

Molecular Epidemiology of Carbapenem-Resistant *Serratia marcescens* Revealing Distinct High-Risk Clones Within a Hospital Setting

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Purpose: Carbapenem-resistant *Serratia marcescens* (CRSM) poses an increasing threat in healthcare settings. This study aimed to investigate the molecular epidemiology of CRSM and the drivers of its dissemination within a hospital setting.

Patients and Methods: Twelve non-duplicate clinical CRSM isolates were collected from a tertiary hospital between 2010 and 2024. Clinical data were retrospectively obtained from electronic medical records. Antimicrobial susceptibility testing was performed using the Vitek-2 system. Whole-genome sequencing (WGS) was conducted to determine resistance and virulence gene profiles, sequence types, and phylogenetic relationships. Pulsed-field gel electrophoresis and conjugation assays were performed to assess clonal relatedness and plasmid transferability.

Results: CRSM infections predominantly occurred in neonates (50.0%) and adults (50.0%), with the respiratory tract as the most common infection site (66.7%). All isolates exhibited multidrug-resistant phenotypes; resistance to β -lactams ranged from 50.0% to 100.0%, while no amikacin-resistant isolates were detected. Two dominant clonal lineages were identified: ST595 (5 strains) uniformly carrying *bla*_{KPC-2}, and ST490 (6 strains, 4 of which harbored *bla*_{NDM-5}). SNP phylogeny identified four distinct, well-supported clades, with ST595 strains showing short branch lengths indicative of clonal expansion events during the study period. The *bla*_{NDM-5} gene was successfully transferred via conjugation from four donor strains, and most transconjugants exhibited growth advantages. Core virulence genes were present in all isolates, while accessory virulence factors showed strain-specific variations.

Conclusion: CRSM dissemination in this hospital is likely driven by two co-circulating high-risk clones (ST595-*bla*_{KPC-2} and ST490-*bla*_{NDM-5}), the high horizontal transfer potential of *bla*_{NDM-5}, and specific patient demographics. Despite the small sample size and single-center design, these findings provide actionable insights for infection control and antimicrobial stewardship.

Keywords: *Serratia marcescens*, MLST, carbapenem-resistant, whole-genome sequencing

Introduction

Serratia marcescens is a Gram-negative bacterium that can be transmitted in healthcare settings through contaminated medical equipment, water sources, or healthcare worker hands. It exhibits high genetic plasticity and strong colonization potential, frequently causing infections and outbreaks.^{1,2} This bacterium has evolved into an opportunistic pathogen primarily infecting immunocompromised individuals such as ICU patients,³ causing pneumonia, bloodstream infections, and urinary tract infections,⁴ and is particularly prevalent in healthcare-associated infections.⁵ The detection rate and resistance rate of *S. marcescens* have been continuously increasing, making it one of the primary pathogens causing hospital-acquired infections.^{6,7} Most *S. marcescens* isolates exhibit multidrug resistance, which presents major obstacles to clinical treatment. These strains exhibit inherent resistance to multiple antibiotics, including ampicillin, macrolides,

and first- and second-generation cephalosporins.⁸ The widespread use of carbapenem antibiotics has led to the emergence of carbapenem-resistant *Serratia marcescens* (CRSM), limiting therapeutic options.⁹

Currently, the development of resistance to β -lactam antibiotics in *S. marcescens* involves multiple mechanisms: production of carbapenemases, loss of outer membrane porins, enhanced efflux pump activity, alterations in antibiotic targets, and the role of integrons.^{10–13} Among these, carbapenemase production is the primary mechanism. Carbapenemases include Class A (KPC), Class B metalloenzymes (NDM, VIM, IMP), and Class D oxacillinases (OXA-48).¹⁴ The ATLAS 2018–2022 global surveillance shows distinct regional distributions of carbapenemases in carbapenem-resistant *Enterobacteriaceae* (*bla*_{KPC} predominant in Europe and North America, *bla*_{NDM} in Asia-Pacific, Latin America, and Middle East and Africa with a global increasing trend) and evolving resistance complexity.¹⁵ Epidemiological analysis indicates *bla*_{KPC} is the predominant carbapenemase in CRSM, with *bla*_{KPC-2}, *bla*_{OXA-48}, and *bla*_{VIM-1} dominating in China, Pakistan, and Germany, respectively. Meanwhile, 76% of *bla*_{NDM}-producing isolates were obtained from North and South America.¹⁶ A meta-study shows that the country/region with the highest carriage rates of the *bla*_{KPC} and *bla*_{NDM} genes is Ecuador; in addition, the United States and China have the largest number of positive strains.^{9,17}

However, systematic data on the molecular epidemiology, resistance gene transmission, and biological characteristics of CRSM in our hospital are still lacking. The prevalence, clonal relatedness, resistance mechanisms, and transmission routes of CRSM in our healthcare setting remain unclear, which hinders the formulation of targeted infection control measures and rational antimicrobial therapy. Given the increasing incidence of CRSM and its significant threat to clinical treatment and patient safety, it is particularly important and urgent to clarify the molecular characteristics and transmission dynamics of CRSM in our institution.

To address these knowledge gaps, we retrospectively collected 12 CRSM clinical isolates from 2010 to 2024. We integrated traditional microbiological methods with advanced molecular biological techniques, including WGS and multiple complementary molecular typing approaches (pulsed-field gel electrophoresis, PFGE; multilocus sequence typing, MLST; single nucleotide polymorphism, SNP-based phylogenetic analysis). This comprehensive strategy was designed to explore the clonal transmission, resistance mechanisms, and horizontal gene transfer capacity of CRSM isolates in our hospital, thereby providing a scientific basis for controlling the emergence and spread of drug-resistant bacteria.

Materials and Methods

Strain Collection and Clinical Characteristics

We collected CRSM isolates (resistant to any of imipenem, ertapenem, or meropenem) from the Medical Laboratory Center of Ningxia Medical University General Hospital between 2010 and December 2024. After excluding duplicate isolates from the same patient and site, 12 strains were obtained as experimental samples. Bacterial identification was performed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF-MS)-based mass spectrometry.¹⁸ Clinical data and characteristics of the 12 CRSM-infected patients were retrospectively collected from the electronic medical record system of the hospital, and statistical analysis was performed. Categorical variables were presented as numbers (percentages, %), and continuous variables were expressed as medians (ranges). The chi-square (χ^2) test was used to evaluate the associations between clinical parameters and clinical outcomes for variables with expected frequency ≥ 5 ; Fisher's Exact Test was used for variables with expected frequency < 5 . The significance level was set at $\alpha = 0.05$.

Antimicrobial Susceptibility Testing (AST) and Detection of Carbapenemase Genes

AST was performed using the Vitek-2 automated system, and minimum inhibitory concentrations (MICs) were interpreted according to Clinical and Laboratory Standards Institute (CLSI) M100 guidelines.¹⁹ *Escherichia coli* (*E. coli*) ATCC 25922 was used as a quality control strain for AST according to CLSI M100 guidelines. Detection of acquired carbapenemase genes was done by PCR analysis (Table 1), including the *bla*_{NDM} and *bla*_{KPC}.^{20,21} PCR amplification was performed with pre-denaturation at 95°C for 2 min, followed by 30 cycles of 94°C for 30s, 60°C for 30s, 72°C for 45s, and a final extension at 72°C for 5 min.

