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Persistence and evolution of linezolid- and methicillin-resistant *Staphylococcus epidermidis* ST2 and ST5 clones in an Italian hospital

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ABSTRACT

Objectives: *Staphylococcus epidermidis* is a member of the human skin microbiome. However, in recent decades, multidrug-resistant and hospital-adapted *S. epidermidis* clones are increasingly involved in severe human infections associated with medical devices and in immunocompromised patients. In 2016, we reported that a linezolid- and methicillin-resistant *S. epidermidis* ST2 clone, bearing the G2576T mutation, was endemic in an Italian hospital since 2004. This study aimed to retrospectively analyse 34 linezolid- and methicillin-resistant *S. epidermidis* (LR-MRSE) strains collected from 2018 to 2021 from the same hospital.

Methods: LR-MRSE were typed by Pulsed-Field Gel Electrophoresis and multilocus sequence typing and screened for transferable linezolid resistance genes. Representative LR-MRSE were subjected to whole-genome sequencing (WGS) and their resistomes, including the presence of ribosomal mechanisms of linezolid resistance and of *rpoB* gene mutations conferring rifampin resistance, were investigated.

Results: ST2 lineage was still prevalent (19/34; 55.9%), but, over time, ST5 clone has been widespread too (15/34; 44.1%). Thirteen of the 34 isolates (38.2%) were positive for the *cfr* gene. Whole-genome sequencing analysis of relevant LR-MRSE displayed complex resistomes for the presence of several acquired antibiotic resistance genes, including the SCC*mec* type III (3A) and SCC*mec* type IV (2B) in ST2 and ST5 isolates, respectively. Bioinformatics and polymerase chain reaction (PCR) mapping also showed a plasmid-location of the *cfr* gene and the occurrence of previously undetected mutations in L3 (ST2 lineage) and L4 (ST3 lineage) ribosomal proteins and substitutions in the *rpoB* gene.

Conclusion: The occurrence of LR-MRSE should be carefully monitored in order to prevent the spread of this difficult-to-treat pathogen and to preserve the efficacy of linezolid.

1. Introduction

Coagulase-negative staphylococci (CoNS), members of the human skin microflora, are increasingly involved in severe hospital-acquired infections. Among them, *Staphylococcus epidermidis* is the most frequent causative agent of severe nosocomial infections associated with the implantation of indwelling prosthetic devices and intravascular catheters and in immunocompromised patients [1].

The treatment of *S. epidermidis* infections is complicated by the ability of the microorganism to form biofilms on medical devices and by its resistance to most clinically relevant antimicrobials. The therapy frequently requires the use of last-resort antibiotics such as glycopeptides, oxazolidinones, or lipopeptides.

Oxazolidinones (i.e., linezolid and tedizolid) inhibit protein synthesis by binding to the peptidyl transferase center of the bacterial ribosome [2]. Currently, several mechanisms of resistance to oxazolidinones have been identified, including ribosomal mutations in 23S rRNA and/or in the L3, and L4, and L22 ribosomal proteins,

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and transferable mechanisms involving the Cfr and Cfr-like methyltransferases and the ABC-F proteins OptrA, PoxtA, and PoxtA2 [3].

Linezolid-resistant *S. epidermidis* isolates have been recognized as an important cause of hospital-associated outbreaks in recent decades worldwide [4–15].

Unlike staphylococci recovered from healthy people exhibiting high diversity [16], the nosocomial *S. epidermidis* isolates belong to few healthcare-associated clones adapted to the hospital environment worldwide [17,18].

Using a whole-genome sequencing (WGS) approach, Lee et al. [19] show that three multidrug-resistant (MDR) hospital-adapted lineages of *S. epidermidis* (two ST2 and one ST23) have emerged and spread globally. These lineages are resistant to rifampicin through the acquisition of specific *rpoB* mutations that have become fixed in the populations [19]. Moreover, in most of the *rpoB* mutants, they observed reduced vancomycin susceptibility by Macromethod E-test (MET), and postulated that in MDR *S. epidermidis*, rifampin resistance mutations might be associated with vancomycin heteroresistance, as reported for *Staphylococcus aureus* [20].

