G2512T, G2513T, and C2610G in enterococci (20, 31). Most bacteria possess multiple copies of the 23S rRNA gene, with strains of *S. aureus* having five or six rRNA operons. The number of rRNA genes mutated depends on the dose and duration of linezolid exposure and has been shown to influence the level of linezolid resistance (3, 15). Also, a linezolid-resis-

tant isolate of *S. pneumoniae* was found to contain a 6-bp deletion in the gene encoding riboprotein L4 (30).

Nonmutational resistance to oxazolidinones has recently been reported in veterinary isolates of staphylococci. In these isolates, a gene encoding an rRNA methyltransferase (designated *cfr*, for chloramphenicol-florfenicol resistance) has primarily been found on plasmids and appears to be capable of horizontal transfer between staphylococci (11). The mechanism of *cfr*-mediated resistance to linezolid and chloramphenicol involves the methylation of A2503 in the 23S rRNA of the large ribosomal subunit (13).

Recently, we reported that linezolid resistance in a clinical human MRSA isolate (designated CM-05) from Colombia was mediated by the presence of the same *cfr* gene (28). The MRSA CM-05 isolate was characterized, and it was found that, unlike the animal isolates, the gene was located in the chromosome but likely was a part of an integrated plasmid possibly capable of excision and mobilization (28). Also, in MRSA CM-05, the *cfr* gene was clustered in the chromosome with the *erm*(B) gene (which encodes another rRNA methylase and which confers resistance to macrolide, lincosamide, and streptogramin B antibiotics), forming a transcriptional unit designated the *mlr* (for modification of the large ribosomal unit) operon (28). The presence of the *mlr* operon rendered the MRSA isolate resistant to all antibiotics whose target is the large ribosomal subunit (28).

In this work, we describe the clinical and microbiological characteristics of *cfr*-mediated linezolid resistance in the

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MRSA CM-05 isolate from Colombia, including an epidemiological investigation performed to track a possible source of the organism.

CASE REPORT

A 52 year-old female with a medical history of chronic renal failure presented to a hospital in Medellín, Colombia, in May 2005 with a right transtrochanteric hip fracture and, 2 days after admission, underwent internal fixation of the right hip. The postoperative course was complicated by severe gastrointestinal hemorrhage requiring laparotomy with the finding of an actively bleeding ulcer at the ileocecal valve. Resection of the bleeding portions and enteral anastomoses were carried out at surgery, and wound closure was delayed. She was transferred to the intensive care unit, and ciprofloxacin (500 mg intravenously [i.v.] every 12 h) and metronidazole (500 mg i.v. every 8 h) were started empirically. After 3 days in the intensive care unit, the patient developed nosocomial pneumonia. Linezolid was prescribed, and two doses of 600 mg were given i.v. One day later, cultures of a tracheal aspirate revealed an MRSA isolate that was resistant to linezolid. Blood and urine cultures remained negative. Antibiotics were switched to vancomycin (1 g i.v. every 12 h) and meropenem (1 g i.v. every 8 h). The patient's condition continued to deteriorate, and i.v. trimethoprim-sulfamethoxazole was added to the regimen (15 mg/kg of body weight of the trimethoprim component divided every 8 h). However, she died on hospital day 27 with multi-organ failure.

MATERIALS AND METHODS

Surveillance for colonization of household contacts by linezolid-resistant *Staphylococcus aureus*. Nasal and inguinal swab specimens for surveillance cultures were obtained from members of the household (three individuals). Contacts with animals by the patient and household members were investigated by direct questioning. Nasal swabs were obtained from both anterior nares and inguinal areas by using a sterile swab (Amies; Becton Dickinson, Franklin Lakes, NJ) and transported to the laboratory, where the samples were placed into Trypticase soy broth (Biomedics, SL, Madrid, Spain) containing 2% NaCl and finally inoculated onto salt mannitol agar (Biomedics, SL). Single colonies were isolated, Gram stained, and tested for the presence of coagulase. Final identification to the species level was performed by a multiplex PCR assay, as described before (16). Phenotypic resistance to methicillin was evaluated in Mueller-Hinton agar (Biomedics, SL) supplemented with 4% NaCl and oxacillin (6 µg/ml), as recommended by the Clinical and Laboratory Standards Institute (CLSI) (5). All individuals were asked to sign an informed consent, and the surveillance protocol was approved by the ethics committees of local institutions.