Table 1 PCR Primers

Primer	Primer Sequence	Target Gene	Product Size (bp)
NDM-F	GGTTTGGCGATCTGGTTTTC	<i>bla_{NDM}</i>	813
NDM-R	CGGAATGGCTCATCACGATC		
KPC-F	CGTCTAGTTCTGCTGCTTG	<i>bla_{KPC}</i>	798
KPC-R	CTTGTCATCCTGTTAGGCG		

Note: Sequences and targets of oligonucleotide primers used for PCR amplification of *bla_{NDM}* and *bla_{KPC}* genes.

Whole-Genome Sequencing and Genome Assembly

Bacterial isolates were cultured overnight, centrifuged, and sent to Novogene (Beijing) for whole-genome sequencing. Genomic DNA was extracted from clinical *S. marcescens* isolates. Qualified DNA was randomly fragmented using a Covaris sonicator, followed by library construction including end-repair, A-tailing, adapter ligation, PCR amplification, and fragment size selection. Qualified libraries were subjected to paired-end sequencing on a high-throughput Illumina sequencing platform. Raw sequencing reads were processed and filtered using fastp software to remove low-quality reads, adapter sequences, and reads with excessive ambiguous bases, generating high-quality clean reads. De novo genome assembly was performed using SOAP denovo 2.04, SPAdes, and ABySS, followed by integration with CISA and gap closure using gapclose 1.12. Sequences shorter than 500 bp were discarded.

Genome assembly quality was evaluated by determining the total genome size, GC content, N50 value, number of contigs (≥ 500 bp), and average sequencing depth. The assembly metrics of 12 CRSM isolates were as follows: the revised genome size ranged from 4.83 Mb to 8.97 Mb (majority at 5.15–5.78 Mb), GC content was 57.35%–60.08%, N50 length varied from 38.27 kb to 283.08 kb, the number of contigs (≥ 500 bp) ranged from 7 to 320, and the average sequencing depth was $50.58\times$ – $52.80\times$. All assemblies met the quality requirements for subsequent bioinformatics analysis.

Bioinformatics Analysis

Whole-genome assemblies were compared to the PubMLST (<https://pubmlst.org/>)²² database to determine haplotypes and sequence types. A minimum spanning tree was generated using GrapeTree software to visualize the genetic relationships among strains. Average nucleotide identity (ANI) analysis was performed with JSpecies (<https://jspecies.ribohost.com/jspeciesws/#analyse>), and results were visualized as heatmaps on Chiplot (<https://www.chiplot.online/>). SNP analysis was conducted by aligning sequencing data to reference genomes using BWA, followed by SNP calling with GATK and filtering, producing a high-quality SNP matrix. A maximum-likelihood phylogenetic tree was built using IQ-TREE, and branch confidence was assessed by 1000 bootstrap replicates. Antibiotic resistance genes (ARGs) were identified with ResFinder (<http://genepi.food.dtu.dk/resfinder>). Virulence factors were predicted with PathogenFinder (<https://cge.food.dtu.dk/services/PathogenFinder/>).

Pulsed Field Gel Electrophoresis (PFGE)

PFGE was performed according to the standardized PulseNet protocol.^{23,24} Genomic DNA embedded in agarose plugs was digested with XbaI (Takara Bio). The restriction fragments were separated by electrophoresis using a CHEF Mapper system (Bio-Rad) under optimized conditions. The resulting macrorestriction patterns were analyzed with BioNumerics software, and genetic similarity was calculated based on criteria defined by Tenoner et al.²⁵

Conjugation Experiment and Antimicrobial Susceptibility Testing

The conjugation experiment was carried out to determine the transferability of carbapenemase genes. The CRSM strains were used as the donors, and rifampin-resistant *E. coli* 600 (EC600) was chosen as the recipient strain (EC600 was donated by Hong Du from the Second Affiliated Hospital of Soochow University). Both the donors and EC600 were grown to the exponential stage (the optical density at 600 nm reached ~ 0.5) and then mixed at a donor/recipient ratio of

1:1. After incubation at 37°C for 24 h, transconjugants were selected on MacConkey agar plates containing 100 mg/L rifampicin and 2 mg/L meropenem. The conjugants were confirmed by PCR and Sanger sequencing. The antimicrobial susceptibility of the transconjugants was determined using the Vitek-2 automated system, with *E. coli* ATCC 25922 used as the quality control strain.

Conjugant Growth Kinetics

Growth curves experiments for the transconjugants were performed in 96-well plates as described previously. The transconjugants and the recipient strain EC600 were diluted in LB broth medium in 96-well microtiter plates and incubated at 37°C for 17 h. OD600 measurements were taken hourly to construct a growth curve. Relative growth rates were measured via R script, and AUC values were calculated using GraphPad Prism software 8.0.2 (GraphPad Software, San Diego, CA, USA). Growth kinetics assays were performed in triplicate.

Results

Clinical Characteristics

The 12 CRSM isolates included in this study were collected from 2010 to 2024, all of which were obtained from clinical infection samples in the hospital. Descriptive statistical analysis was performed on the clinical data of 12 CRSM-infected patients. Categorical variables were presented as counts and percentages (%), and continuous variables were expressed as medians (ranges). The chi-square (χ^2) test was used to evaluate the associations between clinical parameters and clinical outcomes for variables with expected frequency ≥ 5 , while Fisher's Exact Test was used for variables with expected frequency < 5 . The significance level was set at $\alpha = 0.05$.

This study included 12 patients with CRSM infection (Table 2). Nine were male (75.0%), and 3 were female (25.0%). Age distribution exhibited a bimodal pattern: six cases (50.0%) were neonates (≤ 1 day old); 6 cases (50.0%) were adults,

Table 2 Demographic and Clinical Characteristics of Patients

Clinical Information	Patients (n=12)
Gender	
Male	9
Female	3
Age	
≤ 1 day	6
30–45 y	4
> 45 y	2
Separation Department	
Neonatology	6
Critical Care Medicine	3
Hepatobiliary Surgery	2
Respiratory and Critical Care Medicine	1
Strain Source	
Sputum	8
Venous Blood	3
Drainage fluid	1
Laboratory-related indicators	
Fever ($T > 40^\circ\text{C}$)	5
White blood cell count $> 9 \times 10^9/\text{L}$	5
Decreased red blood cell count	11
Decreased hemoglobin	10
Elevated procalcitonin (PCT)	4 (n=12)
Elevated high-sensitivity CRP	6 (n=11)

(Continued)

Table 2 (Continued).

Clinical Information	Patients (n=12)
Antibiotics used prior to isolating CRSM	n=11
Imipenem-cilastatin	4
Cefoperazone/sulbactam sodium	3
Ticarcillin/Clavulanate Potassium	6
Meropenem	1
Ampicillin	1
Piperacillin-tazobactam	1
Adjusting Antibiotics After Isolating CRSM	n=12
Imipenem/Cilastatin	2
Aztreonam	2
Piperacillin-sulbactam	1
Ceftazidime/Avibactam	1
Amikacin	3
Cefoperazone/sulbactam	1
Tigecycline	1
Treatment Cycle	
<20 days	2
20–40 days	5
>40	5
Outcome	
Death	1
Discharged after recovery	8
Other	3

Notes: Clinical features, laboratory findings, and antimicrobial treatment history of the 12 patients from whom CRSM isolates were recovered, and antibiotic adjustments were made based on antimicrobial susceptibility testing results. (n=x) indicates the number of patients for whom data was available for that specific parameter.

including 4 cases (33.3%) aged 30–45 years and 2 cases (16.7%) aged >45 years. Departmental distribution was highly concentrated: Neonatology (6 cases, 50%), Critical Care Medicine (3 cases, 25%). The remaining cases were distributed across Hepatobiliary Surgery (2 cases, 16.7%) and Respiratory and Critical Care Medicine (1 case, 8.3%). Respiratory tract infections predominated (8 cases, 66.7%), followed by bloodstream infections (3 cases, 25.0%) and surgical drainage fluid-related infections (1 case, 8.3%). Five patients (41.7%) developed high fever ($T > 40^{\circ}\text{C}$).

