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ORIGINAL RESEARCH

Diversity in Adaptive Evolution of Methicillin-Resistant *Staphylococcus aureus* Clinical Isolates Under Exposure to Continuous Linezolid Stress in vitro

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Background: Linezolid resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) was reported frequently in recent years, but the mechanism underlying this process was less reported, especially for clinical isolates with different genetic background. Thus, this study aims to explore the adaptive evolution characteristics underlying linezolid resistance in MRSA clinical isolates exposed to continuous induction stress of linezolid in vitro.

Methods: The in vitro susceptibility of 1032 MRSA clinical isolates to linezolid was detected using commercial VITEK-2 equipment via broth microdilution. MRSA isolates with different minimum inhibitory concentration (MIC) values for linezolid were randomly selected to perform the assay of adaptive laboratory evolution with sub-inhibitory concentrations of linezolid. Polymerase chain reaction assays and sequencing techniques were performed to detect well-known molecular determinants related to linezolid resistance, including the expression of *optrA* and *cfr*, mutations of 23S rRNA gene and ribosomal protein (L3, L4, L22) encoding genes (*rplC, rplD, rplV*).

Results: After induction with sequentially increasing concentrations of linezolid, all four MRSA strains (L914, L860, L1096, and L2875) evolved into linezolid-resistant strains over various induction times (480, 384, 288, and 240 h) and universally formed small colony variants. A new mutation in the domain V region of *23S rRNA* gene (C2404T) and one mutation in amino acid sequences of ribosomal protein (Met208Thr) were firstly identified among linezolid-resistant strains. Except G2576T mutations in *23S rRNA* gene, the distribution of other mutations (A2451T, T2504A, C2404T, T2500A, G2447T) exhibited obvious strain heterogeneity. Furthermore, as the MIC to linezolid increased, the copy numbers of point mutations in the V region of *23S rRNA* gene increased correspondingly.

Conclusion: Strain-specific evolution of resistance to linezolid among MRSA clinical isolates was firstly identified in this study. MRSA isolates with higher MICs for linezolid evolved more easily into resistant ones, which calls for precise monitoring of linezolid resistance levels in patients receiving treatment for MRSA infections with linezolid.

Keywords: MRSA, linezolid, inducible resistance, resistance mechanism

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important bacterial pathogen that causes multiple types of invasive infections and increases the mortality of inpatients.[1] The available antimicrobials for treating MRSA infections include vancomycin, daptomycin, and linezolid. The more frequently appearance of linezolid-resistant MRSA worldwide recently is worthy of attention.[2–4] Compared with glycopeptides and quinupristin–dalfopristin antimicrobials, stable resistance in MRSA is more easily achieved upon exposure to continuous linezolid in vitro.[5] Therefore, linezolid resistance in MRSA poses a real challenge for clinicians.

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By binding to 23S rRNA on 50S ribosomal subunits, linezolid can exert its antibacterial effects by preventing the synthesis of bacterial proteins and interfering with the function of the 50S ribosomal subunit.[6,7] The most prevalent mechanism contributing to linezolid resistance in *S. aureus* is point mutations within the domain V region of the 23S rRNA gene.[8–10] The linezolid-binding site at the ribosomal peptidyl transferase center (PTC) is composed entirely of RNA, and mutations within the 23S rRNA gene can block the binding of linezolid to PTC and lead to linezolid resistance. In addition, the *S. aureus* chromosome encodes 5–6 independent rRNA genes (rrn) or operons, and the number of mutations is approximately correlated with the level of linezolid resistance. Another determinant of linezolid resistance in *S. aureus* is mutations in the genes encoding ribosomal proteins. The main parts of ribosomal proteins L3 and L4 are positioned on the surface of the 50S subunit, but a loop ending in two tips extends into the PTC. It is reported that mutations in L3 can alter the conformation of PTC nucleotides by interfering with the binding of the A-site and P-site, further mediating resistance to antimicrobials targeting PTC.[11,12] Furthermore, *cfr* and *optrA* were reported to be correlated with acquired linezolid resistance in *Staphylococcus* spp.[13] cfr RNA methyltransferase can methylate at position 2503 of the 23S rRNA gene, altering the potential of linezolid to bind to its targets and thus mediate bacterial resistance to linezolid.[14] OptrA belongs to the ABC protein family, which is reported to be involved in mediating resistance to oxazolidinones. However, the actual mechanism by which OptrA mediates linezolid resistance remains unclear. Under exposure to repeated linezolid pressure in vivo and in vitro, multiple determinants have emerged that contribute to linezolid resistance in *S. aureus*.[15,16]

To date, most reports have focused on the specific molecular mechanisms that contribute to linezolid resistance among linezolid-resistant MRSA clinical isolates, as described above. Few studies have attempted to elucidate the mechanisms underlying the process of resistance evolution in vivo or in vitro, thereby providing guidance for preventing the emergence of linezolid resistance during linezolid treatment. Wu et al[9] reported the rapid evolution of linezolid resistance in MRSA clinical isolates obtained from a patient receiving long-term treatment with linezolid, with five mutations identified in the domain V region within the 23S rRNA gene, including G2576T, T2537C, G2234A, T1557A, and C253A. Ono et al[17] also reported the rapid acquisition of linezolid resistance in a patient after receiving linezolid treatment for 14 days and identified a common mutation in the 23S rRNA gene (G2576T). Staudacher et al[18] obtained a series of linezolid-resistant *S. aureus* isolates using a stepwise in vitro induction model, and several partially novel single nucleotide variants (SNVs) were detected in the *rplC* gene, which encodes the 50S ribosomal protein L3 in *S. aureus*. The abovementioned three reports revealed a universal phenomenon of induced resistance to linezolid in *S. aureus* and the underlying complex resistance mechanisms. For patients infected with MRSA who are receiving long-term treatment with linezolid, active monitoring of linezolid resistance using phenotypic and genotypic methods appears to be even more important.

To the best of our knowledge, few studies have intensively explored the evolution mechanism of linezolid resistance in MRSA clinical strains under linezolid stress. Therefore, in this study, phenotypic and genetic techniques were employed to explore the evolution mechanism of linezolid resistance in MRSA using stress induction protocols with sequentially increased concentrations of linezolid in vitro.

Materials and Methods

Collection and Identification of MRSA Isolates

In this study, 1032 clinical MRSA isolates were collected from patients admitted to the Affiliated Hospital of Inner Mongolian Medical University between January 2011 and March 2022. All isolates were collected from routine clinical microbiological samples, and the isolates were frozen at −80°C until usage. The isolates were subcultured on blood agar plates during this study, and all isolates were revalidated using EXS 3000 matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) (Zybio, China).

Antimicrobial Susceptibility Test

The phenotypic resistance of all 1032 clinical MRSA isolates to linezolid was detected using an AST-GP67 card on VITEK-2 compact automation equipment, and the isolates with minimum inhibitory concentration (MIC) ≥1 μg/mL to linezolid were further determined using microdilution broth. Furthermore, an antimicrobial susceptibility test and data analysis were performed according to the procedure recommended by the Clinical and Laboratory Standards Institute

(M100 32th edition).[19] According to the definition of CLSI-M100, the breakpoints for sensitive (S) and resistant (R) were set as follows, S: ≤4 μg/mL, R: ≥8 μg/mL. Based on the antimicrobial susceptibility testing results obtained from the VITEK-2 Compact system with a commercial AST-GP67 card (BioMérieux, France), the isolates with a positive cefoxitin screening and oxacillin phenotypic resistance (MIC ≥ 4 μg/mL) were identified as MRSA. The MRSA isolates were confirmed by the detection of the *mecA* gene using PCR.[20]

