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Microbiological Studies on the Mechanisms of Beta-Lactam Resistance and Nosocomial Spread of Clinical Isolates of *Serratia marcescens*

Abstract

Of a dissertation for the award of the educational and scientific degree "Doctor"

Scientific supervisor:

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The dissertation consists of 165 pages and is illustrated with 32 figures and 10 tables. A total of 364 bibliographic references are cited, including 3 in Cyrillic and 361 in Latin script.

The dissertation was discussed at a meeting of the Departmental Council of the Department of Microbiology and Virology, Medical University – Varna, and was approved for public defence before the following Scientific Jury:

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The experimental work was conducted at the Department of Microbiology and Virology, Medical University – Varna, and at the University Hospital "St. Marina" – Varna.

The public defence of the dissertation will take place on …….. at …… in ……

The materials related to the dissertation defence are available for consultation at the Library of the Medical University – Varna.

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List of Abbreviations

AFLP – Amplified Fragment Length Polymorphism

AK – Amikacin

AmpC – Ambler class C β-lactamases

CAZ – Ceftazidime

CIP – Ciprofloxacin

CRO – Ceftriaxone

CTX-M – Cefotaximase-Munich

DDST – Double Disk Synergy Test

DHFR – Dihydrofolate Reductase

DHPS – Dihydropteroate Synthase

ECDC – European Centre for Disease Prevention and Control

ERIC-PCR – Enterobacterial Repetitive Intergenic Consensus – Polymerase Chain Reaction

ESBLs – Extended-Spectrum Beta-Lactamases

ESKAPE – Group of multidrug-resistant nosocomial pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.

EUCAST – European Committee on Antimicrobial Susceptibility Testing

FEP – Cefepime

GM – Gentamicin

HAI – Healthcare-Associated Infection

ICU – Intensive Care Unit

IMP – Imipenemase

IPM – Imipenem

IQR – Interquartile Range

KPC – *Klebsiella pneumoniae* Carbapenemase

LVX – Levofloxacin

MBL – Metallo-β-lactamase

MDR – Multidrug Resistance

MEM – Meropenem

MIC – Minimum Inhibitory Concentration

MLST – Multilocus Sequence Typing

NCBI – National Center for Biotechnology Information

NCIPD – National Center of Infectious and Parasitic Diseases (Bulgaria)

NDM – New Delhi Metallo-β-lactamase

PBP – Penicillin-Binding Proteins

PCR – Polymerase Chain Reaction

PFGE – Pulsed-Field Gel Electrophoresis

RAPD-PCR – Random Amplification of Polymorphic DNA – PCR

SME – *Serratia marcescens* Enzyme

SXT – Trimethoprim/Sulfamethoxazole

TZP – Piperacillin/Tazobactam

VIM – Verona Integron-Encoded Metallo-β-Lactamase

WGS – Whole Genome Sequencing

1. Introduction

Over the years, the etiological spectrum of healthcare-associated infections has shifted, with a growing incidence of infections caused by opportunistic Gram-negative bacteria. Among them, *Serratia marcescens* stands out as a significant nosocomial pathogen. Once considered a harmless saprophyte, it is now known to rarely cause community-acquired infections but frequently leads to hospital outbreaks, especially in immunocompromised patients and those undergoing prolonged hospital stays or invasive procedures. *S. marcescens* is well-adapted to hospital environments and has been implicated in over 100 documented outbreaks between 1968 and 2019 (Tavares-Carreon, 2023), most commonly causing urinary, respiratory, and wound infections, often progressing to sepsis. It consistently ranks among the top ten bacterial species isolated in HAIs (ECDC, 2024).

Numerous reports in the scientific literature describe outbreaks caused by *S. marcescens*—more than 100 such events have been documented between 1968 and 2019 (Tavares-Carreón F, 2023). The most frequently reported clinical manifestations are urinary tract infections, respiratory tract infections, and wound infections, while dissemination of the inflammatory process may present clinically as sepsis. The highest-risk groups for developing *S. marcescens*-associated infections are patients undergoing prolonged antibiotic therapy and extended hospitalisation, immunocompromised individuals, and those subjected to invasive diagnostic and/or therapeutic procedures (Tóth A., 2020). Contemporary aetiological profiles of the most common bacterial pathogens causing life-threatening healthcare-associated infections consistently place *Serratia* spp. among the ten most frequently isolated species (ECDC, 2024).

Equally alarming are data indicating the global rise of antimicrobial resistance, regarded as one of the greatest threats to public health and development. It has been estimated that antimicrobial resistance directly caused 1.27 million deaths worldwide in 2019 and contributed indirectly to an additional 4.95 million deaths (Antimicrobial Resistance Collaborators, 2022). Bulgaria is among the European countries with the highest antibiotic consumption and has shown a clear upward trend during the period 2019–2023, according to the latest ECDC data (ECDC, 2024). β-lactam agents (including higher-generation cephalosporins and carbapenems) are among the most widely used antibiotics in clinical practice in Bulgaria. The emergence of resistance to these agents, as a logical consequence of their irrational use, has significantly reduced their clinical

effectiveness. The intrinsic resistance of *S. marcescens* to strategically important antibiotics, along with the acquisition of novel resistance determinants, therefore represents a potentially serious threat to public health.

Given its widespread distribution and diverse mechanisms for acquiring antimicrobial resistance, *S. marcescens* is of substantial clinical importance. The high incidence of hospital-acquired infections, particularly among high-risk patient groups—immunocompromised individuals, neonates, children, and patients admitted to intensive care units—emphasises the need for rapid and accurate microbiological identification, along with the timely initiation of appropriate antimicrobial therapy guided by local resistance patterns. This underlines the importance of establishing comprehensive datasets on *S. marcescens* susceptibility profiles, detailed investigation of the specific resistance mechanisms involved, and an in-depth analysis of the hospital epidemiology of these infections.

2. Aim and Objectives of the Dissertation

The issues outlined in the literature review defined the following aim of the present study: **To investigate the mechanisms of resistance to β-lactam antibiotics and to analyse the principal aspects of the nosocomial dissemination of clinical *Serratia marcescens* isolates.**

In relation to this aim, the following objectives were set:

1. To investigate the epidemiology of *S. marcescens*-associated infections by: determining their isolation frequency and temporal trends; analysing the association between isolation frequency and colistin consumption; assessing the distribution of isolates according to the type of clinical specimens; characterising the leading clinical syndromes at hospital admission; identifying risk factors for the development of *S. marcescens*-associated infections and evaluating their impact on 30-day mortality.
2. To analyse the dynamics of antimicrobial resistance among 488 clinical *S. marcescens* isolates during the study period, and to evaluate the association between resistance and 30-day mortality, as well as the impact of different therapeutic approaches on clinical outcome.
3. To determine and analyse the in vitro susceptibility of a collection of 200 clinical *S. marcescens* isolates to a panel of antibacterial agents.
4. To investigate, using phenotypic and molecular-genetic methods, the dissemination of genes encoding extended-spectrum β-lactamases among the collected *S. marcescens* isolates.
5. To assess the effectiveness of RAPD and ERIC-PCR as methods for the epidemiological typing of the collected *S. marcescens* isolates.