Laboratory results indicated that 5 out of 12 cases (41.7%) had elevated white blood cell counts ($>9 \times 10^9/\text{L}$) and that 10 out of 12 cases (83.3%) had decreased hemoglobin levels, which is a sign of anemia. Among inflammatory markers, elevated high-sensitivity C-reactive protein (hs-CRP) was observed in 6/11 cases (54.55%), higher than procalcitonin (PCT) elevation (4/12, 33.3%). Eleven patients (91.7%) had prior antimicrobial exposure, with β -lactam/inhibitor combinations accounting for 90.9% (10/11) and carbapenems for 45.5% (5/11). Treatment regimens were predominantly adjusted to multidrug-resistant pathogen combinations. Treatment duration was generally prolonged, with 10 cases (83.3%) lasting ≥ 20 days. Outcomes primarily reflected clinical resolution: 8 patients (66.7%) improved and were discharged, 1 patient (8.3%) died, and 3 patients (25.0%) had other outcomes.

No significant associations were observed between clinical parameters and infection outcomes (all $P > 0.05$) (Table 3). Although no statistically significant associations were identified, the limited sample size ($n=12$) may have constrained the power to detect true differences; thus, these findings should be interpreted as exploratory.

Antibiotic Susceptibility Test and Detection of Carbapenemase Genes

Antimicrobial susceptibility testing was performed on 12 clinical CRSM isolates and 4 transconjugants. Untested isolates (marked with *) were excluded from resistance rate calculation, and all 12 clinical isolates showed a multidrug-resistant

Table 3 Associations Between Clinical Parameters and Clinical Outcomes in CRSM-Infected Patients

Parameter	Improvement (n=8)	Unfavorable Outcome (n=4)	Statistic	p-value
Gender			1.200	>0.05
Male	7 (87.5)	2 (50.0)		
Female	1 (12.5)	2 (50.0)		
Age			7.200	>0.05
≤1 day	6 (75.0)	0 (0.0)		
30–45 years	1 (12.5)	3 (75.0)		
>45 years	1 (12.5)	1 (25.0)		
Separation Department			9.600	>0.05
Neonatology	6 (75.0)	0 (0.0)		
Critical Care Medicine	1 (12.5)	2 (50.0)		
Hepatobiliary Surgery	1 (12.5)	1 (25.0)		
Respiratory and Critical Care Medicine	0 (0.0)	1 (25.0)		
Strain Source			4.800	>0.05
Sputum	7 (87.5)	1 (25.0)		
Venous Blood	1 (12.5)	2 (50.0)		
Drainage Fluid	0 (0.0)	1 (25.0)		
Laboratory-related indicators				
Fever (T > 40°C)			4.571	>0.05
No	3 (37.5)	4 (100.0)		
Yes	5 (62.5)	0 (0.0)		
White Blood Cell Count			0.600	>0.05
Decreased	2 (25.0)	1 (25.0)		
Increased	4 (50.0)	1 (25.0)		
Normal	2 (25.0)	2 (50.0)		
Hemoglobin Count			0.200	>0.05
Decreased	7 (87.5)	3 (75.0)		
Increased	0 (0.0)	0 (0.0)		
Normal	1 (12.5)	1 (25.0)		
Procalcitonin			0.800	>0.05
Negative	0 (0.0)	0 (0.0)		
Increased	2 (25.0)	2 (50.0)		
Normal	6 (75.0)	2 (50.0)		
High-sensitivity CRP			0.071	>0.05
Negative	0 (0.0)	0 (0.0)		
Increased	4 (57.1)	2 (50.0)		
Normal	3 (42.9)	2 (50.0)		

Notes: Categorical data are presented as n (%). The statistic is Pearson chi-square value; p-values are derived from Fisher's Exact Test, all >0.05.

(MDR) phenotype. High overall resistance was observed to β -lactam agents. Resistance rates to imipenem, cefazolin and cefuroxime were 100.0%; resistance rates of other β -lactams ranged from 50.0% to 92.3%. Ampicillin, ampicillin/sulbactam and cefotetan were not tested. Low overall resistance was observed to most non- β -lactam agents. Among aminoglycosides, tobramycin and gentamicin resistance rates were 16.7% (2/12) and 41.7% (5/12), respectively; no amikacin-resistant isolates were detected (0/12). Fluoroquinolone resistance rates were 41.7%–50.0%, trimethoprim/sulfamethoxazole resistance was 8.3% (1/12), and no resistant isolates were found for fosfomycin, tigecycline, doxycycline. Nitrofurantoin resistance was observed in 1 of 1 tested isolate (100.0%); polymyxin B was not tested (Figure 1).

Carbapenemase genes of all strains were detected by PCR amplification in 9 of 12 CRSM isolates (75.0%), revealing that among the nine positive strains, four harbored *bla*_{NDM-5} and five harbored *bla*_{KPC-2}.