Protocol of Linezolid Stress Evolution

All four isolates were subcultured on blood agar and incubated for 24 h at 37°C under aerobic conditions. A single colony on blood agar originated from each isolate was inoculated into 5 mL of TBS liquid medium and aerobically grown at 37°C with agitation at 220 rpm until the exponential phase was reached. The bacterial suspension for each isolate was adjusted to 1.5×10^8 CFU/mL. The initial concentration of the bacterial suspensions used for the resistance induction assay was set to 1.5×10^6 CFU/mL. For each isolate, 0.5× MIC of linezolid was used as the initial induction concentration during the first round of induction generation, and then the induction concentrations of linezolid were increased sequentially to 1×, 2×, 4×, 8×, 16×, 32×, and 64× MICs for each of the remaining rounds of induction generation until the MIC of the resistant isolates eventually reached 256 μg/mL. After the addition of the required quantity of linezolid, bacterial suspensions of 20 mL were subcultured at 37°C for 24 h under agitation (220 rpm). The cultures were observed visually at 24-hour intervals until they were obviously turbid. Then, the next passage of subculture continued with the same initial concentration of bacterial density and linezolid until the MICs of the isolates were increased two-fold compared with the initial concentration within that round of induction generation.[21] For each round of induction generation, the broth microdilution method was employed to detect the linezolid MICs of linezolid-induced derivates at intervals of four passages. In general, one passage will experience at least one 24-hour period of subculture and 4–12 passages were performed for each MRSA isolate during one round of induction generation. To exclude possible contamination during continuous induction culture, MALDI-TOF MS was employed to identify suspected colonies. For each isolate, the resistant derivates produced from the induction assay were cryopreserved in glycerol containing (40%) Mueller–Hinton Broth at −80°C until the end of the study. After the induction assay was completed, representative isolates with the highest level of resistance to linezolid (MIC ≥ 256 μg/mL) were inoculated onto blood agar medium without antibiotics and subcultured sequentially for 100 generations with a subculture period of 24 hours. During this process, the isolates were selected at ten-generation intervals and their MICs to linezolid were determined using the broth microdilution method. The flowchart for induction resistance was presented in Figure 1.

To ensure biosecurity during this study, all resistant strains were preserved in an independent freezer with a lock; upon completion of the study, they were destroyed by autoclaving. All operations were performed in a biosafety level 2 laboratory. After the completion of each experiment, cultures and related materials were sterilized using an autoclave. The sterilized cultures and related materials were further processed as medical waste in accordance with the regulations of Affiliated hospital of Inner Mongolian Medical University.

Detection of Resistance-Related Determinants

Each isolate was inoculated on blood agar medium at 35°C for 24 h, and the colonies were transferred into a 1.5-mL Eppendorf tube with 0.5-mL sterile distilled water. Lysozyme (1 mg/mL) was added to each tube and mixed thoroughly by vortexing. The tubes were then placed in a water bath and incubated at 35°C for 30 min. Total bacterial DNA was extracted using a bacterial genome DNA extraction kit according to the manufacturer's instructions (Beijing Tiangen Company, China). The details of the primers used in this study and polymerase chain reaction (PCR) conditions are presented in Table 1.

The PCR amplification products of the V region of the 23S rRNA gene and ribosomal protein-encoding genes (*rplC, rplD*, and *rplV*) from four parental MRSA strains and their corresponding induction strains were separated by electrophoresis on a 1% agarose gel and purified using a DNA purification kit (Tiangen Biotech Co., Ltd, Beijing). Then, the purified PCR products were sequenced using the same primers used for PCR amplification, and Sanger sequencing was performed using an ABI 3730XL DNA Analyzer (Applied Biosystems, USA). The sequence alignment between the primary strains and their corresponding mutated strains was compared using Sequencher DNA sequence analysis software 5.4.6.

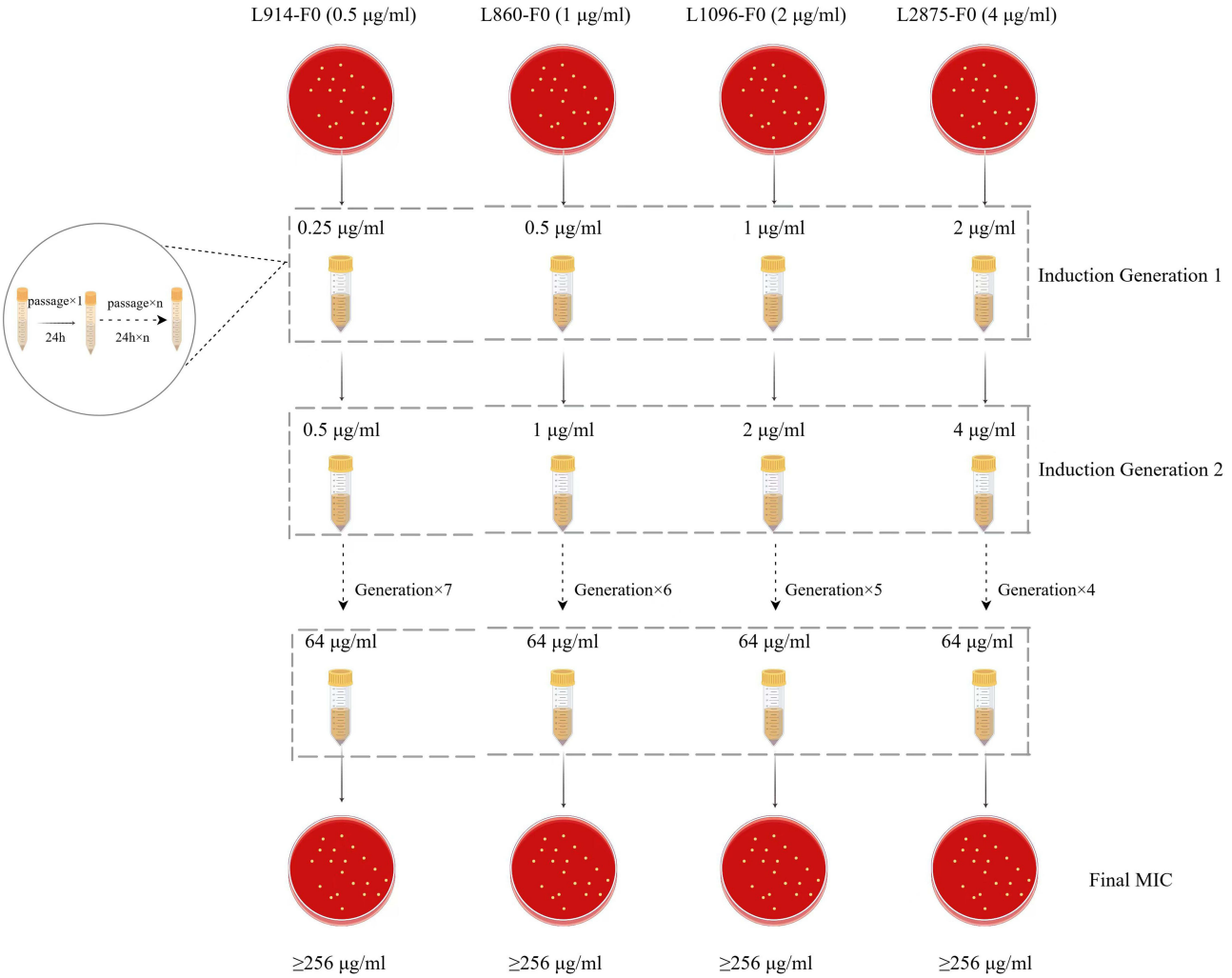

Figure 1 Flowchart for stepwise induction resistance by linezolid among four MRSA isolates.
Note: Each MRSA isolate will experience 6–9 rounds of induction generations until the linezolid MICs of the mutated isolates reached up to ≥256 μg/mL.

To detect the number of copies of six *23S rRNA* operons (rrn1-rrn6) and analyze the possible mutations for mutated MRSA strains originated from each parental MRSA strain, PCR technique was performed according to the procedure introduced by Meka et al.[22] The PCR conditions in detail were listed in Table 1. Subsequent procedures of separation, purification and sequencing of the PCR products were same as that for analysis of the domain V region of *23S rRNA* gene.

Molecular Typing Using MLST

In general, bacterial chromosomal DNA was extracted using a TIANamp Bacterial DNA kit (Tiangen Biotech, China) according to the manufacturer's instructions. The PCR amplification protocol was based on the procedure suggested by Enright et al.[24] Seven PCR assays were performed to amplify seven housekeeping genes for MRSA, including *arc, aroE, glpF, gmk, pta, tpi*, and *yqil*. The PCR products were sequenced using the same primers used for PCR amplification, and Sanger sequencing was performed using an ABI 3730XL DNA Analyzer (Applied Biosystems, USA). The sequences were compared with known alleles in the MLST database (http://saureus.mlst.net). The variable repeat region of the *spa* was amplified using the following primers: *Spa-1113f* (TAAAGAGGATCCTTCGGTGAGC) and *Spa-1514r* (CAGCAGTAGTGCCGTTTGCT).[25] The PCR products were then sequenced an ABI 3730DL DNA Analyzer (Applied Biosystems, USA), and the sequences were analyzed using the Random web server (http://spaserver.ridom.de).