Susceptibility testing. The MICs of linezolid, vancomycin, teicoplanin, trimethoprim-sulfamethoxazole, tetracycline, erythromycin, clindamycin, chloramphenicol, and ciprofloxacin were determined for the clinical isolates by an agar dilution method following the recommendations of the CLSI (5). The clinical *S. aureus* isolates included the 1 from the index case and 16 other isolates recovered from the same hospital during the following 3 months after the isolation of the linezolid-resistant MRSA (LR-MRSA) isolate. In order to compare the performance characteristics of commonly used susceptibility tests, determination of the linezolid MIC for the *cf*r-carrying LR-MRSA isolate (isolate CM-05) was performed both by broth and agar dilution methods (5) and by Etest. Isolates of *S. aureus* recovered from surveillance studies carried out in Colombian hospitals between 2001 and 2004 (1, 6) were investigated for their resistance to chloramphenicol and linezolid (a phenotypic characteristic of staphylococci harboring the *cf*r gene). The double-diffusion test (D-test) was performed by following the recommendations of Montanari et al. (18), with minor modifications. Erythromycin and clindamycin disks (15 and 2 µg, respectively; BBL Microbiology Systems, Cockeysville, MD) were placed 15 mm apart on a Mueller-Hinton agar (Biomedics, SL) plate. A modified D-test with erythromycin and linezolid disks (15 and 30 µg, respectively) was performed with isolate CM-05.

Molecular typing and PCR amplification of the *cf*r and *erm* genes. The total DNA of each isolate was extracted and was digested with SmaI by following previously described protocols (6). After digestion, the total DNAs were separated by agarose electrophoresis on a CHEF-DR II system (Bio-Rad Laboratories, Hercules, CA) with the following conditions: for block 1, a run time of 10 h, a switch time of 5 to 15 s, and a voltage of 6 V/cm; for block 2, a run time of 13 h, a switch time of 15 to 60 s, and a voltage of 6 V/cm. The gels were stained and photographed by using the standard methodology. Cluster analysis of the macrorestriction profiles was performed, and the profiles were analyzed by using the Dice similarity coefficient with pulsed-field gel electrophoresis (PFGE) band similarity software (Fingerprinting II software; Bio-Rad Laboratories) by using a cutoff of 75% as the criterion for cluster formation. Final PFGE interpretation was based on the criteria of Tenover et al. (27). *S. aureus* NCTC 8325-4 was used as a control strain to assess the fragment sizes. Representative MRSA isolates of the most common PFGE clones circulating in South America (HPV120, Iberian clone; HSJ93, Brazilian clone; HDE 3, pediatric clone; and CHL93, Chilean clone) were included for comparison.

Multilocus sequence typing was performed for isolate MRSA CM-05 by following the methodology described previously (7). Briefly, PCR amplifications of seven housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, *yqiL*) were performed, and the amplicons were then sequenced by an automated method. The sequence type (ST) was determined by use of the combination of the seven alleles (7), and each unique allelic profile was designated an ST. SCC*mec* types I to IV were evaluated by a previously reported multiplex PCR assay (19) to determine their staphylococcal chromosomal cassette *mec* type (types I to IV).

Total genomic DNA from strain CM-05 was used as the template for the amplification of the *cf*r gene with the oligonucleotide primers described previously (13) and by using the following conditions: 5 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C; and a final extension at 72°C for 5 min. The presence of erythromycin resistance genes was also determined by PCR. Primer sequences targeting *erm*(A), *erm*(B), and *erm*(C) (which encode methylases) and *msrA* (which encodes an efflux pump) were used according to the recommendations of Martineau et al. (16). Fifteen isolates of MRSA, 1 MSSA isolate recovered in the same unit immediately after MRSA CM-05 was identified, and 68 clinical isolates of chloramphenicol-resistant MRSA collected in Colombian hospitals from 2001 to 2004 (1, 6) were investigated for the presence of the *cf*r gene by PCR.