In 2016, we reported a linezolid- and methicillin-resistant *S. epidermidis* (LR-MRSE) clone endemic in a regional hospital in central Italy since 2004 [12]. Almost all LR-MRSE isolates carried the G2576T mutation in domain V of 23S rDNA, exhibited the same Pulsed-Field Gel Electrophoresis (PFGE) pattern (pulsotype A), and shared the clonal lineage ST2. Five clonal isolates showed a second mutation in L4 ribosomal protein reflecting an evolution of this clone since 2015. Moreover, three LR-MRSE, displaying known amino acid substitutions/insertions in L3 and L4 proteins, belonged to ST5 (pulsotype C) and ST23 (pulsotypes B and B1) [12].

ST2, ST5, and ST23 were previously reported in LR-MRSE with mutation-mediated linezolid resistance in Italy [12,21–24].

The purpose of this study was retrospectively to analyse 34 LR-MRSE strains collected from 2018–2021 from the same hospital.

2. Materials and methods

2.1. Bacterial isolates and susceptibility testing

From August 2018 to October 2021, 34 LR-MRSE isolates were collected from different wards of the Ancona regional hospital. The strains were recovered from several clinical specimens including blood (n = 31), ascitic fluid (n = 1), liquor (n = 1), and central venous catheter (CVC) (n = 1), according to standard methods (Table 1). To prevent duplicate isolates, only one strain for each patient was included in the study. LR-MRSE strains are listed in Table 1 and numbered in chronological order from Se27 to Se60, the first 26 isolates being included in the previous study [12].

All strains were resistant to linezolid (minimum inhibitory concentration [MIC] range, all >4 mg/L) and to oxacillin (MIC range, all >16 mg/L).

Susceptibilities to linezolid and oxacillin provided by the Vitek-2 system (bioMerieux, Marcy-l'Etoile, France) were confirmed by broth microdilution method, using in-house made microplates, according to Clinical & Laboratory Standards Institute guidelines.

The isolates were also tested for their susceptibility to tedizolid by E-test (Liofilchem, Roseto degli Abruzzi, Italy), according to the manufacturer's instructions, and to levofloxacin, vancomycin, tetracycline, gentamycin, erythromycin, clindamycin, and rifampicin, by standard broth microdilution assays.

Susceptibility tests were interpreted according to European Committee on Antimicrobial Susceptibility Testing (version 13.0, www.eucast.org) and Clinical & Laboratory Standards Institute [25] clinical breakpoints. *Staphylococcus aureus* ATCC 29213 was used as quality control.

2.2. Genotypic characterization

Total DNA of LR-MRSE isolates was extracted using QIAcube extraction kit (Qiagen, Germany). The presence of *cfr, optrA*, and *poxtA* genes was investigated by PCR, using primer pairs as previously described [26].

2.3. Typing, WGS, and genome analysis

All strains were typed by SmaI-PFGE and multilocus sequence typing (MLST) assays. PFGE analysis of genomic DNA was performed with the addition of 50 mg/L lysostaphin (Sigma), as previously described [27].

Multilocus sequence typing was carried out as reported by Thomas et al. [28]. Sequence types (STs) were assigned from the MLST database (http://sepidermidis.mlst.net/).

Extracted DNA was subjected to WGS using short-read Illumina MiSeq platform (MicrobesNG, Birmingham, UK) with a 2 × 250 paired-end technology. SPAdes 3.15.2 software was used for the assembly of short reads (http://bioinf.spbau.ru/spades).

Possible mutations in domain V of the 23S rDNA and in L3 and L4 proteins were investigated by aligning obtained sequences with the corresponding ones from the wild-type reference genome of the linezolid-susceptible *S. epidermidis* RP62A (GenBank accession no. CP000029).

The occurrence of substitutions in the *rpoB* gene was assessed by aligning the sequences of selected LR-MRSE subjected to WGS with the rifampicin-susceptible *S. epidermidis* ATCC 14990 (GenBank accession no. NZ_NARC00000000). Genomes were used to draw a phylogenetic tree and calculate SNPs using CSI Phylogeny v.1.4 (https://cge.cbs.dtu.dk/services/CSIPhylogeny/), and to identify the SCCmec cassette via SCCmec finder v.1.2 (https://cge.cbs.dtu.dk/services/SCCmecFinder/). A phylogenetic tree was performed by FigTree v.1.4.4 tool. Moreover, antibiotic resistance genes were found using Resfinder 4.1 (https://cge.cbs.dtu.dk/services/ResFinder/). BLASTN tool (https://blast.ncbi.nlm.nih.gov/BlastAlign.cgi) was performed for comparative analysis and to detect the presence of ribosomal mutations.