Table 1 Primers Used for PCR Amplification of Resistance Related Genes

Primers	Sequence (5′-3′)	Size (bp)	PCR conditions	Reference
23S_rRNA_F	GCGGTCGCCTCCTAAAAG	390	94°C for five minutes, 32 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for one minute	[22]
23S_rRNA_R	ATCCCGGTCCTCTCGTACTA			
optrA_F	TACTTGATGAACCTACTAACCA	422	94°C for five minutes, 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for one minute	[23]
optrA_R	CCTTGAACTACTGATTCTCGG			
cfr_F	TGAAGTATAAAGCAGGTTGGGAGTCA	746		
cfr_R	ACCATATAATTGACCACAAGCAGC			
cfr(B)_F	TGAGCATATACGAGTAACCTCAAGA	293	94°C for five minutes, 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72 °C for one minute	
cfr(B)_R	CGCAAGCAGCGTCTATATCA			
L3_rplC_F	AACCTGATTTAGTTCCGTCTA	822	94°C for 10 minutes, 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for one minute	
L3_rplC_R	GTTGACGCTTTAATGGGCTTA			
L4_rplD_F	TCGCTTACCTCCTTAATG	1200		
L4_rplD_R	GGTGGAAACACTGTAACTG			
L22_rplV_F	CAACACGAAGTCCGATTGGA	350		
L22_rplV_R	GCAGACGACAAGAAAACAAG			
23S_rrn1_F	GCGGTGTTTTGAGAGATTATTTA	3657	94°C for one minute, 32 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 68°C for seven minutes	[22]
23S_rrn1_R	GCTTCATGATATACGCTTCCTTT			
23S_rrn2_F	GAAAGGCGTAACGATTTGGG	1688		
23S_rrn2_R	GATACCGTCTTACTGCTCTTCCT			
23S_rrn3_F	AGGCCGGCAATATGTAAG	5637		
23S_rrn3_R	GTCGTCAAACGGCACTAATA			
23S_rrn4_F	TGTGGACGGTGCATCTGTAG	6337		
23S_rrn4_R	ATCACCCGCTCCATAGATAAT			
23S_rrn5_F	GCCGATAGCTCTACCACTG	5850		
23S_rrn5_R	AGGTGCGATGGCAAAACA			
23S_rrn6_F	GAAAGGCGTAACGATTTGGG	1968		
23S_rrn6_R	CGTTGACATATTGTCATTCAG			

Results

Phenotypic Resistance and Molecular Typing

Of the 1032 MRSA strains, 8.3% (86/1032) and 91.7% (946/1032) were isolated from outpatients and inpatients, respectively. Isolates from male patients accounted for 65.9% of MRSA strains; 34.1% of strains were from female patients. The majority of the strains were isolated from sputum samples (53.8%), followed by wound secretion samples (22.5%), pus samples (6.6%), blood samples (6.2%), urine samples (3.7%), throat swabs (2.8%), drainage (1.1%), bronchoalveolar lavage fluid (0.6%), catheter (0.6%), and others (2.1%). Among these patients, 47.7% were older than 60 years and 38.1% were aged between 18 and 60 years. The remaining patients (14.2%) were younger than 18 years. In addition, the highest proportion of patients (19.0%) was admitted to the neurosurgery department, followed by orthopedics (10.8%), ICU (10.0%), respiratory medicine (9.7%), rehabilitation (8.1%), dermatology (5.7%), pediatrics (5.1%), neurology (5.0%), general surgery (3.1%), cardio-thoracic surgery (3.0%), and others (20.5%). The linezolid MICs of 1032 MRSA clinical isolates were all < 8 μg/mL, and no linezolid resistant isolate was identified. Among them, 65% (670/1032) had an MIC value of 1 μg/mL, 31% (320/1032) had an MIC value of 2 μg/mL, 3% (33/1032) had an MIC value of 0.5 μg/mL, and 1% (9/1032) had an MIC value of 4 μg/mL. Furthermore, among the 1032 MRSA strains collected in this study, nine (0.87%, 9/1032) had MIC values of 4 μg/mL, of which seven were collectively isolated between 2018 and 2022. However, the MICs of these nine strains were <4 μg/mL using the VITEK 2 compact instrument, as shown in Table 2. The MLST and spa types of the nine MRSA isolates with MIC values of 4 μg/mL showed a diverse tendency; ST22-t005, ST239-t030, ST59-t437, and ST72-t548 accounted for 22% (2/9), 22% (2/9), 22% (2/9), and 34% (3/9) of each ST type, respectively.

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Table 2 Phenotypic Resistance and Molecular Typing results of Nine MRSA Isolates With MIC of 4 μg/mL to Linezolid

Isolates	Gender	Age	Department	Sources of Sample	MIC (μg/mL)		Seven Housekeeping Genes Used for MLST							STs	spa
					VITEK-2	BMD	*arcC*	*aroE*	*glp*	*gmk*	*pta*	*tpi*	*yqi*		
L473	M	73	Derm	Wound secretion	2	4	7	6	1	5	8	8	6	22	t005
L552	M	65	Neurology	Sputum	2	4	19	23	15	2	19	20	15	59	t437
L1138	F	74	Neurology	Sputum	2	4	1	4	1	8	4	4	3	72	t548
L1146	M	80	TCM	Sputum	4	4	19	23	15	2	19	20	15	59	t437
L1161	M	74	Neurology	Secretion	2	4	2	3	1	1	4	4	87	239	t030
L1679	F	60	Rehabilitation	Urine	2	4	2	3	1	1	4	4	87	239	t030
L2612	M	64	Mongolian- Medicine	Sputum	2	4	1	4	1	8	4	4	87	72	t548
L2658	F	68	Derm	Secretion	2	4	7	6	1	5	8	8	6	22	t005
L2875	F	75	Neurology	Sputum	2	4	1	4	1	8	4	4	3	72	t548

Abbreviations: M, male; F, female; TCM, Traditional Chinese Medicine; Derm, Dermatology; MLST, multilocus sequence typing; *arcC*, carbamate kinase; *aroE*, shikimate dehydrogenase; *glpF*, glycerol kinase; gmk, guanylate kinase; *pta*, phosphate acetyltransferase; *tpi*, triosephosphate isomerase; *yqiL*, acetyl coenzyme A acetyltransferase; BMD, broth microdilution; STs, sequence types.

Induction of Linezolid Resistance

Four isolates with different MICs to linezolid were randomly selected to undergo induction assays in vitro, and the MICs of these isolates were 0.5 μg/mL (L914-F0), 1 μg/mL (L860-F0), 2 μg/mL (L1096-F0), and 4 μg/mL (L2875-F0). The MICs of the four MRSA strains exceeded 256 μg/mL after continuous induction in vitro; however, resistance induction times for the four isolates varied greatly from 240 to 480 h, as presented in Table 3. To verify the stability of induced resistance, L860-F56 was continuously passaged on blood agar medium without antibiotics, and resistance remained stable for 100 generations (256 μg/mL) as detected via broth microdilution. Excluding changes in phenotypic resistance to linezolid, the resistant strains grew slowly during 24-h culture, showing a feature of small colony variants (SCVs), as shown in Figure 2. Furthermore, SCVs showed decreased hemolytic activity compared with the parental strains.

Table 3 Dynamic Evolution of Phenotypic Resistance of Four MRSA Strains During Adaptive Resistance Evolution

Strains	IC (μg/mL)	IT (h)	CT (h)	MIC (μg/mL)
L914-F4	0.25	96	96	1
L914-F8	0.5	96	192	2
L914-F16	1	192	384	4
L914-F20	2	96	**480**	8
L914-F28	4	192	672	16
L914-F36	8	192	864	32
L914-F40	16	96	960	64
L914-F44	32	96	1056	128
L914-F48	64	96	1152	>256
L860-F4	0.5	96	96	2
L860-F8	1	96	192	4
L860-F16	2	192	**384**	8
L860-F24	4	216	600	16
L860-F28	8	120	720	32
L860-F40	16	336	1056	64
L860-F48	32	192	1248	128

(*Continued*)

Table 3 (Continued).