RESULTS AND DISCUSSION

Linezolid resistance in the absence of oxazolidinone exposure and surveillance. As described previously, MRSA CM-05 did not exhibit any mutations in domain V of the 23S rRNA (28). The identification of the *cf*r gene, which encodes an rRNA methylase, in this isolate recovered from a patient after such a short exposure to linezolid (28) indicates that the gene was also most likely acquired by the isolate under a selective pressure that did not involve exposure to oxazolidinones. An alternative explanation is that the strain was selected in an unidentified patient exposed to linezolid and was then passed on to the case patient. However, we did not find any evidence of the presence of *cf*r in any of the clinical isolates of MRSA previously recovered in Colombia or isolated around the time that the first isolate was discovered (see below), making this alternative possibility less likely. Although linezolid resistance in the absence of oxazolidinone exposure had been documented in *Enterococcus* spp. (2, 10, 21), it has not previously been described in MRSA. In the case of enterococci, linezolid resistance emerged in hospitalized patients but was also described in a patient with no risk factors associated with the selection of resistant organisms (hospitalization, receipt of multiple antibiotics, or comorbidities) (2). As opposed to MRSA CM-05, all linezolid-resistant enterococci described in the absence of oxazolidinone exposure carried the G2576U mutation, suggesting that this rRNA mutation may be selected by factors other than exposure to linezolid.

TABLE 1. Susceptibilities of clinical isolates of *Staphylococcus aureus*

Isolate	Source	MIC ($\mu\text{g/ml}$)/interpretation ^a											
		OXA	VAN	TEI	LNZ	CIP	ERY	CLI	GEN	RIF	CHL	TET	SXT
A	Surgical wound	8/R	0.5/S	0.5/S	2/S	0.5/S	0.5/S	0.12/S	>64/R	0.015/S	8/S	32/R	0.5/9.5/S
B	Soft tissue	0.5/S	0.5/S	1/S	2/S	0.5/S	0.25/S	0.06/S	0.5/S	0.015/S	16/I	0.25/S	0.5/9.5/S
C	Surgical wound	>64/R	1/S	0.5/S	2/S	16/R	>64/R	>32/R	>64/R	0.015/S	16/I	0.12/S	0.5/9.5/S
D	Blood	>64/R	1/S	0.5/S	2/S	32/R	>64/R	>32/R	>64/R	0.015/S	16/I	0.12/S	0.5/9.5/S
E	Bone	>64/R	0.5/S	1/S	2/S	32/R	>64/R	>32/R	>64/R	0.015/S	16/I	0.12/S	0.5/9.5/S
F	Skin abscess	>64/R	0.5/S	1/S	2/S	32/R	>64/R	>32/R	>64/R	0.015/S	16/I	0.12/S	0.5/9.5/S
G	Intraocular fluid	>64/R	0.5/S	1/S	2/S	16/R	>64/R	>32/R	>64/R	0.015/S	16/I	0.12/S	0.5/9.5/S
H	Blood	>64/R	0.5/S	1/S	2/S	16/R	>64/R	>32/R	>64/R	0.015/S	16/I	0.12/S	0.5/9.5/S
I	Blood	>64/R	0.5/S	1/S	2/S	32/R	32/R	>32/R	>64/R	0.015/S	16/I	0.12/S	0.5/9.5/S
J	Soft tissue	>64/R	1/S	1/S	2/S	32/R	>64/R	>32/R	>64/R	0.015/S	8/S	32/R	0.5/9.5/S
K	Surgical wound	>64/R	2/S	0.5/S	2/S	32/R	>64/R	>32/R	>64/R	0.015/S	16/I	64/R	0.5/9.5/S
L ^b	Tracheal aspirate	>64/R	1/S	0.5/S	<u>16/R</u>	>32/R	>64/R	>32/R	>64/R	0.0075/S	>64/R	0.12/S	0.5/9.5/S
M	Urine	>64/R	2/S	1/S	2/S	32/R	>64/R	>32/R	1/S	0.015/S	16/I	0.25/S	0.5/9.5/S
N	Blood	>64/R	1/S	1/S	2/S	32/R	>64/R	>32/R	>64/R	4/R	16/I	64/R	0.5/9.5/S
O	Soft tissue	>64/R	0.5/S	1/S	2/S	16/R	>64/R	>32/R	>64/R	0.015/S	16/I	>64/R	0.5/9.5/S
Q	Bone	>64/R	1/S	1/S	2/S	32/R	>64/R	32/R	>64/R	0.015/S	16/I	0.12/S	1/19/S
R	Peritoneal fluid	>64/R	0.5/S	0.5/S	2/S	16/R	>64/R	>32/R	>64/R	0.12/S	8/S	0.12/S	0.5/9.5/S