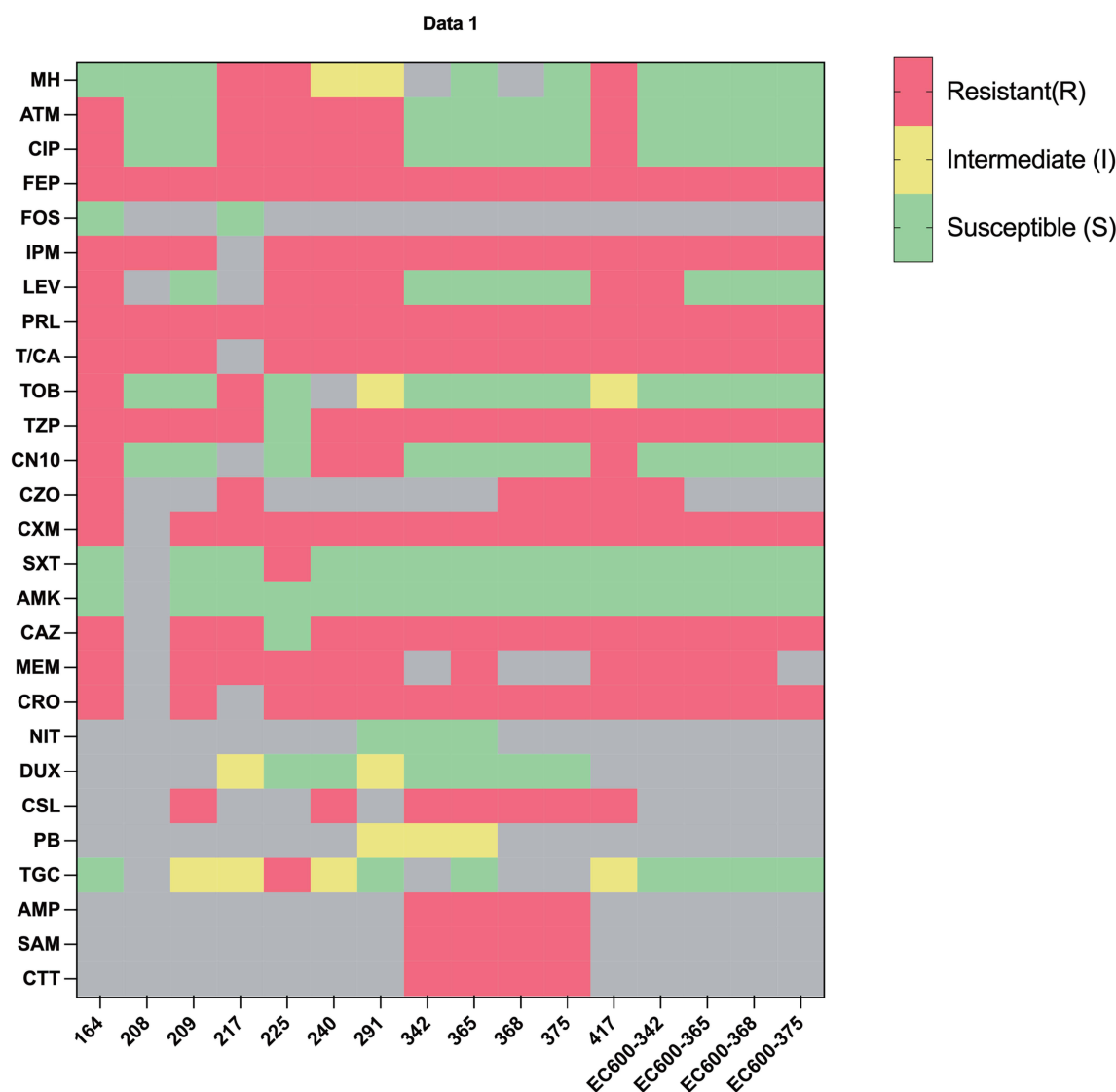


Figure 1 Antimicrobial susceptibility heatmap of 12 CRSM clinical isolates and 4 conjugants.

Note: gray, not tested.

Abbreviations: ATM, aztreonam; CIP, ciprofloxacin; FEP, cefepime; FOS, fosfomycin; IPM, imipenem; LEV, levofloxacin; PRL, piperacillin; T/CA, ticarcillin/clavulanic acid; TOB, tobramycin; TZP, piperacillin-tazobactam; CN10, gentamicin; CZO, ceftazidime; CXM, cefuroxime; SXT, trimethoprim/sulfamethoxazole; AMK, amikacin; CAZ, ceftazidime; MEM, meropenem; CRO, ceftriaxone; NIT, nitrofurantoin; DUX, doxycycline; CSL, Cefoperazone/sulbactam; PB, polymyxin B; TGC, tigecycline; AMP, ampicillin; SAM, ampicillin-sulbactam; CTT, cefotetan; MH, minocycline.

ANI Analysis and PFGE

To assess genetic relatedness between strains at the whole-genome level, we performed ANI analysis. Results showed that all clinical isolates exhibited ANI values above the 95% species-defining threshold relative to the reference *S. marcescens* genome, confirming the accuracy of strain classification (Figure 2A).

The PFGE clustering analysis dendrogram showed that the 12 CRSM strains were grouped into distinct pulsed-field patterns, revealing a clear genetic clustering structure. Specific clustering relationships are as follows: S164 and S217 shared over 95% pattern similarity, indicating high genetic homology and classification into the same clonal lineage, suggesting potential origin from the same transmission chain. S342 and S368 shared over 95% pattern similarity, indicating high genetic homology and classification into the same clonal lineage, suggesting potential origin from the same transmission chain. S208, S209, and S365 also exhibited over 95% pattern similarity, forming another independent clonal lineage, demonstrating a common transmission origin. At higher clustering levels, S164, S217, S417, and S291

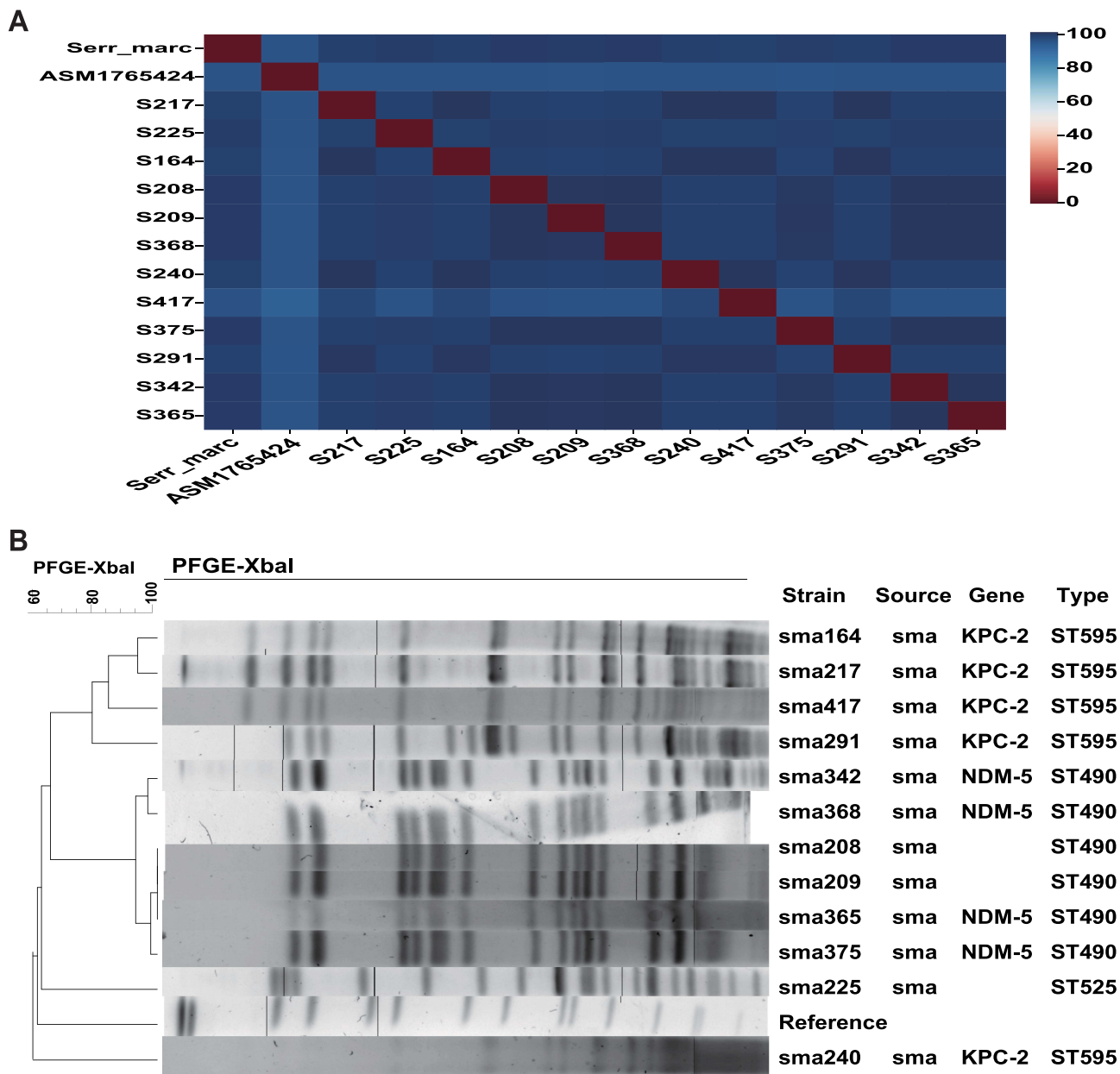


Figure 2 The homology of 12 strains of CRSM. **(A)** Results of ANI Analysis of 12 CRSM; **(B)** Pulsed-field gel electrophoresis (PFGE) typing.

aggregated into the same evolutionary branch with similarity exceeding 80%. Meanwhile, S208, S209, S365, S368, S342, and S375 collectively formed another major evolutionary branch, with a similarity exceeding 80%. The genotypic similarity among strains within each branch ranged from 80% to 94%. This indicates that strains within the same branch may share a common evolutionary ancestor but have accumulated a certain degree of genetic variation during transmission. The genotypic similarity among the remaining strains was below 80%, demonstrating low genetic relatedness. This suggests that these strains belong to different clonal lineages with independent genetic backgrounds (Figure 2B).