Strains	IC (μg/mL)	IT (h)	CT (h)	MIC (μg/mL)
L860-F52	64	192	1440	>256
L1096-F8	1	192	192	4
L1096-F12	2	96	**288**	8
L1096-F20	4	192	480	16
L1096-F28	8	192	672	32
L1096-F36	16	288	960	64
L1096-F44	32	288	1152	128
L1096-F52	64	288	1440	>256
L2875-F8	2	240	**240**	8
L2875-F20	4	336	576	16
L2875-F24	8	144	720	32
L2875-F32	16	240	960	64
L2875-F40	32	192	1152	128
L2875-F48	64	96	1248	>256

Notes: The MICs value of primary susceptible strains (L914, L860, L1096, and L2875) to linezolid was 0.5 μg/mL, 1 μg/mL, 2 μg/mL, and 4 μg/mL, respectively. F stands for "filial generation under induction by continuous linezolid." For example, F4, F8, and F12 represent the fourth, eighth, and twelfth filial generations, respectively. The bold values indicated cumulative induction time (CT) for each MRSA isolate (L914, L860, L1096, and L2875).

Abbreviations: MIC, Minimum inhibitory concentration, IC, induction concentration; IT, induction time; CT, cumulative induction time.

Detection of Linezolid Resistance-Related Mechanisms

In general, none of the resistant strains harbored *optA, cfr*, or *cfr(B)*. Multiple mutations within the V region of the 23S rRNA gene sequences were identified among the resistant strains when the G2576T mutation was universally distributed among the four isolates. The mutations contributing to linezolid resistance were detected among all four MRSA isolates (F0) at the beginning of the induction assay. As shown in Table 4, only the G2523A mutation was observed within the V region of the 23S rRNA gene of L914-F0; however, this mutation was not detected in the other isolates. Based on the results of this study, it is speculated that the G2523A mutation has no or only negligible effects on linezolid resistance in MRSA. Moreover, the four MRSA isolates (F0) did not exhibit any other mutations at the beginning of the induction assay. Interestingly, only one type of point mutation (G2576T) within the V region of the 23S rRNA gene was found among the linezolid resistant generations (F16 to F52) of L860 strain, as shown in Table 4. In contrast, strain-specific mutations were found in the L914 (G2523A and T2504A) and L2875 (C2404T, T2500A, and G2447T) strains. C2404T was identified for the first time in *S. aureus* in this study (Table 4). Furthermore, the number of mutations among resistant strains increased correspondingly as the MICs of linezolid increased, including the types of mutations and copy numbers of the 23S rRNA gene (Table 5).

Moreover, multiple mutations were observed among the other types of resistance-related elements investigated in this study, namely, ribosomal protein-encoding genes. In this study, mutations mainly occurred in L3 (*rplC*) and L4 (*rplD*), and mainly eight types of mutation (Gly152Asp, Asp159Glu, Met208Thr, Phe147Ile, Val154Leu, Met156Thr, Ser158Phe, and Asp159Tyr) and one type of mutation (Lys68Asn) were detected among the L3 and L4, respectively. Except for L1096, the L3-Gly152Asp mutation was universally detected in the other three isolates. The L4-Lys68Asn mutation was detected only in the resistant strain L914-F48. As shown in Table 4, significant heterogeneity in the distribution of L3 mutations was observed among the four strains tested in this study. Except for the L914 and L2875 strains, diverse L3 mutations were identically identified among the resistant strains.

Discussion

In this study, the phenotypic resistance of 1032 MRSA clinical isolates to linezolid was analyzed using a commercial system and broth microdilution method. Nine MRSA clinical isolates with an MIC value of 4 μg/mL were identified

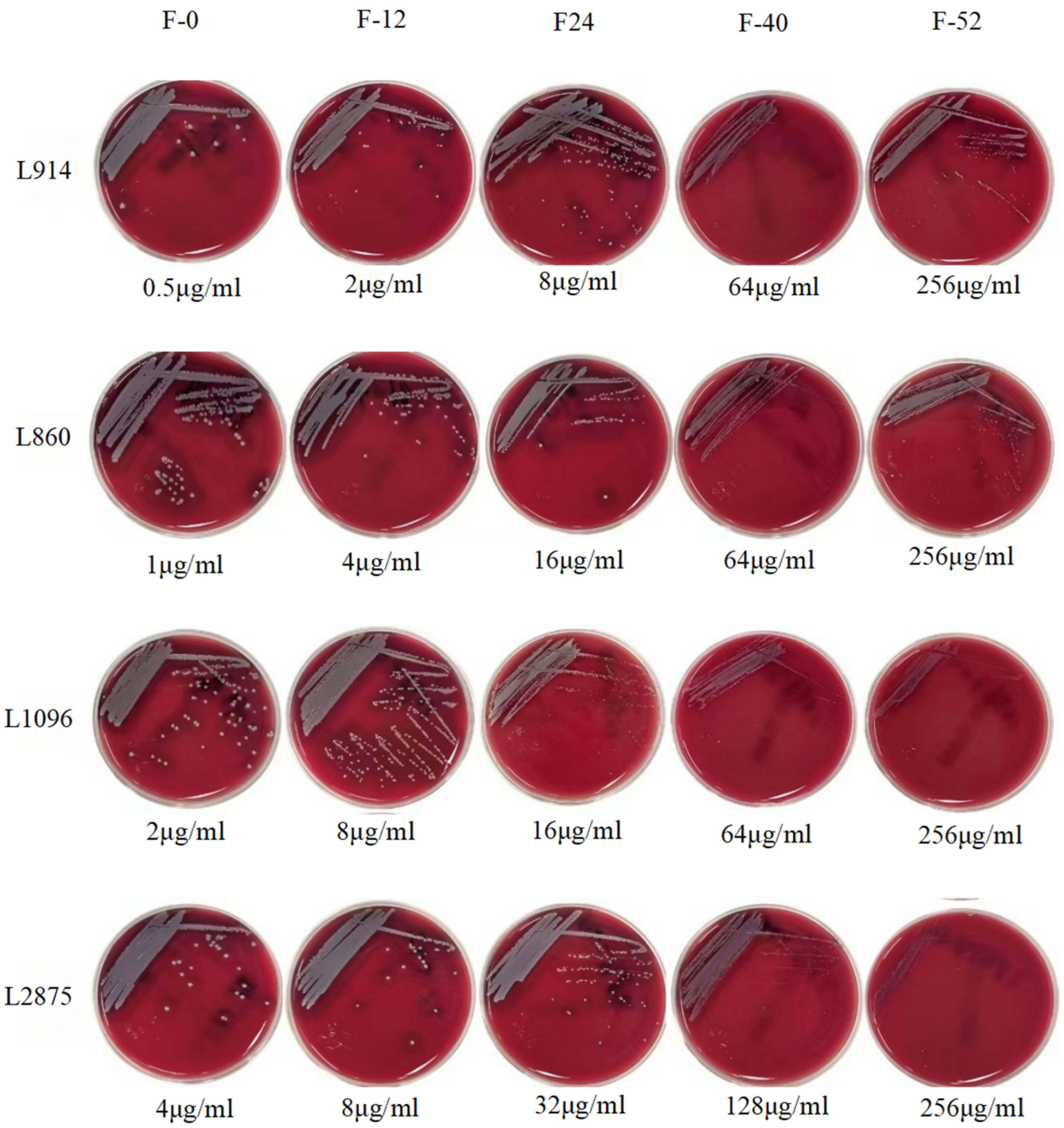

Figure 2 Growth characteristics of the parent MRSA strains and their corresponding derivatives with linezolid resistance.
Notes: F stands for "filial generation under induction by continuous linezolid". Photographs were captured when the blood agar plates were incubated at 37°C for 24 h. The growth rates of the induced derivates from each of the primary isolate slowed gradually as the resistance to linezolid increased. Over time, the hemolytic activities of the linezolid-resistant isolates weakened gradually (F24 and F40) or even disappeared (F52).

between 2018 and 2022, indicating an increasing tendency toward linezolid resistance. A deviation in MIC values between these two methods was observed; the MICs of nine MRSA isolates decreased by at least 2 μg/mL using the VITEK-2 system. In contrast, Yoo et al[23] found that the level of linezolid resistance was overestimated by the VITEK-2 system. Meanwhile, four linezolid-susceptible MRSA isolates evolved into linezolid-resistant isolates after different induction times. The shortest induction time was observed for an MRSA strain with a MIC value of 4 μg/mL, which implies differentiation of the resistance capabilities of MRSA isolates to linezolid. Considering the aforementioned

Table 4 Mutations in Domain V Region of 23S rRNA Gene and Amino Acid Sequences of Ribosomal Proteins (L3 and L4) Among Four MRSA Strains and Their Resistant Derivates