2.4. Screening of vancomycin heteroresistance by population analysis

LR-MRSE isolates were preliminarily screened for vancomycin heteroresistance by population analysis as previously described [29].

Bacteria were grown overnight in tryptic soy broth (TSB) (Liofilchem, Roseto degli Abruzzi, Italy) at 37°C. Broth cultures were adjusted to OD 0,6 at 550 nm (ca. 10^8 -10^9 CFU/ml), then plated (100 µL) in duplicate on TS agar (TSA) (Liofilchem) plates with and without vancomycin. The vancomycin concentration range was 0.25 to 32 mg/L. Plates were incubated at 37°C for 24 h, the colony-forming units (CFUs) were counted, and Population Analysis Profiles (PAPs) were obtained by plotting colony counts against vancomycin concentrations. The homogeneous or heterogeneous phenotype was established from the appearance of the curve, homogeneity usually being characterized by a steep slope which followed an almost horizontal course at the permissive drug concentrations and heterogeneity usually being characterized by one or more inflection points.

2.5. Mating experiments

Conjugative transfer of linezolid resistance genes was assessed by filter mating experiments [30] using *S. epidermidis* RP62A, selected in our laboratory for rifampicin resistance (MIC, >128 mg/L), as recipient. Transconjugants were selected on brain heart infusion agar (BHIA) (Oxoid, Basing-stoke, UK) plates containing linezolid

Table 1
Distinctive features of the 34 LR-MRSE investigate in this study.

LR-MRSE isolate	Isolation data			MIC (mg/L)										Linezolid resistance genes	Molecular typing	
	Year	Source	Ward	LZD	TZD	OXA	LEV	VAN	TE	GEN	ERY	CM	RA		PFGE pulsotype	MLST Sequence type
Se27	2018	Blood	Medicine	128	16	128	128	4	2	16	1	4	>128	-	A	2
Se28	2018	Blood	Medicine	128	16	128	128	4	2	16	1	4	>128	-	A	2
Se29	2018	Blood	ICU	128	16	128	128	4	2	16	128	4	>128	-	A	2
Se30	2018	Ascitic fluid	IDU	128	16	128	128	4	2	16	128	4	>128	-	A	2
Se31	2018	Blood	Cardiology unit	64	16	128	128	4	2	16	128	4	>128	-	A	2
Se32	2018	Liquor	ICU	128	16	128	128	4	2	16	128	4	>128	-	A	2
Se33	2018	Blood	Medicine	> 128	8	128	128	2	2	16	1	4	>128	*cfr*	C1	5
Se34	2019	Blood	Medicine	128	8	128	128	4	2	16	1	4	≤ 0.5	-	C	5
Se35	2019	Blood	Medicine	128	16	128	128	4	2	16	128	4	>128	-	A	2
Se36	2019	Blood	Medicine	128	16	128	128	4	2	16	128	4	>128	-	A	2
Se37	2019	Blood	Medicine	8	1	128	> 128	2	2	16	128	4	≤ 0.5	*cfr*	A1	2
Se38	2020	Blood	Medical Clinic	128	16	128	128	4	2	16	128	4	>128	-	A	2
Se39	2020	Blood	Medicine	> 128	8	128	128	4	2	16	1	4	≤ 0.5	-	C	5
Se40	2020	Blood	ICU	128	16	128	128	4	2	16	128	4	>128	-	A	2
Se41	2020	Blood	IDU	128	16	128	128	4	2	16	128	4	>128	-	A	2
Se42	2020	Blood	Medicine	32	4	128	128	2	2	16	1	4	≤ 0.5	-	C	5
Se43	2020	Blood	IDU	128	16	128	128	4	2	16	128	4	>128	-	A	2
Se44	2020	Blood	Medicine	128	8	128	128	4	2	16	1	4	≤ 0.5	-	C	5
Se45	2020	Blood	Cardiology	128	16	128	> 128	4	2	16	0.5	4	>128	-	A2	2
Se46	2020	CVC	Neurosurgery	> 128	8	128	128	4	2	16	1	4	≤ 0.5	*cfr*	C	5
Se47	2020	Blood	Neurologic unit	> 128	8	128	128	4	2	16	1	4	≤ 0.5	*cfr*	C	5
Se48	2020	Blood	Medicine	> 128	8	128	128	4	2	16	1	4	≤ 0.5	*cfr*	C	5
Se49	2020	Blood	Neurologic unit	> 128	8	128	128	4	2	16	1	4	≤ 0.5	*cfr*	C	5
Se50	2020	Blood	Cardiology unit	128	16	128	128	4	2	16	1	4	≤ 0.5	*cfr*	C	5
Se51	2021	Blood	ICU	> 128	16	128	128	4	2	16	1	4	≤ 0.5	-	C	5
Se52	2021	Blood	Medicine	128	8	128	128	2	2	16	1	4	>128	*cfr*	C1	5
Se53	2021	Blood	Orthopaedic unit	> 128	16	128	128	4	2	16	128	4	>128	-	A	2
Se54	2021	Blood	Medicine	128	8	128	128	2	2	16	1	4	>128	*cfr*	C1	5
Se55	2021	Blood	Medicine	> 128	8	128	128	2	2	16	1	4	>128	*cfr*	C1	5
Se56	2021	Blood	IDU	64	8	128	128	2	2	16	1	4	>128	*cfr*	C1	5
Se57	2021	Blood	UTIC	8	16	128	128	4	2	16	128	4	>128	-	A	2
Se58	2021	Blood	ICU	128	1	128	> 128	2	2	16	128	4	≤ 0.5	*cfr*	A1	2
Se59	2021	Blood	UTIC	8	16	128	128	4	2	16	128	4	>128	-	A	2
Se60	2021	Blood	ICU	16	1	128	> 128	1	2	8	4	4	≤ 0.5	*cfr*	A1	2