3. Materials and Methods

Study Design

The study employed a retrospective design, analysing data related to 10 variables in all patients with clinically manifested *Serratia marcescens*-associated infections treated at the University Multiprofile Hospital for Active Treatment St. Marina University Hospital – Varna during the period 2016–2023. The principal inclusion criterion was the presence of at least one episode of *S. marcescens*-associated infection within the study period. Repeated isolates from the same patient were excluded from the analysis.

Over the entire study period, a collection of 200 non-duplicate clinical *S. marcescens* isolates was selected from 488 different patients. These isolates represented diverse resistance profiles to β-lactam antibiotics and originated from various types of clinical specimens (urine, blood, wound secretions, sputum, and others).

For the purposes of the epidemiological investigation, the medical records of 488 patients hospitalised at St. Marina University Hospital – Varna, between 2016 and 2023, were analysed in detail. The study aimed to examine the epidemiology of *S. marcescens*-associated infections by: determining their isolation frequency and temporal trends; analysing the association between *S. marcescens* isolation and colistin consumption; assessing the distribution of isolates according to the type of clinical specimens; characterising the major clinical syndromes at hospital admission; and identifying the risk factors for *S. marcescens*-associated infections and evaluating their impact on 30-day mortality.

The variables for which data were collected included:

1. Sex
2. Age
3. Duration of current hospitalisation
4. History of prior hospitalisation
5. Surgical treatment
6. Stay in an intensive care unit
7. Type of underlying disease
8. Presence and type of comorbidities
9. Type of antimicrobial therapy administered
10. Clinical outcome

To explore the correlation between colistin exposure and the frequency of *Serratia*-associated infections, data were analysed on the relative frequency of *S. marcescens* isolation (expressed as a percentage of all bacterial isolates recovered from hospitalised patients) and colistin consumption, presented as Defined Daily Doses (DDD), for the period 2016–2023. This parameter was used as an approximate indicator of antibiotic exposure in the hospital setting.

The study was approved by the Ethics Committee for Research with protocol No. 115/31.03.2022.

3.2. Microbiological Methods

Clinical isolates of *Serratia marcescens*

For the purposes of microbiological analysis, a collection of 200 clinical isolates of *S. marcescens* obtained from 200 different patients hospitalised St. Marina University Hospital – Varna during the period 2016–2023 was examined. The study included all clinically significant *S. marcescens* isolates recovered from patients, regardless of their age, sex, diagnosis, or hospital ward. Only one isolate per patient was analysed in order to avoid duplication. All included strains were recovered after 48 hours of hospitalisation, in the absence of evidence of prior *S. marcescens*-associated infection, and were classified as nosocomial.

3.2.1. Culture media and reagents

3.2.2. Species identification methods for *S. marcescens*

- Identification by automated system Phoenix 100 (BD)
- Identification by automated system Vitek 2 (bioMérieux, France)
- Identification by MALDI Biotyper Sirius mass spectrometry system (Bruker, Germany)

3.2.3. Methods for antimicrobial susceptibility testing of *Serratia marcescens*

- Disk diffusion method according to Bauer–Kirby
- Determination of minimum inhibitory concentration (MIC) of colistin by broth microdilution

3.2.4. Methods for β-lactamase detection

3.2.4.1. Phenotypic methods

- Double disk synergy test (DDST, Jarlier method) for ESBL detection (Jarlier V., 1988)
- Modified Hodge test (MHT) for carbapenemase detection (CLSI, 2010)
- Immunochromatographic test RESIST-3 O.K.N. (Coris BioConcept, Belgium)

3.2.4.2. Molecular-genetic methods

- Polymerase chain reaction (PCR) for β-lactamase genes
- Real-time PCR MM GRAM NEG RES (Alifax S.r.l., Italy)

3.3. Methods for epidemiological typing of *Serratia marcescens*

- ERIC PCR
- RAPD PCR

3.4 Statistical methods

- Descriptive
- Hypothesis testing

4. Results and Discussion

4.1. Epidemiology of *Serratia marcescens*-associated infections

The epidemiology of infectious diseases is shaped by a variety of factors, including the characteristics of the patient population, the hospital environment, and the quality of healthcare delivery. In the present retrospective study, the medical records of 488 patients with microbiologically confirmed *S. marcescens* infection, hospitalised at "St. Marina" University Hospital – Varna between June 2016 and January 2023, were analysed, along with systematised data from the Laboratory of Microbiology and Virology.

The main characteristics of patients with confirmed *S. marcescens*-associated infections are presented in Table 1.

Table 1. Main characteristics of the studied patients

Variables	**Total n = 488 (100%)**	**Deceased n = 51 (10.5%)**	**Favorable Outcome n = 437 (89.5%)**	**P**
	488 (100%)	51 (10.5%)	437 (89.5%)	

Age, median (IQR)	66 (49-75)	74 (60 – 77)	65 (48-74)	0.012
Previous Hospitalization				
No	241 (49.3%)	26 (5.3%)	215 (44.1%)	0.73
Yes	247 (50.6%)	23 (4.7%)	224 (45.9%)	
Type of Hospital Ward				
Internal Medicine Clinics	188 (38.5%)	10 (2.1%)	178 (36.5%)	**0.002**
Intensive Care Units	90 (18.4%)	30 (6.14%)	60 (12.3%)	
Surgical Clinics	161 (32.9%)	8 (1.6%)	153 (31.3%)	
Pediatric Clinics	24 (4.9%)	0	24 (4.9%)	
Other (ENT, Psychiatry, Infectious Diseases, Dermatology, Day Hospital, Addiction Medicine)	25 (5.1%)	3 (0.6%)	22 (4.5%)	
Main Clinical Syndrome				
Respiratory	141 (28.9%)	15 (3.1%)	126 (25.9%)	