^a Abbreviations: OXA, oxacillin; VAN, vancomycin; TEI, teicoplanin; LNZ, linezolid; CIP, ciprofloxacin; ERY, erythromycin; CLI, clindamycin; GEN, gentamicin; RIF, rifampin; CHL, chloramphenicol; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole; R, resistant; S, susceptible; I, intermediate.

^b Isolate L is LR-MRSA isolate CM-05, and the MIC of linezolid is underlined.

In Europe, *cfr* was initially found on a plasmid of a bovine isolate of *Staphylococcus sciuri* (14, 24), and its acquisition by animal isolates appears to have been influenced by the use of florfenicol in the veterinary industry (23) and the mobility of the DNA encoding it (12). Chloramphenicol is still used in Colombia, especially in the pediatric population, and although the use of phenicols in the veterinary industry is unknown in Colombia, it is likely that the acquisition of *cfr* might have been influenced by the use of the phenicol group of antibiotics in animals and humans. The pleuromutilins and streptogramins

(which could also select for the presence of *cfr*) are not used in humans in Colombia, and their use in the veterinary industry is also unknown.

Since *cfr* was initially characterized from animal isolates, we sought to determine possible animal contacts by the patient. The patient had no pets at home, and the family reported no visits of animals from surrounding neighbors. The patient lived with her son, a male adult (age, 37 years); his female partner (age, 24 years); and a nephew (age, 6 years). Nasal and inguinal samples revealed the presence of colonization by chloram-