Passages	Domain V of 23S *rRNA**	Ribosomal Proteins**	
		L3	L4
L914-F0 (0.5 μg/mL)	G2523A	–	–
L914-F20 (8 μg/mL)	G2576T, G2523A	Gly152Asp	–
L914-F24 (8 μg/mL)	G2576T, G2523A	Gly152Asp	–
L914-F28 (16 μg/mL)	G2576T, G2523A	Gly152Asp	–
L914-F32 (16 μg/mL)	G2576T, G2523A	Gly152Asp	–
L914-F36 (32 μg/mL)	G2576T, G2523A, A2451T	Gly152Asp	–
L914-F40 (64 μg/mL)	G2576T, G2523A, A2451T	Gly152Asp	–
L914-F44 (128 μg/mL)	G2576T, G2523A, A2451T	Gly152Asp	–
L914-F48 (256 μg/mL)	G2576T, G2523A, A2451T T2504A	Gly152Asp, Asp159Glu	Lys68Asn
L860-F0 (1 μg/mL)	–	–	–
L860-F16 (8 μg/mL)	G2576T	–	–
L860-F20 (8 μg/mL)	G2576T	–	–
L860-F24 (16 μg/mL)	G2576T	–	–
L860-F28 (32 μg/mL)	G2576T	–	–
L860-F32 (32 μg/mL)	G2576T	Gly152Asp	–
L860-F36 (32 μg/mL)	G2576T	Phe147Ile, Met156Thr Met208Thr, Gly152Asp	–
L860-F40 (64 μg/mL)	G2576T	Phe147Ile, Met156Thr Met208Thr, Gly152Asp	–
L860-F44 (64 μg/mL)	G2576T	Phe147Ile, Met156Thr Met208Thr, Gly152Ala	–
L860-F48 (128 μg/mL)	G2576T	Gly152Ala, Phe147Ile, Met156Thr, Met208Thr, Gly155Asp	–
L860-F52 (>256 μg/mL)	G2576T	Gly152Ala, Phe147Ile, Val154Leu Met156Thr, Met208Thr, Gly155Asp	–
L1096-F0 (2 μg/mL)	–	–	–
L1096-F12 (8 μg/mL)	G2576T	Phe147Ile, Val154Leu Met156Thr	–
L1096-F16 (8 μg/mL)	G2576T	Phe147Ile, Val154Leu Met156Thr	–
L1096-F20 (16 μg/mL)	G2576T, A2451T	Phe147Ile, Val154Leu Met156Thr	–
L1096-F24 (16 μg/mL)	G2576T, A2451T	Phe147Ile, Val154Leu Met156Thr	–
L1096-F28 (32 μg/mL)	G2576T, A2451T	Phe147Ile, Val154Leu Met156Thr	–
L1096-F32 (32 μg/mL)	G2576T, A2451T	Phe147Ile, Val154Leu Met156Thr	–
L1096-F36 (64 μg/mL)	G2576T, A2451T	Phe147Ile, Val154Leu Met156Thr	–
L1096-F40 (64 μg/mL)	G2576T, A2451T	Phe147Ile, Val154Leu Met156Thr	–
L1096-F44 (128 μg/mL)	G2576T, A2451T	Phe147Ile, Val154Leu Met156Thr	–
L1096-F48 (128 μg/mL)	G2576T, A2451T	Phe147Ile, Val154Leu Met156Thr, Ser158Phe	–
L1096-F52 (>256 μg/mL)	G2576T, A2451T	Phe147Ile, Val154Leu Met156Thr, Ser158Phe	–

(*Continued*)

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Table 4 (Continued).

Passages	Domain V of 23S *rRNA**	Ribosomal Proteins**	
		L3	L4
L2875-F0 (4 μg/mL)	–	–	–
L2875-F8 (8 μg/mL)	G2576T	–	–
L2875-F16 (8 μg/mL)	G2576T	–	–
L2875-F20 (16 μg/mL)	G2576T	–	–
L2875-F24 (32 μg/mL)	G2576T, C2404T, T2500A	–	–
L2875-F28 (32 μg/mL)	G2576T, C2404T, T2500A	–	–
L2875-F32 (64 μg/mL)	G2576T, C2404T, T2500A	–	–
L2875-F36 (64 μg/mL)	G2576T, C2404T, T2500A	–	–
L2875-F40 (128 μg/mL)	G2576T, C2404T, T2500A	–	–
L2875-F44 (128 μg/mL)	G2576T, C2404T, T2500A G2447T	Gly152Asp	–
L2875-F48 (>256 μg/mL)	G2576T, C2404T, T2500A G2447T	Gly152Asp, Asp159Tyr	–

Notes: *Mutations in domain V region of 23S rRNA gene; **Mutations in amino acid sequences of ribosomal proteins (L3 and L4).

Table 5 Distribution of Point Mutations in Domain V Region of 23S rRNA Gene Copies (rrn1-rrn6)

Passages	MIC (μg/mL)	Point Mutations in Domain V of 23S rRNA Gene Copies					
		*rrn1**	*rrn2**	*rrn3**	*rrn4**	*rrn5**	*rrn6**
L914-F0	0.5	–	–	–	–	–	–
L914-F20	8	–	–	G2576T	G2523A	–	G2576T
L914-F24	8	–	–	G2576T	G2523A G2576T	–	G2576T
L914-F28	16	–	–	G2576T	G2523A G2576T	G2576T	G2576T
L914-F32	16	G2576T	–	G2576T	G2523A G2576T	G2576T	G2576T
L914-F36	32	G2576T A2451T G2523A	–	G2576T	A2451T G2523A G2576T	G2576T	G2576T
L914-F40	64	G2576T A2451T G2523A	–	G2576T	A2451T G2523A G2576T	A2451T G2523A G2576T	G2576T
L914-F44	128	G2576T A2451T G2523A	–	G2576T	A2451T G2523A G2576T	A2451T G2523A G2576T	G2576T
L914-F48	256	G2576T A2451T G2523A	–	G2576T T2504A	A2451T G2523A G2576T	A2451T G2523A T2504A G2576T	G2576T T2504A
L860-F0	1	–	–	–	–	–	–
L860-F16	8	–	–	–	G2576T	G2576T	–
L860-F20	8	G2576T	–	–	G2576T	G2576T	–
L860-F24	16	G2576T	–	–	G2576T	G2576T	–
L860-F28	32	G2576T	–	–	G2576T	G2576T	–
L860-F32	32	G2576T	–	G2576T	G2576T	G2576T	–
L860-F36	32	G2576T	–	G2576T	G2576T	G2576T	–

(*Continued*)

Table 5 (Continued).

Passages	MIC (μg/mL)	Point Mutations in Domain V of 23S rRNA Gene Copies					
		*rrn1**	*rrn2**	*rrn3**	*rrn4**	*rrn5**	*rrn6**
L860-F40	64	G2576T	–	G2576T	G2576T	G2576T	–
L860-F44	64	G2576T	–	G2576T	G2576T	G2576T	–
L860-F48	128	G2576T	–	G2576T	G2576T	G2576T	–
L860-F52	>256	G2576T	–	G2576T	G2576T	G2576T	–
L860-F56	>256	G2576T	–	G2576T	G2576T	G2576T	–
L1096-F0	2	–	–	–	–	–	–
L1096-F12	8	G2576T	–	–	–	–	
L1096-F16	8	G2576T	–	–	G2576T	G2576T	–
L1096-F20	16	G2576T	G2576T	–	A2451T G2576T	G2576T	–
L1096-F24	16	G2576T	G2576T	–	A2451T G2576T	G2576T	–
L1096-F28	32	G2576T	G2576T	–	A2451T G2576T	G2576T	–
L1096-F32	32	G2576T	G2576T	–	A2451T G2576T	G2576T	–
L1096-F36	64	G2576T	G2576T	–	A2451T G2576T	G2576T	–
L1096-F40	64	G2576T	G2576T	–	A2451T G2576T	G2576T	–
L1096-F44	128	G2576T	G2576T	–	A2451T G2576T	G2576T	–
L1096-F48	128	G2576T	G2576T	–	A2451T G2576T	G2576T	–
L2875-F0	4	–	–	–	–	–	–
L2875-F16	8	–	G2576T	–	–	–	–
L2875-F24	32	G2576T	G2576T	–	–	–	–
L2875-F28	32	C2404T T2500A	G2576T C2404T T2500A	–	–	–	C2404T T2500A
L2875-F32	64	C2404T T2500A	G2576T C2404T T2500A	–	–	–	C2404T T2500A
L2875-F36	64	C2404T T2500A	G2576T C2404T T2500A	–	–	–	C2404T T2500A
L2875-F40	128	C2404T T2500A	G2576T C2404T T2500A	–	–	–	C2404T T2500A
L2875-F44	128	C2404T T2500A G2447T	G2576T C2404T T2500A G2447T	–	–	G2447T	C2404T T2500A
L2875-F48	>256	C2404T T2500A G2447T	G2576T C2404T T2500A G2447T	–	–	G2447T	C2404T T2500A

Note: *Copies of 23S rRNA gene operons.