Cardiovascular	19 (3.9%)	3 (0.6%)	16 (3.3%)	**0.015**
Gastrointestinal	51 (10.5%)	5 (1%)	46 (9.4%)	
Urological	43 (8.8%)	10 (2.1%)	33 (6.8%)	
Toxic-infectious	193 (39.5%)	19 (3.9%)	174 (35.7%)	
Central Nervous System	52 (10.7%)	16 (3.3%)	36 (7.4%)	
Skin and Soft Tissue	103 (21.1%)	9 (1.9%)	94 (19.3%)	
Comorbidities				
Cardiovascular	321 (65.8%)	31 (6.3%)	290 (59.4%)	0.13
Respiratory	48 (9.8%)	6 (1.2%)	42 (8.6%)	
Urological	68 (13.9%)	8 (1.6%)	60 (12.3%)	
Endocrinological	154 (31.6%)	12 (2.5%)	142 (29.1%)	
Gastrointestinal	63 (12.9%)	14 (2.9%)	49 (10%)	
Central Nervous System	60 (12.3%)	11 (2.3%)	49 (10%)	
Other	45 (9.2%)	6 (1.2%)	39 (8%)	
Infection Acquisition setting				
Community-acquired	6 (1.2%)	1 (0.2%)	5 (1%)	0.485
healthcare-associated (nosocomial)	482 (98.7%)	50 (10.3%)	433 (88.7%)	

Type of Treatment				
Surgical treatment	152 (31.1%)	5 (1%)	147 (30.1%)	**0.003**
Treatment in Intensive Care Unit	62 (12.7%)	25 (5.1%)	37 (7.6%)	
Surgical and ICU treatment	34 (6.9%)	8 (1.6%)	26 (5.3%)	
Conservative treatment	239 (48.9%)	12 (2.5%)	237 (48.6%)	

4.1.1 Isolation Frequency

The frequency of *S. marcescens*-associated infections during the period 2016–2023 at St. Marina University Hospital, Varna is presented in Figure 1.

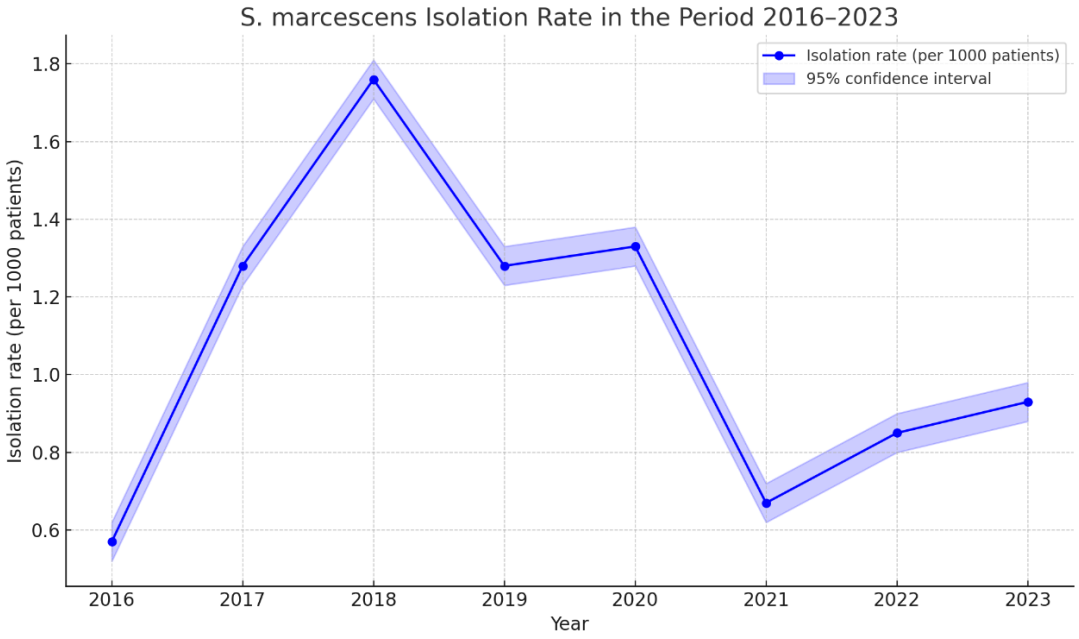


Figure 1. Isolation frequency of *S. marcescens*, 2016–2023, n (%)

The analysis of the relative frequency of *S. marcescens* isolation per 1,000 hospitalised patients demonstrated distinct trends over the study period. From 0.58 in 2016, the frequency increased markedly in 2017 to 1.27 per 1,000 patients, reaching a

peak value of 1.76 per 1,000 patients in 2018. During 2019–2021, a substantial decline was observed – to 1.28 (2019) and 0.67 (2021). In contrast, the years 2022–2023 showed a moderate increase – to 0.85 (2022) and 0.93 (2023) per 1,000 patients.

Discussion

Between 2019 and 2021, the frequency of *S. marcescens* isolation decreased significantly, which may be explained by the restrictions imposed during the COVID-19 pandemic, including a reduction in elective hospitalisations and restricted visitor access. In 2022–2023, a moderate rise was recorded (0.85 in 2022 and 0.93 in 2023 per 1,000 patients), coinciding with the restoration of normal hospital activities and the increased circulation of *S. marcescens* within hospital facilities.

It is noteworthy that the shaded area around the line in the graphical representation of isolation frequency corresponds to the 95% confidence interval. The narrow width of the interval at peak values indicates a high degree of precision of the estimates.

Analysis of the dynamics of *Serratia* spp. isolation in UMHAT "St. Marina" – Varna (2016–2023) revealed that in 2017, *Serratia* spp. ranked 11th among all isolates (1.6%), while in 2018 it rose to 9th place (2.1%). In the same year, it reached 5th place among all blood isolates – the highest ranking during the study period. In subsequent years, the frequency gradually declined, falling to 1.5% in 2019 and 1.4% in 2020. In 2023, it was 1.3% and 11th place among all isolates, with persistent presence in blood and urine (12th and 11th positions, respectively) (unpublished data, Hospital Journal 2016–2023). Within the aetiological structure of bacteraemias, not only during the study period but also over a ten-year window (2011–2021), *S. marcescens* was consistently among the ten most common causative agents (Stoeva T., 2023).

According to ECDC data, the overall incidence of healthcare-associated infections in Europe varied significantly during the same period. Similar to our findings, there was a temporary decline associated with pandemic-related measures, followed by a sharp increase upon the resumption of routine hospital activities (ECDC, 2024).

Conclusion

The frequency of *S. marcescens* isolation varied considerably during the study period, peaking in 2018, followed by a pronounced decrease in 2021 coinciding with reduced elective hospitalisations during the COVID-19 pandemic, and a moderate increase in 2022–2023, in parallel with the restoration of regular hospital activity.