CM, clindamycin; ERY, erythromycin; ICU, intensive care unit; IDU, infectious diseases unit; GEN, gentamycin; LEV, levofloxacin; LZD, linezolid; VAN, vancomycin; OXA, oxacillin; TE, tetracycline; RA, rifampicin; TZD, tedizolid; UTIC, cardiological intensive care unit.
The grayscale differentiates resistant (dark grey), intermediate (light grey), and susceptible (white) isolates.

(4 mg/L) and rifampicin (32 mg/L). Plates were incubated at 37°C for 24 h and then examined for the presence of transconjugants.

Conjugation frequencies were expressed as ratio of cell number (CFU/mL) of transconjugants to recipient. Transconjugants were evaluated for their susceptibility to linezolid, tedizolid, and tested by PCR for the presence of the oxazolidinone resistance genes. SmaI-PFGE patterns were analysed to confirm the genetic background of transconjugants. Mating experiments were done at least three times.

2.6. Curing assays

LR-MRSE isolates carrying linezolid resistance genes have been subjected to curing assays. LR-MRSE were grown in BHIA (without linezolid) at 37°C for seven passages. After each passage, ten colonies were picked up, and their DNA was extracted and screened by PCR for the presence of linezolid resistance genes. In case of negative testing, the strain was regarded as possibly cured.

2.7. Nucleotide sequence accession numbers

The WGS data of the seven 8 LR-MRSE isolates are available under the BioProject ID PRJNA996505 (accession numbers of the genomes: JAUOOB000000000, JAUOOC000000000, JAUOOD000000000, JAUOOE000000000, JAUOOF000000000, JAUOOG000000000, JAUPBK000000000, and JAUOBL000000000).

3. Results

3.1. Molecular typing, susceptibility, and detection of linezolid resistance genes

Fifteen of 34 LR-MRSE exhibited the same PFGE pattern (pulsotype A), 3 LR-MRSE (Se37, Se58, and Se60), and Se45 exhibited the closely related A1 and A2 pulsotypes, respectively. All nineteen strains (19/34; 55.9%) shared the clonal lineage ST2 (Table 1).

Table 2
Linezolid resistance markers and molecular typing of eight selected LR-MRSE isolates.

LR-MRSE isolate	MIC (mg/L)			Linezolid resistance markers				Rifampicin resistance markers	Molecular typing	
				Linezolid resistance genes	Domain V of 23S rDNA	Ribosomal proteins		*rpoB* mutations	PFGE pulsotype	MLST sequence type
	LZD	TZD	RA			L3	L4			
Se37	8	1	≤0.5	*cfr*	WT	WT	WT	I527M	A1	ST2
Se43	128	16	>128	-	G2576T	M156T	WT	D471E I527M	A	ST2
Se45	128	16	>128	-	G2576T	M156T	WT	D471E I527M	A2	ST2
Se59	8	16	>128	-	G2576T	M156T	WT	D471E I527M	A	ST2
Se60	16	1	≤0.5	*cfr*	WT	WT	WT	I527M	A1	ST2
Se42	32	4	≤0.5	-	WT	H146Q/A157R	Ins71GGR72/N158S	-	C	ST5
Se51	> 128	16	≤0.5	-	WT	H146Q/A157R	Ins71GGR72/N158S	-	C	ST5
Se54	128	8	>128	*cfr*	WT	H146Q/A157R	Ins71GGR72/N158S	S486Y	C1	ST5

The grayscale differentiates resistant (dark grey), intermediate (light grey), and susceptible (white) isolates.