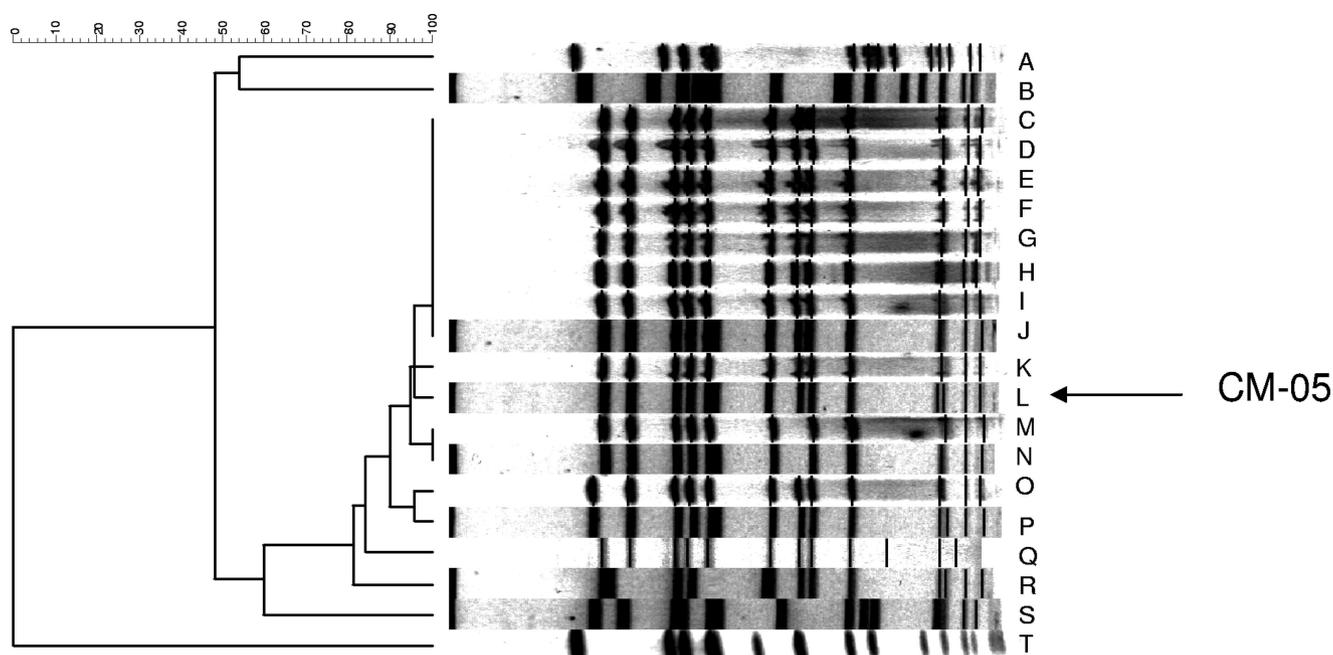


FIG. 1. PFGE of MRSA isolates recovered after identification of the LR-MRSA isolate (isolate CM-05; arrow). The isolate nomenclature is the same as that in Table 1. Additional isolates include isolate P, MRSA CHL93, representative of the Chilean clone; isolate S, MRSA HDE3, representative of the pediatric clone; and isolate T, *S. aureus* NCTC 8325, used as a control.

phenicol-susceptible MSSA in the three individuals. Susceptibility to chloramphenicol indicated that these isolates did not carry *cfr*, since this gene confers resistance to both chloramphenicol and florfenicol (12). In support of this conclusion, we were unable to detect the *cfr* gene by PCR in any of these isolates. Similarly, we screened a total of 16 *S. aureus* clinical isolates recovered from patients in the same unit where the index patient was hospitalized for the presence of the *cfr* gene by PCR. The isolates were recovered after the identification of MRSA CM-05 (Table 1). These nosocomial isolates were susceptible to linezolid, did not exhibit high-level resistance to chloramphenicol (Table 1), and also lacked the *cfr* gene.

In order to determine if *cfr* was prevalent in earlier MRSA isolates from Colombia, we performed a PCR assay targeting the *cfr* gene using 68 chloramphenicol-resistant MRSA isolates recovered in Colombian hospitals between 2001 and 2004 (1, 6). Again, we were unable to detect the presence of *cfr* in these clinical isolates, indicating that the gene is rare in clinical isolates of Colombian MRSA and that the acquisition of this gene might have occurred recently. This finding is in agreement with the findings of studies performed in Germany, where *cfr* was first identified, which reported that the gene was not found in any of 114 human isolates of *S. aureus* studied but was present in 11 of 188 staphylococci from animal sources (12).

Phenotypic and genotypic characteristics of LR-MRSA CM-05. As was shown previously (28), linezolid-resistant clinical isolate CM-05 was found to be resistant to ciprofloxacin, erythromycin, clindamycin, gentamicin, chloramphenicol, and linezolid (Table 1). MRSA CM-05 exhibited the constitutive macrolide-lincosamide-streptogramin B (cMLS_B) phenotype, and we were able to detect both the *erm(A)* and the *erm(B)* genes. This was an unusual finding, since previous studies have shown that macrolide resistance in Colombian nosocomial isolates of MRSA was mediated by the presence of the *erm(A)* gene, the *erm(C)* gene, or a combination of the *erm(A)* and *msr(A)* genes but not the *erm(B)* gene (all isolates exhibited the cMLS_B phenotype) (22). Conversely, macrolide resistance mediated by the *erm(B)* gene was found in all erythromycin-resistant enterococci (22). This finding is consistent with our previous hypothesis that *cfr* might have originated from an enterococcal isolate (28). The horizontal transfer of the *vanA* operon from *Enterococcus faecalis* to MRSA has been described previously (4), and this could represent another example of the horizontal exchange of antibiotic resistance genes between enterococcal and staphylococcal species.

PFGE of isolate MRSA CM-05 and 16 other *S. aureus* isolates from the same unit where the patient was hospitalized revealed that CM-05 and 88% of the other *S. aureus* isolates had an electrophoretic pattern identical to that of the isolates belonging to the Chilean clone (Fig. 1), but only CM-05 was resistant to linezolid. Multilocus sequence typing of isolate CM-05 indicated that it belonged to ST5 (allelic profile 1-4-14-4-12-2-10) and carried staphylococcal chromosomal cassette *mec* type I (ST5-MRSA-I). This ST5-MRSA-I clone initially emerged in the southern cone of South America (26) and appears to have subsequently spread to the northern areas of the continent (6). The molecular typing information confirmed that the acquisition of *cfr* is likely to be a recent event in Colombia.