4.1.2. Association between the frequency of *Serratia marcescens* isolation and colistin consumption

Given the intrinsic resistance of bacteria of the genus *Serratia* to polymyxins, we analysed the potential association between the frequency of *S. marcescens* isolation and colistin consumption, expressed in defined daily doses (DDD), over the study period (Figure 2).

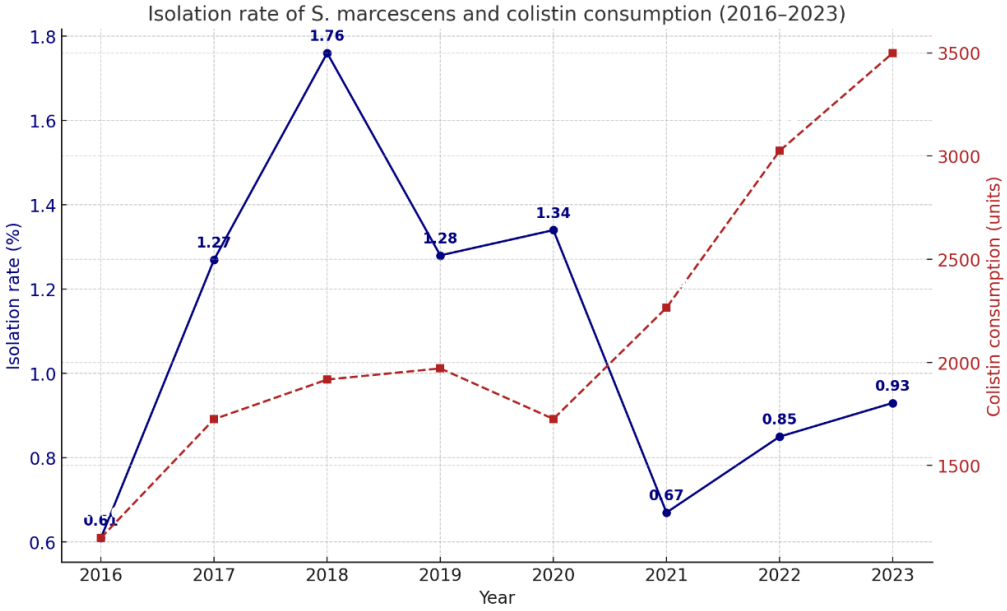


Figure 2. Frequency of *Serratia marcescens* isolation and colistin consumption (DDD), 2016–2023

The Pearson correlation coefficient between colistin consumption and the frequency of *S. marcescens* isolation was –0.16 (p = 0.697), indicating a weak and statistically non-significant negative association. Spearman's correlation coefficient was –0.07 (p = 0.867), further confirming the absence of a statistically significant non-linear relationship between the two variables.

Lag regression analysis was conducted with 1-, 2-, and 3-year shifts in colistin consumption, but none of these lagged variables demonstrated a statistically significant impact on the frequency of *S. marcescens* isolation. The strongest (yet still non-significant) negative associations were observed with 2- and 3-year lags (r = –0.73, p = 0.096 and r = –0.69, p = 0.190, respectively). The overall explanatory power of the model (R-squared) was 0.497, indicating that the model failed to adequately explain the observed variation in isolation frequency.

Overall, the analysis did not identify a statistically significant relationship between colistin consumption, expressed as DDD, and the frequency of *S. marcescens* isolation. Nevertheless, a distinct concordant trend between the two variables was evident in the last three years of the study period (2021–2023).

Discussion

At St. Marina University Hospital – Varna colistin has been used since 2016 for the treatment of severe, life-threatening infections caused by MDR Gram-negative bacteria. Until 2020, colistin use remained infrequent, with minor annual variations, but after 2021 its use increased by more than 100%, persisting at elevated levels through the end of the study period in 2023. We consider that this necessitates continued monitoring, as the analysed time window may be insufficient to capture potential dependencies. More robust conclusions will require studies with a longer observation period, inclusion of other antimicrobial agents, and analysis of additional infection control-related factors.

In human medicine, colistin use was initially restricted to topical applications due to its nephrotoxic and neurotoxic effects when administered systemically (European Medicines Agency, 2016). In veterinary practice, it remains widely used for treating *Enterobacterales* infections in livestock, where increasing rates of resistant isolates have been reported in treated versus untreated animals (Burow E., 2019). In clinical settings, colistin is currently regarded as a last-resort drug for infections caused by MDR pathogens, including *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and carbapenemase-producing *Enterobacterales*. Both the World Health Organization (WHO) and government agencies such as Health Canada have reclassified colistin as an "antimicrobial of highest priority" for human medicine (WHO, 2018; CIPARS, 2014).

At present, particular concern arises from plasmid-mediated transmissible resistance to colistin (primarily associated with *mcr* genes), since unlike chromosomally mediated resistance, these determinants can spread readily across different *Enterobacterales* species, predominantly in livestock settings. It may be assumed that with ongoing efforts to reduce colistin consumption in veterinary medicine under the One Health approach, the prevalence of *mcr*-carrying plasmids will gradually decrease to a low but stable level. In the absence of colistin as a selective pressure, bacteria are expected to eliminate these plasmids due to the energy cost of their replication. Conversely, chromosomally mediated colistin resistance has been increasingly observed in clinical

Enterobacterales isolates (including *Serratia* spp.), particularly in human medicine where colistin is increasingly used as a last-resort therapy for MDR pathogens.

In addition to the dramatic global rise of acquired colistin resistance among certain bacterial species—especially within the ESKAPE group—numerous studies highlight that selective pressure from colistin use poses a serious risk of increasing infections caused by bacteria intrinsically resistant to colistin, such as *S. marcescens*, *Morganella morganii*, *Proteus mirabilis*, and *Burkholderia* spp. (Poirel L., 2017; Olaitan AO, 2014; Carroll LM, 2019). At present, however, there is no systematic monitoring of these processes.

Conclusion

This study did not identify a statistically significant association between colistin consumption, expressed in DDD, and the frequency of *S. marcescens* isolation during the period 2016–2023. Despite the absence of a confirmed correlation, the parallel upward trend observed in both variables during the last three years of the study (2021–2023) suggests a potential relationship that warrants continued monitoring and further investigation over a longer time frame.

4.1.3. Distribution of *Serratia marcescens* isolates by type of clinical specimen

In the present study, the highest proportion of *S. marcescens* isolates was recovered from urine samples – 30.5% (n = 149), followed by wound secretions – 26.8% (n = 131) and respiratory secretions – 25.8% (n = 126). Blood isolates accounted for 10.0% (n = 45), while specimens from other clinical materials (skin secretions, ocular secretions, punctates, faeces) represented 6.8% (Figure 3).