Multilocus Sequence Typing (MLST)

Based on whole-genome sequencing data, we performed multi-locus sequence typing on all 12 isolates. Analysis revealed three distinct ST types among the 12 CRSM strains. Among these, ST490 (6 strains) and ST595 (5 strains)

constituted the predominant clonal groups, forming two major clonal clusters. One strain belonged to ST525, which was clustered into the ST595-dominant Clade I in the SNP phylogeny, indicating a close evolutionary relationship with the ST595 lineage. This finding demonstrates genetic diversity among the collected isolates (Figure 3A).

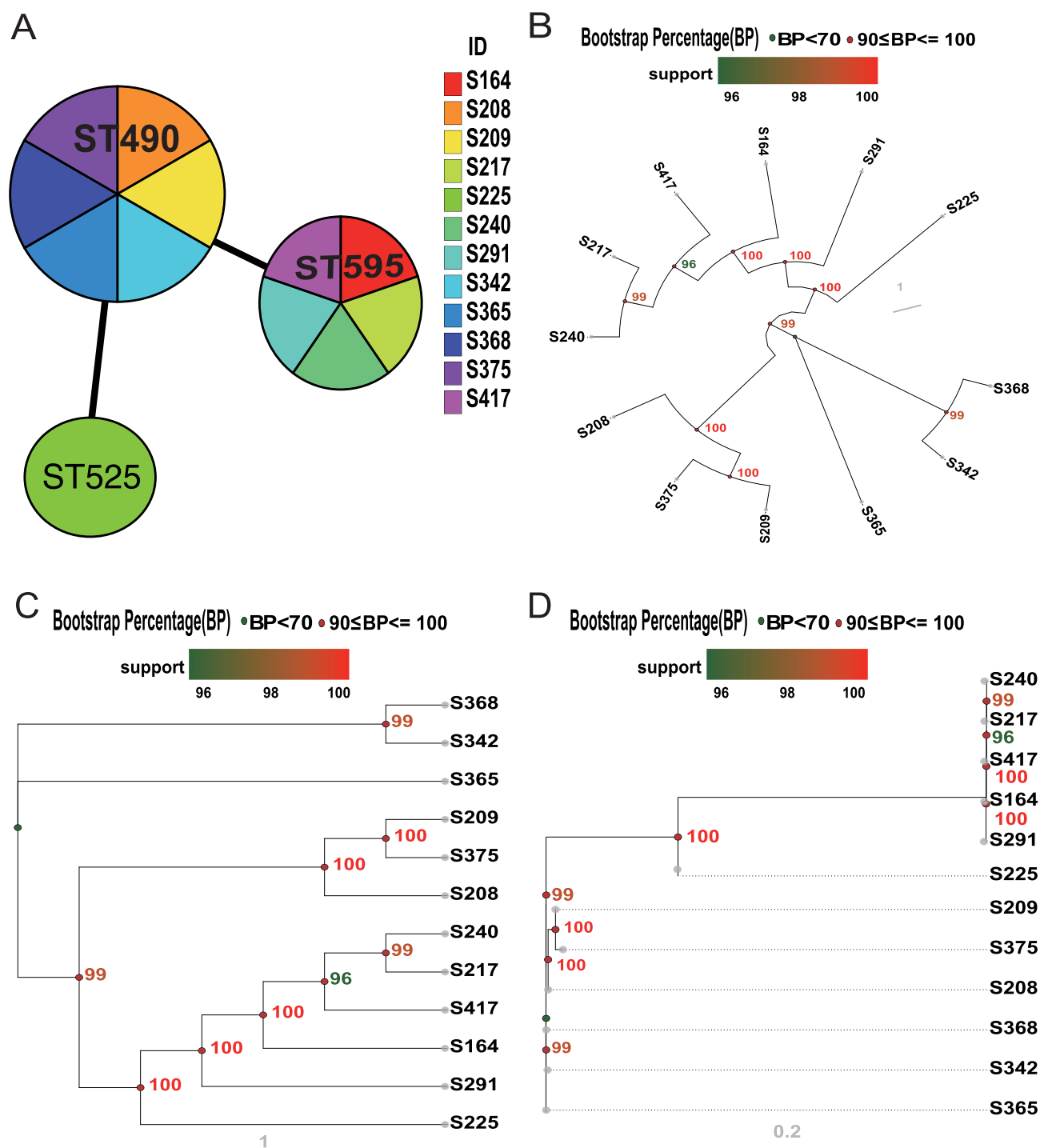


Figure 3 Molecular typing and phylogenetic analysis revealing the coexistence and clonal transmission of ST595 and ST490 in CRSM.

Notes: (A) MLST types of 12 CRSM isolates. The isolates were divided into three sequence types: ST490, ST595, and ST525. (B–D) SNP phylogenetic trees. All approaches revealed four well-supported clades (Clade I, Clade II, Clade III, and a single-strain clade).

SNP Phylogenetic Trees

To analyze the population genetic structure of the strains, multiple phylogenetic and typing methods were employed, including three SNP-based phylogenetic trees (a phylogram, a circular tree, a standardized cladogram) and a multilocus sequence typing–based minimum spanning tree (MLST-MST) (Figure 3A–D). All approaches consistently revealed four well-supported clades (Clade I, Clade II, Clade III and a single-strain clade), confirming the presence of distinct evolutionary lineages within the collection.

The SNP phylogeny showed four strongly supported clades (bootstrap = 100% for all lineage-differentiating core nodes). Clade I, comprising strains S240, S217, S417, S164, S291, and S225, displayed short branch lengths, indicating a notable clonal expansion event during the study period; only one secondary node within this clade (between S217 and S417) showed a slightly lower bootstrap support of 96%, while all other internal nodes reached 100% support. In contrast, Clade II (strains S208, S375, S209), Clade III (strains S368, S342), and the single-strain independent clade (strain S365) exhibited longer branch lengths, with the S365 clade presenting the greatest genetic distance from all other strains, reflecting greater genetic diversity and deeper evolutionary divergence within this group. These patterns were visually reinforced in the circular tree and clarified in the branch-length-standardized cladogram.

The MLST-MST analysis corroborated the whole-genome SNP phylogeny: Clade I corresponded predominantly to ST595, with the exception of ST525 strain S417 and ST490 strain S291, while Clade II, Clade III, and the single-strain clade all belonged exclusively to ST490. This concordance between genome-wide SNP data and multilocus sequence typing validates the strong robustness of the four-clade population structure observed in this study.

ARG and Virulence Factor Encoding Genes of CRSM

Whole-genome sequencing of 12 CRSM isolates revealed clone-specific resistance profiles. The ST595 clone uniformly carried *bla*_{KPC-2}, ESBL genes, aminoglycoside resistance genes, and *qnrS1*. Isolate S417 within this clone uniquely harbored additional genes (*bla*_{OXA-23}, *bla*_{TEM-1D}, *tet*(B), *msr*(E), *mph*(E), *armA*) indicating a separate horizontal gene transfer event. In contrast, the ST490 clone primarily utilized *bla*_{NDM-5} for carbapenem resistance, carried *bla*_{SRT-2} and *aac*(6′)-Ic, but lacked *bla*_{KPC} and ESBLs. Sporadic strains (ST525) possessed unique combinations, such as *bla*_{CTX-M-3} (Figure 4A).