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results found in this study, it is reasonable to speculate that for MRSA-infected patients for whom linezolid treatment was initiated, active monitoring of phenotypic resistance to linezolid is essential, and classical broth microdilution method is strongly recommended, particularly for medical institutions with higher linezolid consumption.

To better understand the mechanisms underlying linezolid resistance in MRSA when exposed to continuous linezolid stress in vitro, several well-known resistance determinants related to linezolid resistance were investigated, including mutation in the V region of the *23S rRNA* gene; ribosomal proteins encoding genes, L3 (*rplC*), L4 (*rplD*), and L22 (*rplV*); and the presence of plasmid-carrying genes, such as *potrA, cfr*, and *cfr (B)*. Mutations in the V region of the *23S rRNA* gene and ribosomal proteins encoding genes (*rplC, rplD*) were found to be the main resistance mechanisms with isolate-specific differences.

Considering the linezolid resistance mechanism mediated by the 23S rRNA gene, the distances between specific 23S rRNA nucleotides and PTC are a key factor in determining the strength of action mediated by point mutations within the 23S rRNA gene. For instance, the binding pocket is lined with the universally conserved nucleotides G2061, A2451, C2452, A2503, U2504, G2505, U2506, and U2585, which can interact directly with linezolid.[26] In contrast, A2062, G2447, A2453, C2499, U2500, and G2576 are located more distally, and mutations in these nucleotides may affect the bacterial resistance by regulating the affinity between the above nucleotide sites and antibiotics.[27] The types of mutations detected in the 23S rRNA gene in this study were strongly consistent with those found in previous studies,[8,10,24] where the G2576T mutation was still the most prevalent, regardless of the genetic background of the MRSA isolates. Base G2576 can interact with U2506 and G2505, which constitute the majority of contact points in the oxazolidinone binding site.[26,28] Another prevalent mutation, T2504C,[29] was not detected in this study; however, a similar mutation, T2504A, was identified in *S. aureus* (L914-F48). This mutation was frequently detected among *S. epidermidis* clinical isolates, which are considered to be associated with high levels of linezolid resistance.[30] Another mutation, G2447U, may form a new base pair with A2451, thereby limiting the flexibility of A2451 and influencing drug binding.[31] Coincidentally, in this study, mutations were detected at both bases, namely, A2451T and G2447T. Furthermore, the T2500A mutation of 23S rRNA exclusively appeared in resistant L2875 strains from generations F28 to F48. This type of mutation was found to be capable of altering the environment of the PTC, thus interfering with drug binding.[32] However, the resistance phenotype resulting from T2500A mutation was considered to be unstable;[22] thus, it is difficult to speculate on the actual role of T2500A in this study considering its specific distribution among linezolid-resistant derivates from L2875. To the best of our knowledge, the additional C2404T mutation identified in this study has also not been reported previously and was exclusively identified among linezolid-resistant derivates of the L2875 strain. The relationship between mutations at this site and resistance requires further exploration.

As reported by Wilson et al,[33] the linezolid MIC values of *S. aureus* isolates increased as the number of mutant 23S rRNA genes and their copy numbers increased.[27] However, this gene dosage effect has also been reported in some other common clinical bacterial pathogens, such as *Enterococcus faecium* and *E. faecalis*.[34] In this study, the increase in the linezolid MIC value in MRSA strains generated from the corresponding parental isolate was accompanied with increases in the number of mutation sites in 23S rRNA as well as copy numbers of this gene. However, the copy numbers of this gene in linezolid-resistant strains were not always consistent with the resistance levels of MRSA to linezolid. Thus, it is difficult to use MIC value as a precise index to predict the resistance of MRSA isolates to linezolid among strains obtained from different origins.

Another important mechanism that contributes to linezolid resistance is mutations in ribosomal proteins, including L3 and L4. Ribosomal proteins L3 and L4 are located further away from the bound drug, and their effects on linezolid are also indirectly achieved through PTC. Most of the known L3 mutations are found together with other resistance determinants, mainly mutations in the domain V region of 23S rRNA.[35,36] Although previous studies have found that L3 mutations alone can cause an increase in the MIC of linezolid for MRSA,[37] it is mostly considered to play an auxiliary role in the development of MRSA resistance to linezolid. In this study, the Gly152Asp mutation in L3 was universally identified in highly linezolid-resistant derivates generated from three MRSA isolates (L914, L860, and L2875) but not in linezolid-susceptible isolates. The mechanism of this mutation was likely to be correlated with the loss of oxazolidinone affinity by indirectly disrupting bases 2505 and 2506 of 23S rRNA, which is analogous to the G2576T mutation.[38] Similarly, Baos et al reported that linezolid-resistant *S. epidermidis* strains with a combination of the G2576T and Gly152Asp mutations exhibited higher linezolid MIC.[39] Furthermore, previous reports have shown that mutations in the L3 ribosomal protein alone can induce high levels of linezolid resistance in *S. aureus* (16 μg/mL).[40] Mutations in Gly155Arg and Ser158Phe of L3 ribosomal proteins have previously been

reported as independent factors leading to linezolid resistance in *Staphylococcus aureus*.[41] These mutations have been implicated in mediating linezolid binding by disrupting U2504 and were suggested to indirectly disturb the conformation of 23S rRNA G2576, resulting in high levels of resistance. Mutations in these two sites were also found in this study. Moreover, the Met156Thr mutation of L3 has been observed together with *cfr* and 23S rRNA G2576,[42] which was also found in the resistant derivates in this study. Furthermore, two *S. epidermidis* isolates with Asp159Tyr and Ser158Phe mutations in L3 resulted in an increase in linezolid MIC, which were accompanied by *cfr*.[43] A number of mutations and a deletion have been found in L3 at position 147 in *S. epidermidis* and *S. aureus* strains with reduced susceptibility to linezolid.[44] In *Mycobacterium tuberculosis*, the Cys154Arg mutation in L3 was reported to be correlated with linezolid resistance.[45] The Val154Leu point mutation was also observed among linezolid-resistant strains in this study. It is worth noting that the Met208Thr mutation of the L3 protein is reported for the first time in this study. As this site is far from the PTC, the role of the Met208Thr mutation in mediating linezolid resistance in *S. aureus* requires further study. As another important ribosomal protein, the Lys68Gln mutation of the L4 protein was closely correlated with oxazolidinone resistance to *S. aureus*.[38] Coincidently, this mutation was detected in this study among linezolid-resistant derivates. In this study, Lys68Asn was also detected among the linezolid-resistant strains. Based on the results of this study, mutations in the ribosomal proteins of L3 or L4 are more crucial role for determining the degree of MRSA resistance to linezolid.

The set of mutants with stronger resistance to linezolid varied among different isolates. It is not reasonable to compare the resistance levels among different isolates as the distribution of mutations varies greatly among them. However, for each of the four isolates, the set with the maximum number of mutations in the domain V of 23S rRNA operons and ribosomal protein-encoding genes (*rpl3* and *rpl4*) showed higher resistance to linezolid than those with fewer mutations. The assumptions for this phenomenon may be explained as follows. First, it is well-recognized that a series of mutations within the domain V region of the 23S rRNA gene is the dominant mechanism for blocking the binding of linezolid to PTC, including those mutations detected in this study. Second, an increase in the copy number of the 23S rRNA gene with point mutations in MRSA was positively associated with linezolid resistance, which serves as a mechanism to promote linezolid-mediated resistance.[46] Third, a mutation in the gene encoding ribosomal L3 protein was recently recognized to be an independent factor mediating linezolid resistance in MRSA,[40] the emergence of which is usually accompanied with mutations in the 23S rRNA gene. Thus, it is reasonable to speculate that strains with mutations in the domain V region of the 23S rRNA gene and ribosomal protein-encoding genes are more resistant to linezolid. These two mutations play dominant and auxiliary roles, respectively, in the process of linezolid resistance in MRSA.