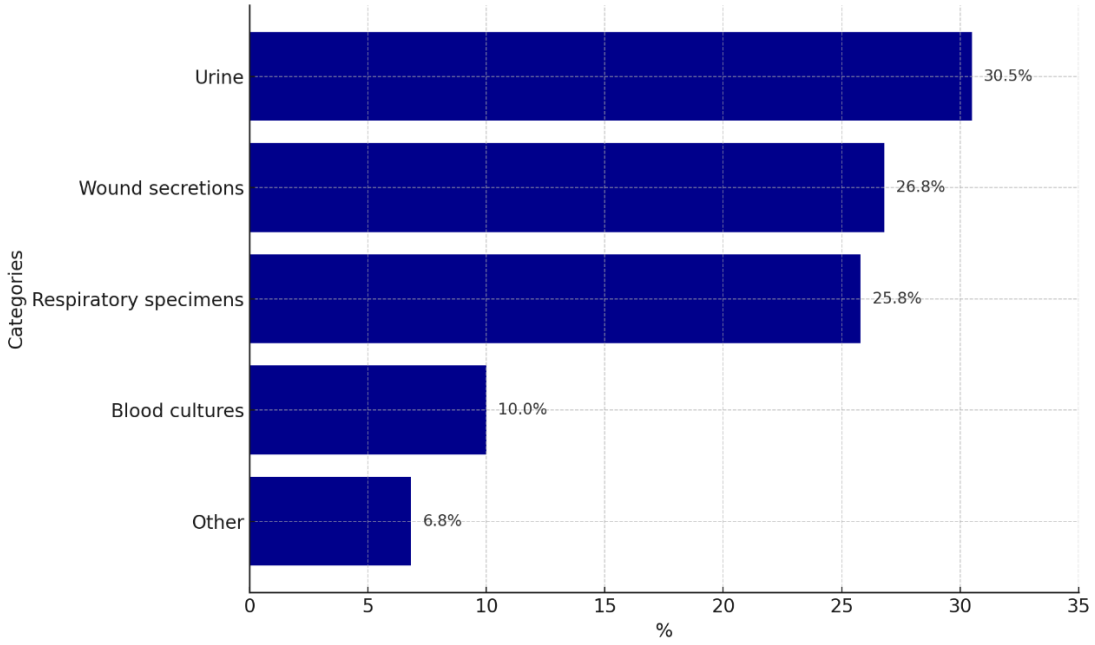


Figure 3. Distribution of *S. marcescens* by anatomical site of isolation (in %); (others – skin swabs, ocular, fecal, puncture fluids)

Analysis of the relative proportion in relation to all positive samples of the respective specimen type demonstrated that the highest frequency of *S. marcescens* isolation was from respiratory secretions – 3.1%, followed by wound secretions – 3.0% and urine – 1.8%. The lowest frequency was observed among blood isolates – 0.8% (Figure 4).

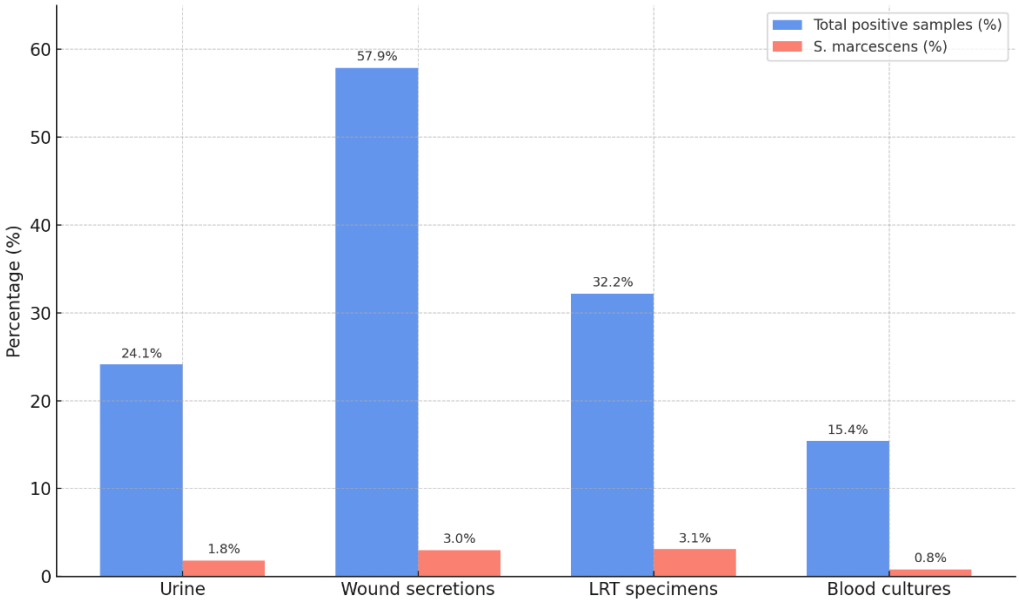


Figure 4. Relative proportion of positive clinical samples by specimen type and proportion of samples positive for *S. marcescens*, %

Discussion

In contrast to our results, the systematic review by Zivkovic Zaric et al. (2023) reported that *S. marcescens* was most frequently isolated from blood (45%), urine (14%), and cerebrospinal fluid (12%). That study included 57 cases of invasive infections, with the most common clinical manifestations being sepsis, endocarditis, and meningitis, while the mortality rate reached 31.6% (Zivkovic Zaric R., 2023).

A more recent investigation (2024) found that *S. marcescens* was most commonly isolated from blood (24.2%), wound secretions (23.3%), urine (20.7%), and endotracheal aspirates (12.8%) (Sannathimmappa MB, 2024). Another study covering the period 2015–2022 and including 396 *S. marcescens* isolates reported bronchial aspirates and sputum as the leading sources (31.6%), followed by blood (21.5%) (Cosimato I., 2024). A Turkish study involving 158 clinical isolates showed that the predominant sources were blood (35.4%) and sputum (24.6%) (Şimşek M., 2019).

According to ECDC data, *S. marcescens* was among the ten most common causative agents of urinary tract infections, respiratory infections, and bloodstream infections acquired in intensive care units in Europe in 2020, and accounted for 3% of all bacteraemias across the continent (ECDC, 2023).

These findings clearly demonstrate that the distribution of *S. marcescens* across clinical specimens varies substantially depending on geographic region, patient population, and healthcare settings. In this context, our results reflect the local epidemiological trend and complement the available international data.