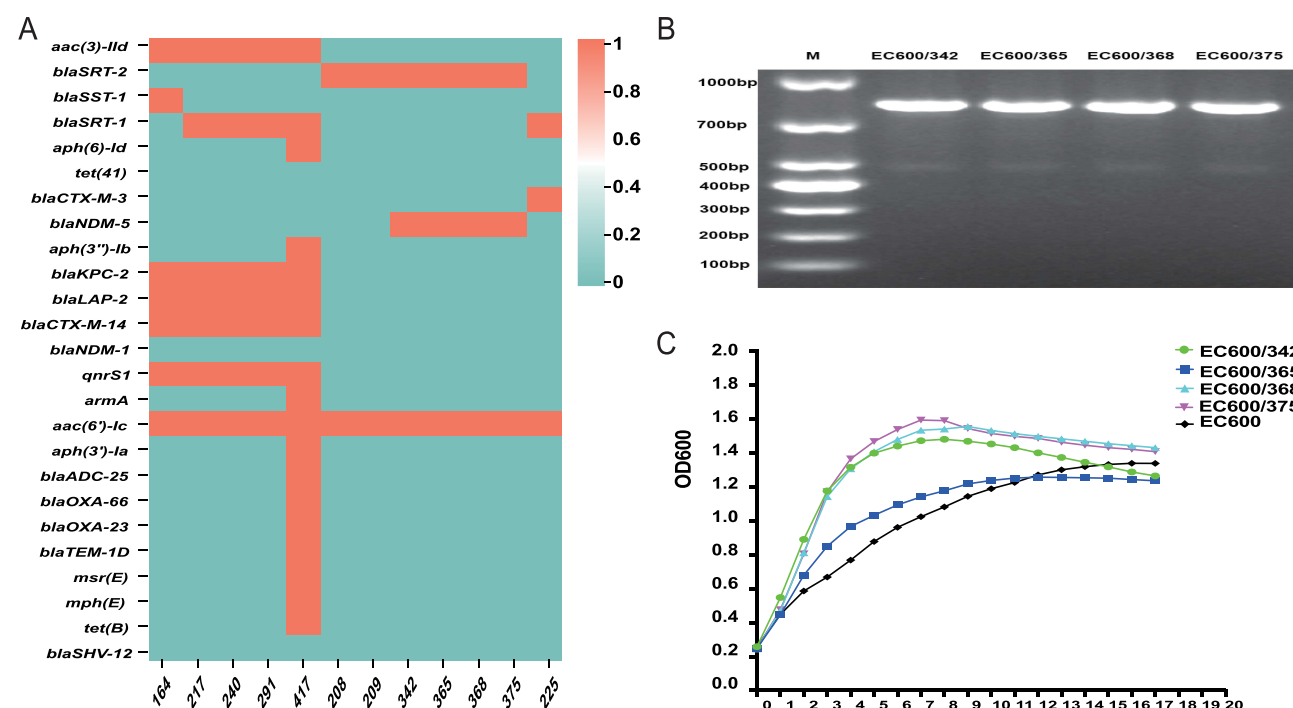


Figure 4 Genomic characterization of antimicrobial resistance, conjugative transfer, and physiological fitness of 12 CRSM isolates.

Notes: (A) Genomic characteristics of antimicrobial resistance genes in 12 CRSM isolates. (B) Confirmation of drug resistance genes in four conjugant strains. (C) Growth curves of four conjugants and the recipient strain EC600.

Table 4 Distribution of Virulence Factors in 12 CRSM Strains

Virulence Category	Virulence Factor	No. of Positive Strains (n=12)	Frequency (%)
Adherence	<i>E. coli</i> common pilus (ECP)	11	91.7
Adherence	Tap type IV pili (<i>Aeromonas</i>)	12	100.0
Adherence	Type I fimbriae (<i>Aeromonas</i>)	12	100.0
Adherence	Type IV pili biosynthesis (<i>Pseudomonas</i>)	3	25.0
Adherence	Type IV pili (<i>Yersinia</i>)	3	25.0
Iron uptake	Pyochelin receptor (<i>Pseudomonas</i>)	11	91.7
Iron uptake	Pyoverdine (<i>Pseudomonas</i>)	12	100.0
Regulation	RcsAB (<i>Klebsiella</i>)	11	91.7
Toxin	Alpha-hemolysin	11	91.7
Biofilm formation	PNAG (<i>Acinetobacter</i>)	11	91.7
Cell surface components	GPL locus (<i>Mycobacterium</i>)	11	91.7
Enzyme	Non-hemolytic phospholipase C (<i>Pseudomonas</i>)	11	91.7

Notes: Only virulence factors with a detection rate $\leq 91.7\%$ are presented. Virulence factors detected in all 12 isolates (100%): Hemorrhagic *E. coli* pilus (HCP), P fimbriae, Type I fimbriae, iron uptake systems (except pyochelin receptor and pyoverdine), PhoPQ regulation, secretion systems, capsule-related antiphagocytosis, efflux pumps, endotoxin, exoenzyme, fimbrial determinants, immune evasion factors, transport systems, motility, metabolism and stress adaptation factors.

A comprehensive analysis of virulence-associated genes across the 12 CRSM revealed a largely conserved core virulence arsenal with notable strain-specific variations. Genes encoding key adherence structures, including the Hemorrhagic *E. coli* pilus (HCP), P fimbriae, and Type I fimbriae, were ubiquitous (12/12 strains). Similarly, a complete suite of iron acquisition systems (eg, aerobactin, enterobactin, and heme uptake), secretion systems (T2SS, T4SS, T6SS, and flagella), and stress adaptation factors (catalases and superoxide dismutase) was present in all isolates, underscoring a robust pathogenic foundation.

Notable variations were observed in several virulence determinants. Genes associated with *Pseudomonas*-like Type IV pili biosynthesis and *Yersinia*-like Type IV pili were identified in only 3 of 12 strains (25.0%). The RcsAB regulatory system, commonly found in *Klebsiella* spp, was detected in 11 of 12 isolates (91.7%). The alpha-hemolysin gene (*hlyB*) and genes for the siderophores pyochelin were present in 11 strains (91.7%), while genes for the siderophore pyoverdine were present in 10 strains (83.3%) (Table 4).

No significant association was found between virulence gene profiles and sequence types (STs) or clinical outcomes. Given the limited sample size (n=12) and the near-ubiquitous presence of most core virulence factors across isolates, the ability to detect such associations was constrained; thus, these findings should be interpreted as descriptive rather than definitive.

Conjugation Experiments

The transferability of the *bla*_{NDM-5} gene was confirmed by conjugation assays from four strains (S342, S365, S368, and S375): EC600/342, EC600/365, EC600/368, and EC600/375. The results showed that *bla*_{NDM-5} could be successfully transferred to EC600. Conjugants carrying *bla*_{NDM-5} were confirmed by PCR and Sanger sequencing (Figure 4B). Susceptibility testing showed that conjugants were resistant to carbapenems (imipenem, meropenem), cephalosporins (cefepime, ceftriaxone, ceftazidime), and β -lactamase inhibitors (piperacillin-tazobactam), consistent with the resistance phenotype observed in the parental strains (Figure 1).