Fifteen LR-MRSE isolates (15/34; 44.1%), showing the pulsotype C (n = 10) and the closely related pulsotype C1 (n = 5), belonged to ST5 (Table 1).

The 34 *S. epidermidis* isolates were all resistant, besides linezolid (MIC range, 8–128 mg/L) and oxacillin (MIC range, all 128 mg/L), to tedizolid (MIC range, 1–16 mg/L), levofloxacin (MIC range, 128–128 mg/L), gentamycin (MIC range, 8–16 mg/L), and clindamycin (MIC range, all 4 mg/L). All LR-MRSE strains were intermediate to tetracycline (MIC range, all 4 mg/L) and susceptible to vancomycin (MIC range, 1–4 mg/L); 16 strains showed resistance to erythromycin (MIC range, all 128 mg/L) (Table 1).

Interestingly, the LR-MRSE isolates belonging to A (n = 15), A2 (n = 1), and C1 (n = 5) phenotypes were resistant to rifampicin (MIC range, all >128 mg/L), whereas those assigned to C (n = 10) and A1 (n = 3) pulsotypes were susceptible (MIC range, all <0.5 mg/L) (Table 1).

Thirteen LR-MRSE were found positive for the presence of the *cfr* gene (Table 1).

3.2. WGS analysis

Some isolates were further investigated. The choice of strains to undergo WGS was driven by: (*i*) sequence type (ST); (*ii*) pulsotype; (*iii*) presence/absence of the *cfr* gene; and (iv) linezolid MIC value. Based on these criteria, the genomes of height LR-MRSE were sequenced and analysed (Table 2).

Regarding mutations, in domain V of the 23S rDNA, three isolates (Se43, Se45, and Se59) shared the G2576T mutation also detected in our previous study in all ST2 isolates [12]; the other five strains showed a wild-type gene (Table 2).

A search for mutations in *rplC* and *rplD* genes, encoding L3 and L4 proteins, respectively, revealed the presence of known amino acid substitutions/insertions in both genes [31]. The same three LR-MRSE isolates exhibiting the G2576T in domain V of the 23S rDNA also showed the mutation M156T in L3; conversely, three isolates (Se42, Se51, and Se54) belonging to ST5 displayed both H146Q/A157R in L3 and ins71GGR72/N158S in L4 (Table 2).

The dual D471E and I527M substitutions in the *rpoB* gene, known to be associated with rifampicin resistance [19], were detected in the three ST2 rifampicin-resistant LR-MRSE (Se43, Se45, and Se59); rifampicin-susceptible Se37 and Se60 strains showed the I527M mutation (Table 2).

The presence of the S486Y mutation explained rifampicin resistance for Se54; a wild-type *rpoB* gene was detected in the remaining two rifampicin-susceptible isolates (Se42 and Se51) (Table 2).

ResFinder analysis of the height genomes revealed complex resistomes for the presence of several acquired antibiotic resistance genes. All isolates showed *mecA* and *blaZ* (resistance to β-lactams) and *fosB* (resistance to fosfomycin) genes (Table 3). Interestingly, ST2 isolates displayed a SCC*mec* type III (3A), while ST5 strains harboured a SCC*mec* type IV (2B). Furthermore, all LR-MRSE belonging to ST2 shared *aadD* and *aac(6′)-aph(2′′)* (resistance to aminoglycosides) and *bleO* (resistance to bleomycin) genes (Table 3). The isolates Se37 and Se60 (pulsotype A1) also carried *erm*(C) (resistance to macrolides, lincosamides, and streptogramins A), *lsa*(B) (resistance to lincosamides), *cfr* (resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramins A), and *qacA* (resistance to chlorhexidine and mupirocin) genes. The *qacA* gene was also present in Se59 and Se45, exhibiting A and A2 pulsotypes, respectively; Se43, in addition to the seven resistance genes shared by all ST2 isolates, showed *mrs(A)* (resistance to macrolides and streptogramins B) (Table 3).