Conclusion

The highest proportion of *S. marcescens* isolates in our study was observed in urine samples (30.5%), followed by wound secretions (26.8%) and respiratory secretions (25.8%), while blood isolates were considerably less frequent (10.0%). Isolates from other clinical specimens (skin secretions, ocular secretions, punctates, faeces) accounted for 6.8%. When analysed relative to all positive samples of the respective type, the highest frequency of *S. marcescens* isolation was observed from respiratory secretions (3.1%), followed by wound secretions (3.0%) and urine (1.8%). The lowest frequency was observed in blood samples (0.8%).

4.1.4. Leading clinical syndromes at hospital admission

The largest proportion of patients with *S. marcescens*-associated infections were hospitalised due to diseases of the urinary system (n = 129; 26.4%), cardiovascular system

(n = 97; 19.9%), and respiratory system (n = 65; 13.3%) (Figure 5). However, the highest mortality rates were observed among patients with underlying central nervous system diseases (28.6%) and malignant neoplasms (17.1%) (Figure 6).

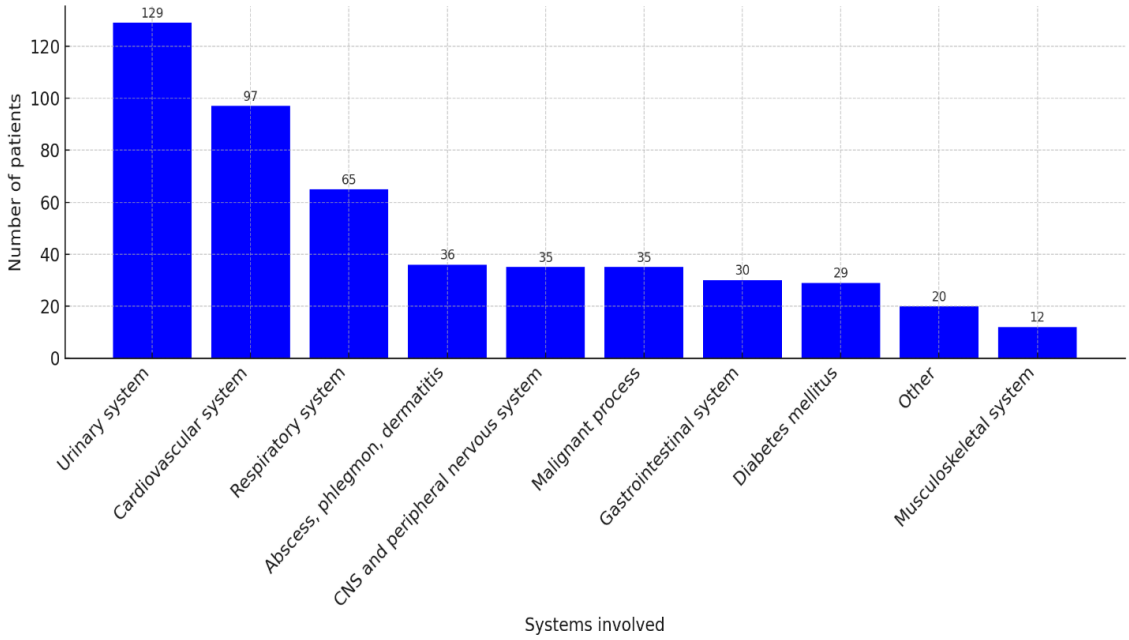


Figure 5. Distribution of patients with *Serratia marcescens*-associated infections according to the leading underlying disease (by system).

The lethality rates across the different groups are presented in the following Figure 6**.**

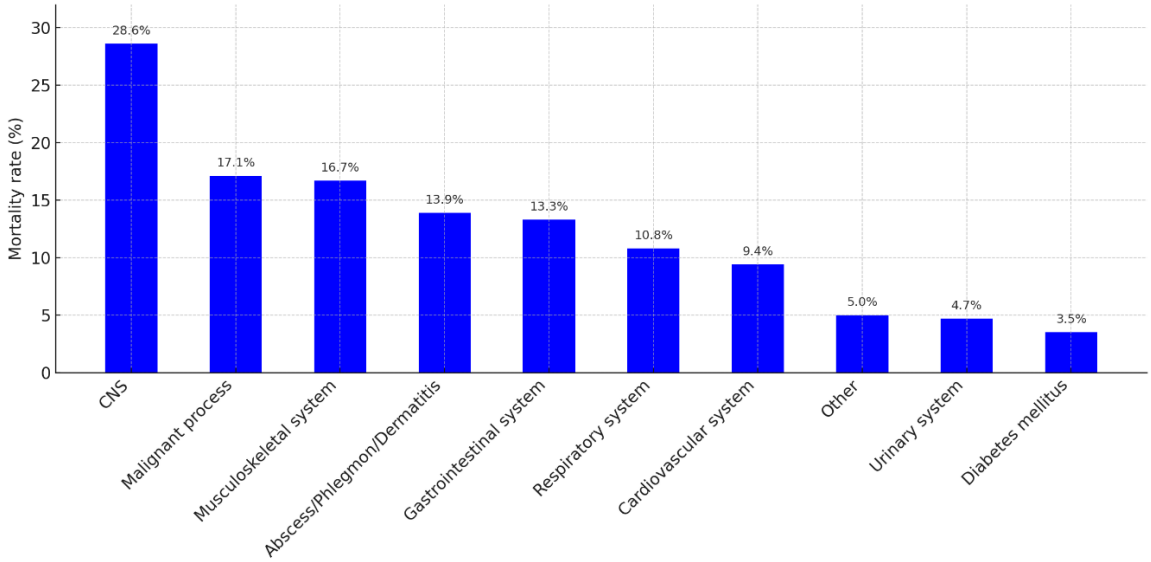


Figure 6. Case Fatality by Primary Disease Categories

The difference between the groups and clinical outcome was statistically significant ($\chi^2 = 44.20$, $p = 0.0197$), indicating that central nervous system (CNS) diseases

and malignant neoplasms were the leading diagnoses most frequently associated with fatal outcomes in patients with *Serratia marcescens* infections.

Discussion

It should be noted that in the group of patients with severe neurological conditions (mainly acute cerebrovascular accidents), intensive care management, prolonged catheterisation, and mechanical ventilation were frequently required—factors that predispose to the development of hospital-acquired infections and worsen the prognosis. In oncological patients, the high mortality was associated with immunosuppression, resulting both from the underlying disease and from the administered therapy, which makes infection control considerably more challenging.