Adaptive Analysis of Conjugants

The growth kinetics of four *bla*_{NDM-5} conjugants (EC600/342, EC600/365, EC600/368, EC600/375) were compared to the recipient strain, EC600. Three conjugants (EC600/342, EC600/368, EC600/375) demonstrated a notable growth advantage. During the exponential phase (2–5 h), their growth rates surpassed that of EC600, reaching OD₆₀₀ values >1.3 at 4 h compared to 0.769 for the recipient. Their final stationary-phase densities (1.4–1.6 OD₆₀₀) were also higher than

that of EC600 (1.3–1.35 OD₆₀₀). In contrast, conjugant EC600/365 exhibited a marked growth defect, with delayed entry into exponential phase, a reduced growth rate, and a lower final biomass (1.17 OD₆₀₀), indicating strain-specific differences in growth performance among the conjugants (Figure 4C).

Discussion

This study provides a comprehensive genomic and phenotypic characterization of CRSM isolates from a hospital setting, revealing a complex epidemiology underpinned by clonal expansion and the high horizontal transfer potential of resistance genes. Our findings highlight the convergence of high-risk bacterial clones, carbapenemase dissemination, and specific patient demographics, painting a concerning picture for infection control.

The clinical epidemiology of our cohort, with a bimodal age distribution (neonates and adults) and concentration in high-dependency units (neonatology, critical care medicine), aligns with established risk factors for healthcare-associated *S. marcescens* infections.²⁶ The bimodal pattern observed in this study suggests that CRSM infection affects two distinct vulnerable populations: neonates, likely associated with perinatal transmission or neonatal intensive care unit exposure, and adults, potentially linked to prior antibiotic use or invasive procedures. The prevalence of *S. marcescens* in neonatal units has been widely reported. The neonatal departments of tertiary hospitals in countries such as Spain²⁷ and Singapore²⁸ are facing the dilemma of outbreaks of *S. marcescens* infections. However, reports on the outbreak of CRSM infections in the neonatal department are relatively scarce. A longitudinal detection report²⁹ on CRSM reported the transmission route of this bacterium and made arrangements for its prevention and control. Many studies on *S. marcescens* have confirmed that this bacterium is likely to infect immunocompromised populations.³⁰ As mentioned in the research of Zhu et al, the distribution of cases across diverse departments, particularly surgical wards and the ICU, underscores the nosocomial nature of *S. marcescens* and its ability to affect a wide range of patient populations.⁴ The predominance of respiratory tract infections further underscores the pathogen's role in ventilator-associated or nosocomial pneumonia. A hospital in Dublin also reported sputum as the most common isolation source of *S. marcescens* in their epidemiological investigation,²³ consistent with the predominance of respiratory tract infections observed in our cohort. The high rate of prior broad-spectrum antibiotic exposure, particularly to β -lactam/inhibitor combinations and carbapenems, creates a potent selective pressure that likely facilitated the emergence and dominance of the CRSM clones described herein.

Our genomic analysis definitively resolves the population structure. While PFGE suggested recent clonal transmission within specific strain pairs (eg, S342/S368), MLST revealed a more nuanced landscape. This clonal transmission pattern resembles the dissemination dynamics of highly prevalent clones like ST258 observed in carbapenem-resistant *Klebsiella pneumoniae* (CRKP),³¹ indicating that certain CRSM clonal lineages with successful adaptive traits also constitute significant sources of hospital infections.

The core-genome SNP phylogeny established four distinct, evolutionarily differentiated clades, including three multi-strain clades (Clade I, Clade II, Clade III) and one single-strain clade (strain S365). Clade I, predominantly comprising ST595 strains (with the exception of ST525 strain S417 and ST490 strain S291) with short branch lengths, is strongly indicative of a successful clonal expansion event during the study period within the hospital, likely explaining a subset of epidemiologically linked cases. Globally, CRSM demonstrates significant clonal diversity and heterogeneity in resistance mechanisms, driven by the independent acquisition of carbapenemase genes among multiple sequence types (STs), facilitating their nosocomial spread. Although ST595 has not been widely reported as a dominant international lineage, ST595 has been identified as the dominant epidemic clone in this study. These lineages exhibited multifactorial resistomes with universal carriage of *bla*_{KPC-2} and additional resistance genes, as well as the lineage-enriched virulence trait pleN in ST595.³² However, no statistically significant association was found between the overall virulence gene profiles and STs or clinical outcomes, likely due to the limited sample size and near-ubiquitous core virulence factors across isolates. In contrast, the genetic characteristics varied among the other three clades: Clade II (ST490 strains S208, S375, S209) and Clade III (ST490 strains S368, S342) showed moderate branch lengths, indicating relatively recent clonal expansion, while the single-strain clade (ST490 strain S365) had the longest branch, reflecting the greatest genetic distance from other strains. Collectively, Clade II, Clade III, and the single-strain clade (all belonging to ST490) exhibit higher overall genetic diversity and longer average branch lengths compared to Clade I, suggesting either multiple

independent introductions or a longer history of endemic circulation with subsequent diversification. This pattern—where recent outbreak lineages coexist with more diverse, established reservoirs (Clade II, Clade III, and S365 clade)—is a hallmark of challenging multidrug-resistant pathogens in the hospital ecosystem.³³

The discordance between the clear clonal framework defined by SNP analysis (four distinct clades) and the relative uniformity of MLST-derived STs (predominantly ST490 and ST595) is particularly instructive. Specifically, SNP analysis differentiated three distinct clades within ST490 (Clade II, Clade III, S365 clade) and identified two exceptional strains (S417, ST525; S291, ST490) within the predominantly ST595 Clade I, while MLST failed to distinguish these fine-scale evolutionary differences. Specifically, the clustering of ST490 strain S291 and ST525 strain S417 into the ST595-dominant clade suggests that these strains may have originated from the ST595 lineage, with subsequent mutations or recombination events in the MLST housekeeping genes leading to new ST assignments, while the majority of their genomic background remains consistent with ST595. It demonstrates that local microevolution (eg, point mutations or recombination at MLST loci) occurs actively within successful high-risk clones during their spread. This underscores a critical limitation of MLST alone for outbreak investigation in rapidly evolving species and affirms the necessity of WGS for accurate transmission tracing.³⁴

The clone-specific resistance gene profiles elucidate the drivers of carbapenem resistance. The stark dichotomy between the *bla*_{KPC-2}-carrying ST595 clone and the *bla*_{NDM-5}-harboring ST490 clone demonstrates two independent pathways to resistance dominance. The high resistance to β -lactams (50.0–100.0%) and complete susceptibility to amikacin (0/12, 0%) observed in this study are consistent with previous reports of CRSM from China, where amikacin has been shown to retain moderate activity against multidrug-resistant *S. marcescens* isolates.⁴ In China, *bla*_{KPC} is the predominant carbapenemase among CRSM, followed by *bla*_{NDM},³⁵ which aligns with our finding that 5 out of 9 carbapenemase-positive isolates harbored *bla*_{KPC}. Notably, no tigecycline-resistant isolates were detected in this study, which was lower than that reported in some prior studies from tertiary hospitals in China,³² which may reflect institutional differences in antibiotic usage or strain background. The successful in vitro conjugation of the *bla*_{NDM-5} gene from four donor strains demonstrates its high horizontal transfer potential, and this mobile genetic element poses a potential direct threat of further dissemination to other *Enterobacteriaceae* within the gut microbiome of patients. One study was the first to isolate both *E. coli* and *C. freundii* strains carrying *bla*_{NDM-5} from one single patient, indicating the possibility of *bla*_{NDM-5} transmission among diverse species.³⁶ Some studies reported several sporadic clinical infection cases related to *E. coli* ST167 carrying *bla*_{NDM-5} in various regions of China,^{37,38} two of them have shown that the horizontal transfer of the *bla*_{NDM-5} gene often occurs through plasmids.