Another phenomenon observed in this study was the global emergence of SCVs accompanied by linezolid resistance, indicating a slower growth of resistant colonies. A similar phenomenon was observed in an MRSA reference strain, ATCC29213, the reason of which was speculated to be associated with the inductive appearance of the SNVs p.G152D and p.G155R in the *rplC* gene.[18] However, the type of mutations in the *rplC* gene among linezolid-resistant colonies varied greatly among different strains, which implies that the evolution of SCVs from a normal form of *S. aureus* under exposure to linezolid stress did not result from specific mutations in the *rpl* gene. As an important phenotype of *S. aureus, S. aureus* in the SCV form is closely correlated with recurrent infections.[47–49] Based on the results of this study and other related studies, it is reasonable to speculate that the appearance of SCVs is the result of adaptive evolution in *S. aureus* upon exposure to continuous linezolid. In contrast, tedizolid exposure was recently reported to be a potent inducer in promoting SCV formation in methicillin sensitive *Staphylococcus aureus* (MSSA), the mechanism of which was probably related to the appearance of the SNVs p.G152D and p.G155R in *rplC* under exposure to continuous tedizolid.[18] In contrast, owing to their reduced growth rate and fundamental metabolic changes, *S. aureus* in the SCV form displays elevated resistance levels to multiple types of antimicrobials or even alters the function of antibiotics by mediating specific metabolic pathways.[48] Thus, the appearance of SCVs in *S. aureus* is an inevitable consequence of the continuous and stepwise induction with linezolid. More attention should be paid not only on the evolution of resistance in MRSA during linezolid treatment but also on virulence or survival fitness.

Conclusion

In conclusion, for resistant strains produced via stepwise induction, the major resistance mechanism was mutations in the domain V region of the 23S rRNA gene and ribosomal protein-encoding genes. The G2756T mutation within the 23S rRNA gene was found to be the most critical and prevalent resistance determinant in this study. Meanwhile, the cumulative increment in the

number of mutations in rRNA gene operons resulted in cumulative effects in promoting linezolid resistance. Another important resistance determinant, mutations in the ribosomal protein-encoding genes, was closely correlated with elevated linezolid resistance, and the role of this determinant in mediating linezolid resistance in *S. aureus* may have previously been underestimated. Because significant strain-specific resistance induction effects of linezolid on MRSA clinical isolates were observed in this study, it is essential to pay close attention to MRSA strains with higher MICs during clinical treatment with linezolid. Furthermore, linezolid exposure can induce the emergence of SCVs, further promoting the possibility of recurrent infections.

Data Sharing Statement

Available on request.

Ethics Approval

This study complies with the Declaration of Helsinki. The bacterial strains used in this study were isolated from the routine biological specimens, which were obtained during the clinical diagnosis and management of the patients. Also, rights and health of the subjects were not under threat, and no personal identifying information was used during this study. The mutated isolates were destroyed using autoclaving device and finally processed as medical wastes, according to the regulation of Affiliated hospital of Inner Mongolian Medical University. Thus, the exemption of written informed consent and ethical review were approved by ethical committee of Inner Mongolian medical university (No. YKD202201166) according to the national regulation on ethical review (No. 2016–11, 12/01/2016).

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests in this work.

References

1. Woodford N, Livermore DM. Infection caused by gram-positive bacteria: a review of the global challenge. *J Infect*. 2009;59(Suppl 1):S4–16. doi:10.1016/S0163-4453(09)60003-7
2. Leach KL, Brickner SJ, Note MC, et al. Linezolid, the first oxazolidinone antibacterial agent. *Ann N Y Acad Sci*. 2011;1222:49–54. doi:10.1111/j.1749-6632.2011.05962.x
3. AbdAlhafiz AI, Elleboudy NS, Aboshanab KM, Aboulwafa MM, Hassouna NA. Phenotypic and genotypic characterization of linezolid resistance and the effect of antibiotic combinations on methicillin-resistant Staphylococcus aureus clinical isolates. *Ann Clin Microbiol Antimicrob*. 2023;22(1):23. doi:10.1186/s12941-023-00574-2
4. Saravolatz LD, Pawlak JM, Wegner C. Delafloxacin activity against Staphylococcus aureus with reduced susceptibility or resistance to methicillin, vancomycin, daptomycin or linezolid. *J Antimicrob Chemother*. 2020;75(9):2605–2608. doi:10.1093/jac/dkaa209
5. Drago L, Nicola L, Vecchi ED. A comparative in-vitro evaluation of resistance selection after exposure to teicoplanin, vancomycin, linezolid and quinupristin-dalfopristin in Staphylococcus aureus and Enterococcus spp. *Clin Microbiol Infect*. 2008;14(6):608–611. doi:10.1111/j.1469-0691.2008.01993.x
6. Bozdogan B, Appelbaum PC. Oxazolidinones: activity, mode of action, and mechanism of resistance. *Int J Antimicrob Agents*. 2004;23(2):113–119. doi:10.1016/j.ijantimicag.2003.11.003