Conclusion

Our data demonstrate that the largest proportion of patients with *S. marcescens*-associated infections were hospitalised due to diseases of the urinary (26.4%), cardiovascular (19.9%), and respiratory systems (13.3%). The highest mortality rates were observed among patients with underlying CNS diseases (28.6%) and malignant neoplasms (17.1%).

4.1.5. Risk factors for morbidity and 30-day mortality associated with *Serratia marcescens*

- **Impact of demographic factors – sex and age**

The gender distribution of patients with confirmed *S. marcescens*-associated infections was 60% versus 40% in favour of males, a pattern consistently observed throughout the study period (Figure 7).

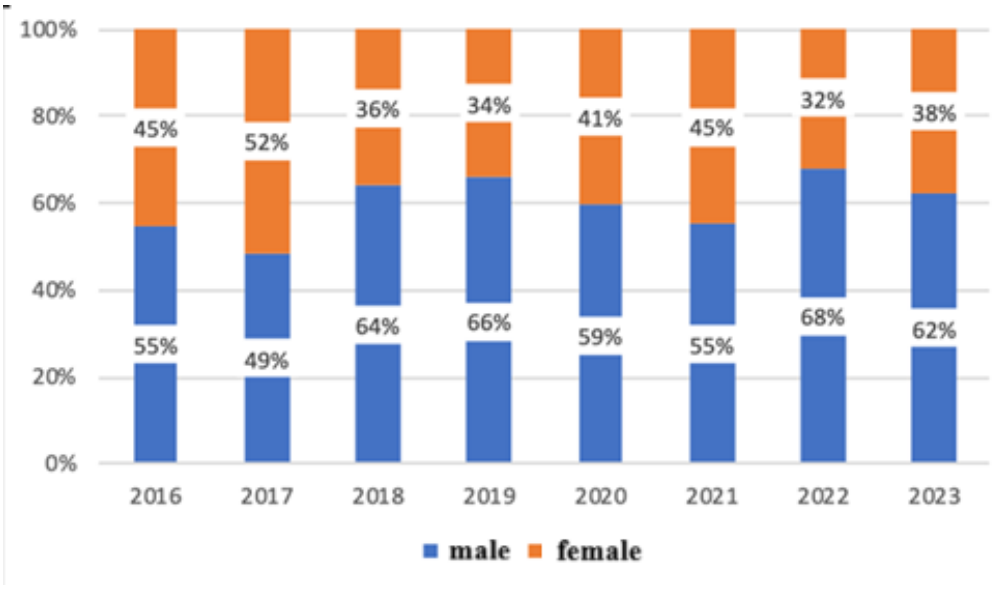


Figure 7. Gender distribution of patients with *S. marcescens*-associated infections during the study period

The analysis did not demonstrate a statistically significant difference in sex distribution between the individual years of the observation period (χ^2 = 10.448, p = 0.315), nor was any association established between sex and the risk of developing *Serratia marcescens*-associated infection or mortality.

The distribution of patients by age groups is presented in Figure 8.

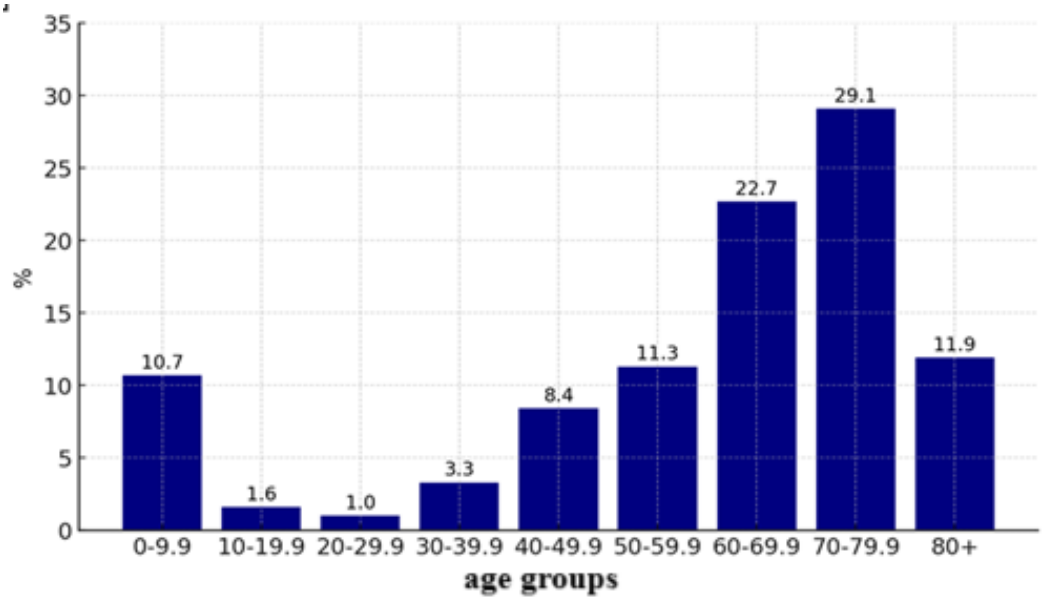


Figure 8. Age group distribution of patients with *S. marcescens*-associated infections

The median age of patients was 58 years (interquartile range 49–75 years). No statistically significant difference in age between sexes was observed: 58.4 years (SD 24.0) for females and 57.8 years (SD 24.8) for males (Student's t = –0.309, p = 0.757).

The highest morbidity was recorded in the 70–79 age group (29.1%), while the lowest was in the 20–29 age group (1%). This difference was statistically significant, identifying age as an important risk factor for acquiring infection ($p < 0.042$).

A considerable proportion of cases also occurred in childhood – up to 10 years of age, accounting for 10.7% of infections. Of note, the highest relative proportion of *S. marcescens*-associated infections was concentrated in the 0–3 month age group, with chi-square analysis demonstrating a highly significant difference between age subgroups ($\chi^2 = 72.0$, $p < 0.001$). A more detailed distribution is shown in Figure 9.