Notably, the fact that most conjugants (EC600/342, EC600/368, EC600/375) exhibited no fitness cost but rather a growth advantage is alarming. This suggests that the acquisition of this resistance plasmid can be selectively neutral or even beneficial under certain conditions, contrary to the conventional view that acquiring mobile genetic elements often impairs host fitness³⁹ and potentially explaining its stable maintenance and spread. The pronounced growth defect exhibited by one transconjugant (EC600/365), however, confirms that the adaptive impact is highly strain-specific. This variability underscores that the fitness outcome is determined by complex interactions following horizontal gene transfer, as detailed in studies of compensatory evolution;⁴⁰ the ultimate fate of a newly acquired genetic element depends on the specific genetic backgrounds of the donor and recipient. The observed growth advantages in most transconjugants may reflect a well-adapted, compatible relationship between the acquired DNA and the host, which would enhance the element's persistence and dissemination within the microbial community.

The integration of phenotypic and genomic data robustly explains the observed multidrug resistance and delineates distinct evolutionary strategies among the dominant clones. The high resistance rates to broad-spectrum β -lactams, particularly carbapenems, are directly attributed to the clonal carriage of *bla*_{KPC-2} (ST595) or *bla*_{NDM-5} (ST490). The concordance between in vitro susceptibility (eg, to amikacin, tigecycline) and the absence of corresponding high-level resistance genes underscores the predictive value of genomics. Furthermore, the discovery of complex, strain-specific resistance gene arrays within established clones, such as in ST595 isolate S417, indicates ongoing horizontal gene transfer. This illustrates a dual threat: the expansion of successful clones is compounded by the continual remodeling of their resistomes, challenging long-term infection control.

The universal conservation of a core arsenal for adherence, iron acquisition, and secretion confirms a formidable innate pathogenicity in *S. marcescens*. The identified strain-level variations in accessory factors (eg, specific pilus types) likely contribute to niche adaptation but were not exclusive to the high-risk clones ST595 or ST490. Several virulence genes identified in this study are typically associated with other bacterial genera. For instance, genes encoding the pyochelin and pyoverdine siderophore systems, originally described in *Pseudomonas aeruginosa*,⁴¹ were present in 11 of 12 isolates (91.7%) and 10 of 12 isolates (83.3%). Respectively, the *rcsA* regulatory gene, commonly found in *Klebsiella spp.*,⁴² was also detected in 11 of 12 isolates. This cross-genus distribution suggests that horizontal gene transfer (HGT) may have contributed to the acquisition of these virulence determinants in *S. marcescens*. Previous studies have demonstrated that *S. marcescens* can acquire genetic material from other genera via conjugative plasmids and outer membrane vesicles,⁴³ and that mobile genetic elements such as transposons can facilitate the dissemination of regulatory systems across species boundaries.⁴⁴ The presence of such cross-genus virulence genes may enhance the pathogenic adaptability of CRSM, although the functional consequences warrant further investigation. While *plcN* was enriched in the ST595 lineage, no significant association was found between the overall virulence gene profiles and sequence types (STs) or clinical outcomes. Given the limited sample size (n=12) and the near-ubiquitous presence of most core virulence factors across isolates, the ability to detect such associations was constrained; thus, these findings should be interpreted as descriptive rather than definitive. This suggests that the pronounced hospital spread of these clones is primarily fueled by their efficient resistance gene carriage and transmissibility, rather than by the acquisition of unique, clone-defining virulence determinants.

Limitations

Several limitations of this study should be acknowledged. The sample size was small (n=12), which limited statistical power to detect significant associations between clinical parameters, virulence gene profiles, and clinical outcomes. Moreover, the near-ubiquitous distribution of core virulence factors across isolates further constrained the ability to identify meaningful correlations involving accessory virulence determinants, sequence types, or clinical outcomes. Accordingly, the exploratory findings should be interpreted with caution. As a single-center study, the findings may not be directly generalizable to other healthcare settings with distinct patient populations, antibiotic prescribing practices, or endemic resistance patterns. Clinical data were collected retrospectively, and certain laboratory parameters (eg, PCT, hs-CRP) were incomplete for a subset of patients, potentially introducing information bias. While multiple molecular typing methods (PFGE, MLST, SNP phylogeny) were applied, plasmid characteristics—including replicon typing, conjugation efficiency, and plasmid stability—were not comprehensively characterized, leaving the mechanisms underlying *bla*_{NDM-5} dissemination incompletely defined. Virulence gene detection relied solely on genomic presence without functional validation (eg, biofilm formation assays, hemolysis tests, or in vivo pathogenicity models); therefore, the potential impact of horizontal gene transfer on virulence remains speculative. Although growth differences were observed among transconjugants, these were not further investigated at the molecular level, and their clinical relevance remains unclear. Despite the study period spanning several years, formal temporal trend analyses (eg, changes in strain distribution, resistance mechanisms, or clonal dynamics over time) were not conducted, owing to the limited number of isolates per year and the retrospective study design. Notwithstanding these limitations, this study provides valuable insights into the molecular epidemiology of CRSM within a high-risk hospital setting and underscores the importance of ongoing surveillance and infection control measures.

Conclusion

This single-center, small-sample study systematically characterizes the clinical epidemiological features, antimicrobial resistance (AMR) profiles, and molecular evolutionary mechanisms of CRSM in our hospital. Clinically, CRSM infections exhibited a bimodal age distribution (with neonates and adults as high-risk populations), predominantly affecting the respiratory tract. Most patients had prior antimicrobial exposure history and prolonged therapeutic courses. Antimicrobial susceptibility results showed high resistance to β -lactams (including carbapenems), while no amikacin-resistant strains were detected—providing direct evidence for rational clinical antimicrobial use.

Molecular analysis confirmed that CRSM in the hospital was predominantly dominated by two co-circulating dominant clonal lineages: ST595 (uniformly harboring *bla_{KPC-2}*) and ST490 (4 of 6 strains carrying *bla_{NDM-5}*). The clonal expansion of ST595 during the study period indicates an elevated risk of clustered infections, whereas ST490 has persisted long-term with strain diversification. The *bla_{NDM-5}* gene was successfully transferred horizontally via conjugative plasmids in vitro, imposing no apparent fitness cost and even conferring growth advantages in most transconjugants, which may be an important mechanism for the dissemination of this resistance gene in clinical settings. All isolates harbored core virulence genes, and the prevalence of dominant clones was primarily attributed to their AMR traits rather than acquisition of unique virulence factors.

In summary, CRSM dissemination is jointly driven by exposure of clinically high-risk populations, antimicrobial selection pressure, and horizontal transfer of resistance genes. Hospital infection control should focus on high-risk groups and respiratory tract infection prevention, optimize antimicrobial stewardship (AMS, preferentially selecting sensitive agents such as amikacin), and incorporate WGS-based surveillance for monitoring clonal transmission and resistance gene dynamics. While the conclusions should not be overgeneralized, this study offers actionable insights for targeted infection control and antimicrobial management in our institution, as well as regional reference data for analogous healthcare settings globally.

Statement Covering Patient Data Confidentiality

To protect patients' personal information and maintain the security of General Hospital of Ningxia Medical University's patient information, we are committed to fulfilling our obligation to keep patients' personal information confidential.

Data Sharing Statement

The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors: Wei Jia (13519299090@126.com) and Jia Tao (taojia880625@163.com).

Ethics Statement

In accordance with the Declaration of Helsinki, this retrospective study was permitted by the Ethics Committee of the General hospital of Ningxia medical university (Code KYLL-2025-2239), and the requirement to obtain informed written consent was waived.

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Disclosure

All authors declare no conflicts of interest related to this work.

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