LR-MRSE belonging to the ST5 exhibited a less complex resistome. All strains showed *mecA, blaZ*, and *fosB* genes; *aac(6′)-aph(2′′)* and *qacA* were detected in Se51 and Se54, while the *lsa*(B) gene was found in Se42 and Se54; the latter strain also showed *cfr* and *vga(A)-LC* (resistance to lincosamides, pleuromutilins, and streptogramins A) (Table 3).

Whole-genome sequencing analysis revealed the presence of a *cfr*-carrying plasmid (40,135 bp) in Se37, Se54, and Se60. This plasmid was almost identical (identity 99%, coverage 100%) to the pSh-*cfr* plasmid (accession no. ON953151) first described in a *Staphylococcus hominis* strain recovered from a patient admitted to the sub-intensive general medicine unit of the Ancona regional Hospital [32].

In order to detect the pSh-*cfr*-like plasmid in the *cfr*-carrying isolates not subjected to WGS, PCR mapping experiments were performed using primer pairs targeting four plasmid regions (Table S1). The plasmid was detected in the remaining ten *cfr*-positive LR-MRSE isolates.

Phylogenetic analysis indicated that the LR-MRSE isolates belonging to the lineage ST2 (A, A1, and A2 pulsotypes) constituted a unique cluster (SNPs min 22, max 1,530), as well as the ST5 LR-MRSE strains (C and C1 pulsotypes) (SNPs min 41, max 128). Between ST2 and ST5, a SNP difference from 4728 to 4,833 has emerged. The ST2 isolates constituted a different branch of the phylogenetic tree (Fig. 1) and demonstrated a higher diversity compared with the ST5 isolates, as also confirmed by the different SCC*mec* type; these findings suggested an intra-hospital spread of two clonal lineages.

3.2. Vancomycin heteroresistance in rifampicin-resistant isolates showing the dual D471E and I527M RpoB substitutions

The relationship between the dual D471E and I527M RpoB substitutions and a vancomycin heteroresistance phenotype was not

Table 3
Resistome and type of SSCmec element of eight selected LR-MRSE isolates.

LR-MRSE isolate	Molecular typing		Acquired resistance genes[a]											
	PFGE pulsotype	MLST sequence type												SCC*mec*
Se37	A1	ST2	*mecA*	*blaZ*	*fosB*	*aadD*	*aac(6′)-aph(2′′)*	*bleO*	-	*erm*(C)	*lsa*(B)	-	*cfr*	*qacA*	type III (3A)
Se43	A	ST2	*mecA*	*blaZ*	*fosB*	*aadD*	*aac(6′)-aph(2′′)*	*bleO*	*mrs*(A)	-	-	-	-	-	type III (3A)
Se45	A2	ST2	*mecA*	*blaZ*	*fosB*	*aadD*	*aac(6′)-aph(2′′)*	*bleO*	-	-	-	-	-	*qacA*	type III (3A)
Se59	A	ST2	*mecA*	*blaZ*	*fosB*	*aadD*	*aac(6′)-aph(2′′)*	*bleO*	-	-	-	-	-	*qacA*	type III (3A)
Se60	A1	ST2	*mecA*	*blaZ*	*fosB*	*aadD*	*aac(6′)-aph(2′′)*	*bleO*	-	*erm*(C)	*lsa*(B)	-	*cfr*	*qacA*	type III (3A)
Se42	C	ST5	*mecA*	*blaZ*	*fosB*	-	-	-	-	-	*lsa*(B)	-	-	-	type IV (2B)
Se51	C	ST5	*mecA*	*blaZ*	*fosB*	-	*aac(6′)-aph(2′′)*	-	-	-	-	-	-	*qacA*	type IV (2B)
Se54	C1	ST5	*mecA*	*blaZ*	*fosB*	-	*aac(6′)-aph(2′′)*	-	-	-	*lsa*(B)	*vga(A)-LC*	*cfr*	*qacA*	type IV (2B)

[a] *mecA* and *blaZ* (resistance to β-lactams); *fosB* (resistance to fosfomycin); *aadD* and *aac(6′)-aph(2′′)* (resistance to aminoglycosides); *bleO* (resistance to bleomycin); *erm*(C) (resistance to macrolides, lincosamides and streptogramins A); *lsa*(B) (resistance to lincosamides); *cfr* (resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramins A); *qacA* (resistance to chlorhexidine and mupirocin); *vga(A)-LC* resistance to lincosamides, pleuromutilins and streptogramins A).