7. Shinabarger D. Mechanism of action of the oxazolidinone antibacterial agents. *Expert Opin Investig Drugs*. 1999;8(8):1195–1202. doi:10.1517/13543784.8.8.1195
8. Han X, Zou G, Liu J, et al. Mechanisms of linezolid resistance in Staphylococcus capitis with the novel mutation C2128T in the *23S rRNA* gene in China. *BMC Microbiol*. 2022;22(1):203. doi:10.1186/s12866-022-02616-9
9. Wu DD, Yan BY, Yang XJ, et al. Whole-genome sequencing for the detection of linezolid resistance in a patient with persistent methicillin-resistant Staphylococcus aureus infection during linezolid exposure. *Int J Antimicrob Agents*. 2020;55(1):105819. doi:10.1016/j.ijantimicag.2019.09.023
10. Liu WT, Chen EZ, Yang L, et al. Emerging resistance mechanisms for 4 types of common anti-MRSA antibiotics in Staphylococcus aureus: a comprehensive review. *Microb Pathog*. 2021;156:104915. doi:10.1016/j.micpath.2021.104915
11. Petrov A, Meskauskas A, Dinman JD. Ribosomal protein L3: influence on ribosome structure and function. *RNA Biol*. 2004;1(1):59–65. doi:10.4161/rna.1.1.957
12. Hashemian SMR, Farhadi T, Ganjparvar M. Linezolid: a review of its properties, function, and use in critical care. *Drug Des Devel Ther*. 2018;12:1759–1767. doi:10.2147/DDDT.S164515
13. Wang Y, Lv Y, Cai JC, et al. A novel gene, *optrA*, that confers transferable resistance to oxazolidinones and phenicols and its presence in Enterococcus faecalis and Enterococcus faecium of human and animal origin. *J Antimicrob Chemother*. 2015;70(8):2182–2190. doi:10.1093/jac/dkv116
14. Locke JB, Finn J, Hilgers M, et al. Structure-activity relationships of diverse oxazolidinones for linezolid-resistant Staphylococcus aureus strains possessing the *cfr* methyltransferase gene or ribosomal mutations. *Antimicrob Agents Chemother*. 2010;54(12):5337–5343. doi:10.1128/AAC.00663-10
15. Pitcher NJ, Feder A, Bolden N, et al. Parallel evolution of linezolid-resistant Staphylococcus aureus in patients with cystic fibrosis. *Microbiol Spectr*. 2023;11(5):e0208423. doi:10.1128/spectrum.02084-23
16. Tsakris A, Pillai SK, Gold HS, et al. Persistence of rRNA operon mutated copies and rapid re-emergence of linezolid resistance in Staphylococcus aureus. *J Antimicrob Chemother*. 2007;60(3):649–651. doi:10.1093/jac/dkm246
17. Ono Y, Kikuchi K, Jin J, Hori S, Hiramatsu K. Mechanism of linezolid resistance in methicillin-resistant Staphylococcus aureus isolated from a patient with mediastinitis. *Juntendo Med J*. 2011;57(4):370–376. doi:10.14789/pjmj.57.370
18. Staudacher M, Hotz JF, Kriz R, et al. Differences in oxazolidinone resistance mechanisms and small colony variants emergence of Staphylococcus aureus induced in an in vitro resistance development model. *Emerg Microbes Infect*. 2024;13(1):2292077. doi:10.1080/22221751.2023.2292077
19. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing. CLSI Supplement M100*. 32th ed. Wayne, PA: CLSI; 2022.
20. Cikman A, Aydin M, Gulhan B, et al. Absence of the *mecC* gene in methicillin-resistant Staphylococcus aureus isolated from various clinical samples: the first multi-centered study in Turkey. *J Infect Public Health*. 2019;12(4):528–533. doi:10.1016/j.jiph.2019.01.063
21. Yao WM, Xu GJ, Bai B, et al. In vitro-induced erythromycin resistance facilitates cross-resistance to the novel fluoroketolide, solithromycin, in Staphylococcus aureus. *FEMS Microbiol Lett*. 2018;365(12):1–6. doi:10.1093/femsle/fny116
22. Meka VG, Pillai SK, Sakoulas G, et al. Linezolid resistance in sequential Staphylococcus aureus isolates associated with a T2500A mutation in the *23S rRNA* gene and loss of a single copy of *rRNA*. *J Infect Dis*. 2004;190(2):311–317. doi:10.1086/421471
23. Yoo IY, Kan OK, Shim HJ, Huh HJ, Lee NY. Linezolid resistance in methicillin-resistant Staphylococcus aureus in Korea: high rate of false resistance to linezolid by the VITIEK 2 system. *Ann Lab Med*. 2020;40(1):57–62. doi:10.3343/alm.2020.40.1.57
24. Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of Staphylococcus aureus. *J Clin Microbiol*. 2000;38(3):1008–1015. doi:10.1128/JCM.38.3.1008-1015.2000
25. Mellmann A, Friedrich AW, Rosenkötter N, et al. Automated DNA sequence-based early warning system for the detection of methicillin-resistant Staphylococcus aureus outbreaks. *PLoS Med*. 2006;3(3):e33. doi:10.1371/journal.pmed.0030033
26. Wilson DN, Schluenzen F, Harms JM, Starosta AL, Connell SR, Funcini P. The oxazolidinone antibiotics perturb the ribosomal peptidyl-transferase center and effect tRNA positioning. *Proc Natl Acad Sci U S A*. 2008;105(35):13339–13344. doi:10.1073/pnas.0804276105
27. Long KS, Vester B. Resistance to linezolid caused by modifications at its binding site on the ribosome. *Antimicrob Agents Chemother*. 2012;56(2):603–612. doi:10.1128/AAC.05702-11
28. Ippolito JA, Kanyo ZF, Wang D, et al. Crystal structure of the oxazolidinone antibiotic linezolid bound to the 50S ribosomal subunit. *J Med Chem*. 2008;51(12):3353–3356. doi:10.1021/jm800379d
29. Livermore DM, Warner M, Mushtaq S, North S, Woodford N. In vitro activity of the oxazolidinone RWJ-416457 against linezolid-resistant and -susceptible Staphylococci and Enterococci. *Antimicrob Agents Chemother*. 2007;51(3):1112–1114. doi:10.1128/AAC.01347-06
30. Liakopoulos A, Neocleous C, Klapsa D, et al. A T2504A mutation in the *23S rRNA* gene responsible for high-level resistance to linezolid of Staphylococcus epidermidis. *J Antimicrob Chemother*. 2009;64(1):206–207. doi:10.1093/jac/dkp167
31. Eyal Z, Matzov D, Krupkin M, et al. Structural insights into species-specific features of the ribosome from the pathogen Staphylococcus aureus. *Proc Natl Acad Sci USA*. 2015;112(43):E5805–5814. doi:10.1073/pnas.1517952112
32. Davidovich C, Bashan A, Yonath A. Structural basis for cross-resistance to ribosomal PTC antibiotics. *Proc Natl Acad Sci USA*. 2008;105(52):20665–20670. doi:10.1073/pnas.0810826105
33. Wilson P, Andrews JA, Charlesworth R, et al. Linezolid resistance in clinical isolates of Staphylococcus aureus. *J Antimicrob Chemother*. 2003;51(1):186–188. doi:10.1093/jac/dkg104
34. Marshall SH, Donskey DJ, Hutton-Thomas R, Salata RA, Rice LB. Gene dosage and linezolid resistance in Enterococcus faecium and Enterococcus faecalis. *Antimicrob Agents Chemother*. 2002;46(10):3334–3336. doi:10.1128/AAC.46.10.3334-3336.2002
35. Kosowska-Shick K, Julian KG, McGhee PL, Appelbaum PC, Whitener C. Molecular and epidemiologic characteristics of linezolid-resistant coagulase-negative Staphylococci at a tertiary care hospital. *Diagn Microbiol Infect Dis*. 2010;68(1):34–39. doi:10.1016/j.diagmicrobio.2010.05.007
36. de Almeida LM, de Araújo MR, Sacramento AG, et al. Linezolid resistance in Brazilian Staphylococcus hominis strains is associated with L3 and 23S rRNA ribosomal mutations. *Antimicrob Agents Chemother*. 2013;57(8):4082–4083. doi:10.1128/AAC.00437-13
37. Klitgaard RN, Ntokou E, Norgaard K, et al. Mutations in the bacterial ribosomal protein L3 and their association with antibiotic resistance. *Antimicrob Agents Chemother*. 2015;59(6):3518–3528. doi:10.1128/AAC.00179-15
38. Locke JB, Hilgers M, Shaw KJ. Novel ribosomal mutations in Staphylococcus aureus strains identified through selection with the oxazolidinones linezolid and torezolid (TR-700). *Antimicrob Agents Chemother*. 2009;53(12):5265–5274. doi:10.1128/AAC.00871-09

39. Baos E, Candel FJ, Merino P, Pena I, Picazo JJ. Characterization and monitoring of linezolid-resistant clinical isolates of Staphylococcus epidermidis in an intensive care unit 4 years after an outbreak of infection by cfr-mediated linezolid-resistant Staphylococcus aureus. *Diagn Microbiol Infect Dis*. 2013;76(3):352–359. doi:10.1016/j.diagmicrobio.2013.04.002
40. Zanfardino A, Napoli MD, Migilore F, et al. Characterization of linezolid-analogue L3-resistance mutation in Staphylococcus aureus. *Microorganisms*. 2023;11(3):700. doi:10.3390/microorganisms11030700
41. Miller K, Dunsmore CJ, Fishwick CWG, Chopra I. Linezolid and tiamulin cross-resistance in Staphylococcus aureus mediated by point mutations in the peptidyl transferase center. *Antimicrob Agents Chemother*. 2008;52(5):1737–1742. doi:10.1128/AAC.01015-07
42. Rajan V, Kumar VGS, Gopal S. A cfr-positive clinical Staphylococcal isolate from India with multiple mechanisms of linezolid-resistance. *India J Med Res*. 2014;139(3):463–467.
43. Mendes RE, Deshpande L, Rodriguez-Noriega E, Ross JE, Jones RN, Morfin-Otero R. First report of Staphylococcal clinical isolates in Mexico with linezolid resistance caused by *cfr*: evidence of in vivo cfr mobilization. *J Clin Microbiol*. 2010;48(8):3041–3043. doi:10.1128/JCM.00800-10
44. Locke JB, Hilgers M, Shaw KJ. Mutations in ribosomal protein L3 are associated with oxazolidinone resistance in Staphylococci of clinical origin. *Antimicrob Agents Chemother*. 2009;53(12):5275–5278. doi:10.1128/AAC.01032-09
45. Zhang ZJ, Pang Y, Wang YF, Liu CT, Zhao YL. Beijing genotype of mycobacterium tuberculosis is significantly associated with linezolid resistance in multidrug-resistant and extensively drug-resistant tuberculosis in China. *Int J Antimicrob Agent*. 2014;43(3):231–235. doi:10.1016/j.ijantimicag.2013.12.007
46. Suzuki K, Saito M, Hanaki H. Increased copy number of 23S ribosomal RNA gene with point mutation in MRSA associated with linezolid resistance in a patient treated with long-term linezolid. *J Infect Chemother*. 2023;29(5):481–484. doi:10.1016/j.jiac.2023.01.019
47. Lee J, Zilm PS, Kidd SP. Novel research models for Staphylococcus aureus small colony variants (SCV) development: copathogenesis and growth rate. *Front Microbiol*. 2020;28(11):321. doi:10.3389/fmicb.2020.00321
48. Zhou S, Rao YF, Li J, Huang Q, Rao X. Staphylococcus aureus small-colony variants: formation, infection, and treatment. *Microbiol Res*. 2022;260:127040. doi:10.1016/j.micres.2022.127040
49. Kittinger C, Toplitsch D, Folli B, Landgraf LM, Zarfel G. Phenotypic stability of Staphylococcus aureus small colony variants (SCV) isolates from cystic fibrosis (CF) patients. *Int J Environ Res Public Health*. 2019;16(11):1940. doi:10.3390/ijerph16111940