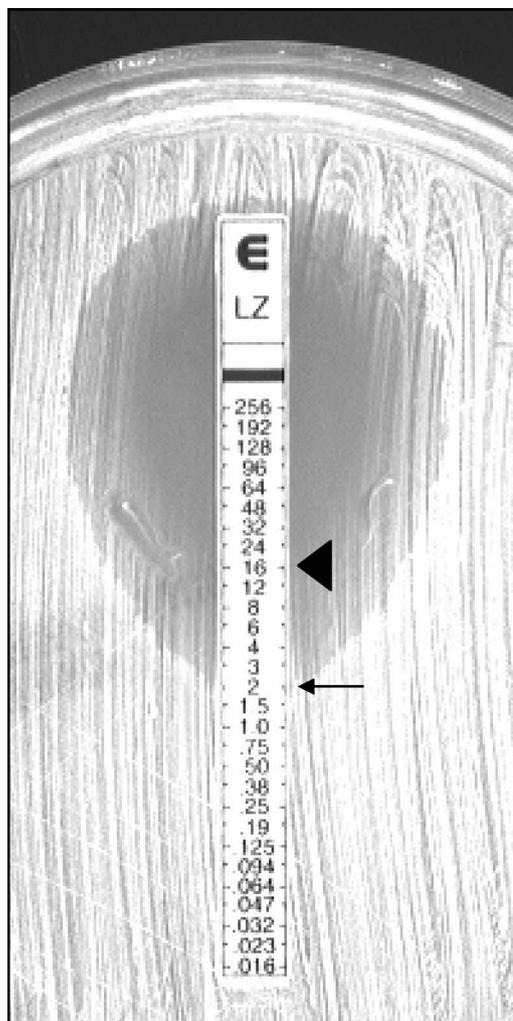


FIG. 2. Etest of LR-MRSA CM-05. The first halo of inhibition was evident at 24 h (interpreted as an MIC of 2 µg/ml; black arrow). After an additional 24 h of incubation, a second halo of growth was identified (MIC, 16 µg/ml; arrowhead).

Detection of linezolid resistance. The MRSA CM-05 isolate had a linezolid MIC of 16 µg/ml and was initially detected by an automated method (Vitek system; BioMérieux, Marcy l'Etoile, France). Subsequently, both the broth and the agar dilution methods yielded similar results. When the organism was tested by Etest, it was reported to be susceptible, with an MIC of 2 µg/ml after 24 h of incubation. A second halo of inhibition was observed after 48 h of incubation, with an MIC of 16 µg/ml (Fig. 2). A modified D-test with a disk of linezolid (30 µg) next to a disk of erythromycin (15 µg) showed no evidence of induction (the D-test was negative), and the result of the disk diffusion test was interpreted as susceptible at 24 h of incubation. The results indicate that disk diffusion susceptibility tests or Etest might not detect *cfr*-mediated linezolid resistance when standard procedures are used and that a longer time of incubation may be needed. The appearance of a double zone of inhibition by Etest may indicate that expression of the *mlr* operon is under controlled regulation and requires the presence of unknown additional factors. The pres-

ence of a “heterogeneous” subpopulation of linezolid-resistant cells within MRSA CM-05 could also be possible, since the differential expression of resistance genes is a common feature of MRSA (9).

In summary, we describe the clinical and microbiological aspects of the first human clinical isolate of MRSA exhibiting resistance to linezolid mediated by the *cfr* gene, which encodes a 23S rRNA methyltransferase; resistance emerged in a country where linezolid usage is very limited. Detection of the resistance phenotype might be compromised by the use of regular disk diffusion tests or Etest. The surveillance studies indicate that this mechanism of resistance is still extremely rare in MRSA but that clinicians should be aware of the potential for the dissemination from animals to humans due to the ability for horizontal gene transfer to occur between staphylococcal and enterococcal isolates of both animal and human origin.

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