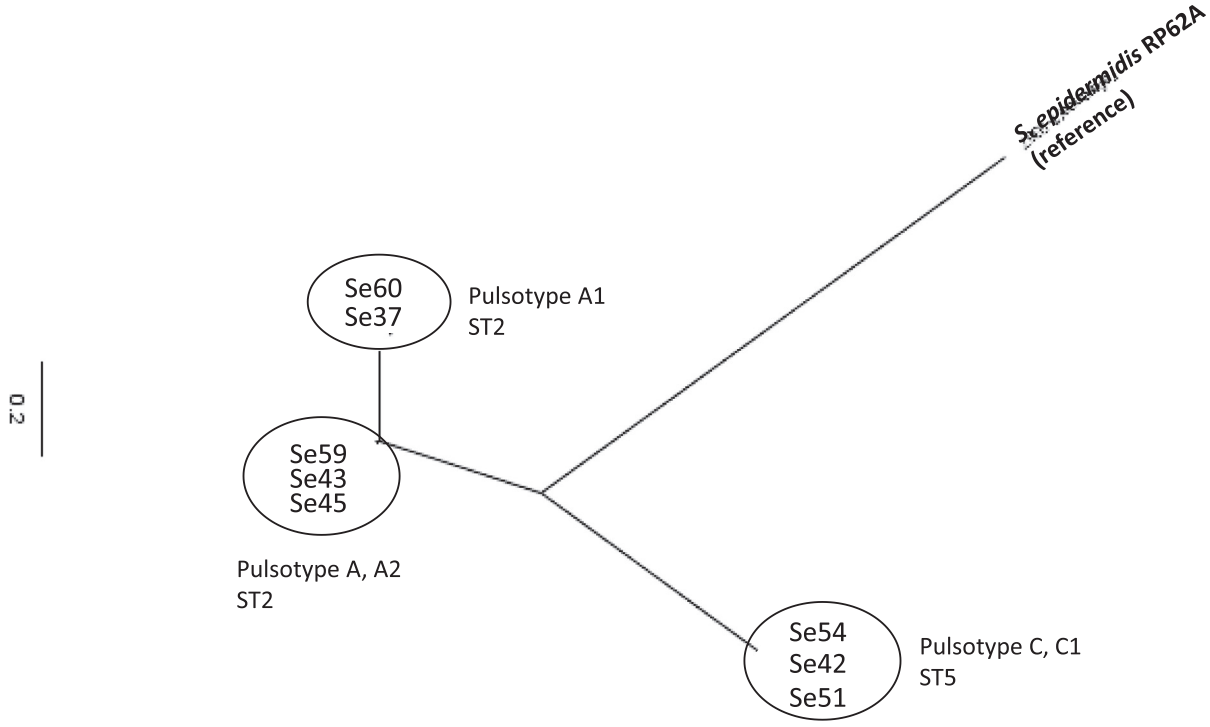

Fig. 1. Maximum likelihood phylogenetic tree of the eight LR-MRSE strains. The Pulsed-Field Gel Electrophoresis pulsotype and the sequence type (ST) of each isolate are shown. *Staphylococcus epidermidis* RP62A was used as the reference genome.

demonstrated in the three vancomycin- and rifampicin-resistant LR-MRSE isolates (Se43, Se45, and Se59) (Table 2). All strains exhibited homogenous population profiles with no subpopulation able to grow at vancomycin concentrations over the MIC values of each strain, which were all included in the vancomycin susceptibility range.

3.3. Transferability and curing assays of the *cfr* gene

The Se60 isolate has been selected as donor in filter mating experiments. This strain was chosen because it is representative of LR-MRSE isolates harbouring the pSh-*cfr*-like plasmid. Despite several attempts, the *cfr* transfer to the *S. epidermidis* RP62A recipient was unsuccessful.

The Se37, Se54, and Se60 isolates, grown on antibiotic-free BHIA at 37°C, were already negative for the presence of the *cfr* gene by PCR after three or four days.

4. Discussion

Antibiotic resistance and biofilm formation are the most important obstacles to treating *S. epidermidis* infections. Healthcare-associated *S. epidermidis* exhibits resistance to various antimicrobials, and usually 60% to 70% of isolates are resistant to commonly

used first-line antibiotics, including beta-lactam antibiotics [33]. Indeed, even the 34 LR-MRSA included in this study were also resistant to levofloxacin, gentamycin, and clindamycin, and most exhibited resistance to erythromycin and rifampicin. Whole-genome sequencing analysis of selected LR-MRSE displayed complex resistomes for the presence of several acquired antibiotic resistance genes, including SCC*mec* type III (3A) and SCC*mec* type IV (2B) in ST2 and ST5 isolates, respectively.

In recent decades, linezolid is recommended for difficult-to-treat infections due to MDR *S. epidermidis* isolates, and although the linezolid resistance remains uncommon among *S. epidermidis*, LR-MRSE outbreaks are increasing worldwide.

In Italy, previous studies indicated the hospital diffusion of three major LR-MRSE clones (ST2, ST5, and ST23) mainly with mutation-mediated linezolid resistance. The *cfr* gene, first found in Italy in a ST23 isolate, was later detected in ST2 and ST5 strains, indicating its capability to transfer from one background to another [21–24]. In 2016, we reported a LR-MRSE ST2 clone endemic in a regional hospital in central Italy since 2004 [12]; here, we confirm the persistence of this linage at the same hospital and show its evolution.

The ST2 lineage, prevalent in our previous study [12], is still the most widespread; moreover, ST5 clonal isolates (only one LR-MRSE was isolated from 2004–2016) have also become established in the hospital. On the other hand, both ST2 and ST5 lineages belong to clonal complex (CC) 5, the predominant and geographically widespread CC of *S. epidermidis* known to be involved in nosocomial infections [14]. Our findings indicate that, over time, ST2 and ST5 clones have evolved by acquiring other mechanisms of linezolid resistance. Thirteen isolates, belonging to both clones, have indeed acquired the *cfr* gene, showing, based on the WGS and PCR mapping data, a plasmid-location. The occurrence of the same plasmid in different lineages of *S. epidermidis* and in *S. hominis* [32] strongly suggests an intra- and inter-specific spread of pSh-*cfr* in the hospital setting. However, in vitro filter mating experiments failed to demonstrate the *cfr*-plasmid transfer to the *S. epidermidis* recipient; these results were consistent with the findings reported by Coccitto et al. [32]. Genomics analysis also displays that, compared with the previously described ST2 strains bearing the G2576T mutation in domain V of 23S rDNA, in some LR-MRSE lately isolated, a new mutation, M156T in L3 protein, has appeared. Similarly, in three ST5 isolates, the N158S mutation in L4 [31,34] was, in addition to H146Q/A157R and Ins71GGR72, already detected in LR-MRSE recovered in 2015.

In France, Dortet et al. described an outbreak caused by three LRSE clones (ST2, ST5, and ST22) [13], whereas Côrtes et al. highlighted that an MDR ST2 *S. epidermidis* lineage carrying a *cfr*-plasmid was able to persist and spread for several years in the same hospital [15]. Over a seven-year study period, a endemic situation caused by a ST2 LR-MRSE clone was detected in a tertiary hospital in Spain [35]. Another Spanish study showed that the LR-MRSE isolates were closely related according to PFGE patterns and selected isolates belonged to the ST2 clone [36]. An outbreak, due to an ST2 clone, has been described in the Republic of Ireland [11]. LRSE isolates were also reported to cause outbreaks in German hospitals, with most of the strains belonging to the ST22 lineage [9]. In the United States, unlike prior studies where ST2 was predominant among LRSE strains [37], Tewhey et al. showed that 42% of the LRSE isolates investigated belonged to the ST23 clone, and no isolates were ST2 [38].

So, even if the prevalence may vary from state to state, only a few linezolid-resistant lineages have been proven to co-circulate within and between several hospitals worldwide. Moreover, these MDR clones, which persist and adapt to hospital settings through recombination events and exchange of genetic mobile elements [14], also represent an important reservoir of antibiotic resistance genes that can be transferred to different staphylococcal species, including *S. aureus* [39]. n conclusion, this report documents a 17-year ST2 clone dissemination in our hospital, as well as the emergence and spread of the LR-MRSE ST5 lineage, highlighting the urgent need for control measures to preserve the therapeutic efficacy of oxazolidinones.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2024.01.020.

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