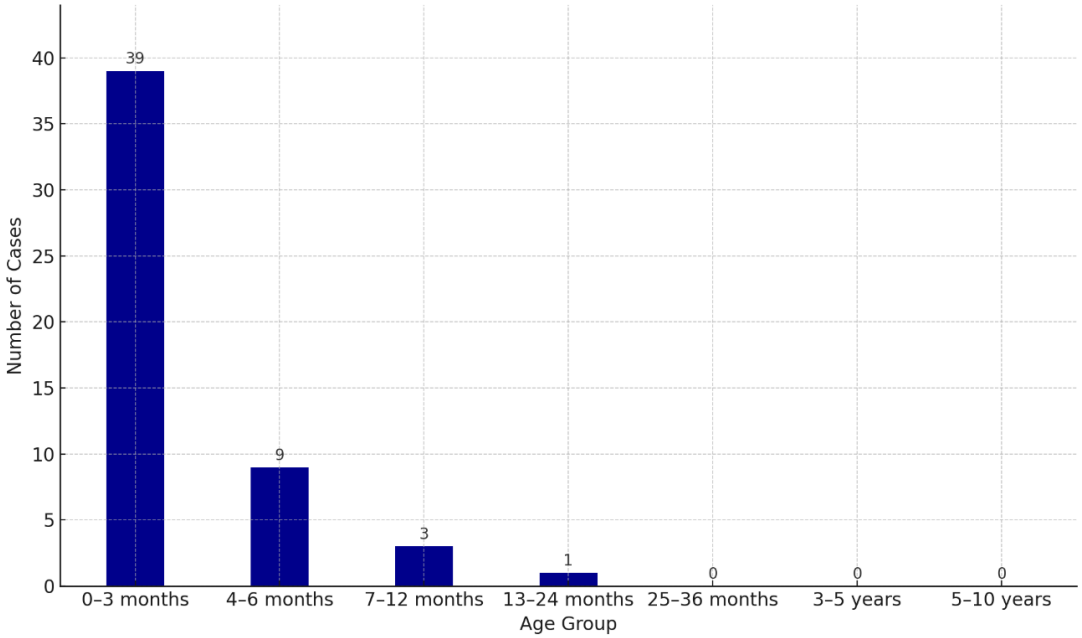


Figure 9. Incidence by Age in the 0–10 Years Age Group

The distribution of deceased patients by age group is presented in Table 5. The highest mortality was observed in the 60–79 age group (12.6%), while the lowest was in the 20–39 age group (4.8%). The mean age of deceased patients was 63.6 years, which was 6.2 years higher than that of survivors (57.4 years), although the difference did not reach statistical significance ($p = 0.086$). The difference in mortality between males and females was also not statistically significant ($p > 0.05$).

Table 2. 30-Day Mortality by Age Group and Sex

	men		women		Total	
Age	%	95% CI	%	95% CI	%	95% CI
0-19	13.5	2.5 -24.5	4.3	0.0-12.6	10.0	3.8-20.5
20-39	0		9.1	2.3-41.3	4.8	0.1-23.8
40-59	5.3	1.1-14.6	5.1	0.6-17.3	5.2	0.2-11.7
60-79	10.1	5.9-15.9	16.8	9.9-25.9	12.6	8.8-17.4
80+	13.3	3.8-30.7	10.7	2.3-28.2	12.1	4.9-23.3
Total	9.6	6.5-13.6	11.7	7.6-17.1	10.5	7.9-13.5

Discussion

The present study did not demonstrate a statistically significant association between sex and the risk of *Serratia*-associated infection or infection-related mortality. Nevertheless, accumulating evidence highlights sex-related disparities in susceptibility to infectious diseases, with males showing increased vulnerability to both bacterial and viral pathogens. This phenomenon is explained by several biological mechanisms: testosterone exerts immunosuppressive effects by reducing pro-inflammatory cytokine production and impairing macrophage and neutrophil activity, both essential for the early host response. By contrast, oestrogens enhance Toll-like receptor expression (e.g. TLR4 and TLR7), promoting a more rapid and effective activation of innate immunity. In addition, many immune-regulatory genes are located on the X chromosome, providing females with a genetic advantage in immune responsiveness. Epidemiological studies corroborate these mechanistic insights, showing that men are at higher risk of severe bacterial infections, including pneumonia, urinary tract infections, and bacteraemia (Dias SP, 2022).

A notable finding in our cohort was the disproportionately high prevalence of *S. marcescens*-associated infections among infants aged 0–3 months. This observation is consistent with the established link between immune system immaturity in early life and increased susceptibility to bacterial and viral pathogens. After the third month, infection rates declined progressively, with a marked reduction beyond the first year of life, reflecting the maturation of both innate and adaptive immune mechanisms and improved responsiveness to environmental antigens. An evident epidemiological trend was

identified, indicating that early infancy represents a critical window of vulnerability that necessitates enhanced clinical surveillance and preventive measures during the first months of life. These results are consistent with numerous reports of *S. marcescens* outbreaks in neonatal and paediatric wards, where risk factors such as immune immaturity, very low birth weight (<1500 g), prolonged hospitalisation, and broad-spectrum antimicrobial exposure have been consistently reported (Cristina ML, 2019). Severe outbreaks, some with fatal outcomes, have been linked to contaminated antiseptics and medical devices, as well as insufficient hand hygiene and inadequate surface disinfection (Morillo A, 2018; Madani TA, 2021; Muyldermans A, 2021; Ministry of Health, 2007). The most severe complications included sepsis, meningitis, pneumonia, and conjunctivitis, with fatalities documented within the first 24 hours after infection onset (Casolari C, 2005; Redondo-Bravo L, 2019).

From a demographic perspective, age-related vulnerability must also be considered. In Bulgaria, life expectancy in 2020 was 73.6 years, the lowest in Europe (Eurostat Database, 2021). National surveillance data demonstrated that elderly individuals were disproportionately affected by severe infections and associated mortality, particularly in the context of the COVID-19 pandemic (NCIPD, 2020). Comparable international findings reinforce this observation: a 14-year analysis of bloodstream infections identified advanced age as an independent predictor of adverse outcomes (Kontula KSK, 2021), while a large-scale study of over one million sepsis cases confirmed a marked increase in mortality with advancing age, with individuals >85 years at the greatest risk (Seymour CW, 2017). By contrast, our results did not confirm age as a predictor of mortality; although advanced age was associated with increased infection risk, it was not predictive of fatal outcome in this cohort.

Conclusion

This study demonstrated no statistically significant sex-related differences in the incidence or outcome of *S. marcescens*-associated infections. The median patient age was 58 years, with no sex-based variation. Morbidity was highest among patients aged 70–79 years and lowest among those aged 20–29 years, establishing age as a significant risk factor for infection acquisition. A considerable proportion of cases occurred in children under 10 years, with the greatest burden concentrated in the 0–3 month subgroup, a finding of high statistical significance. Mortality was highest in patients aged 60–79 years

and lowest in the 20–39 age group. Although deceased patients were on average 6.2 years older than survivors, this difference did not reach statistical significance.

- **Previous Hospitalization**

"Previous hospitalisation" was defined as admission to the same or another hospital within 30 days prior to the current hospital stay.

Out of the 488 patients included, 247 had a history of previous hospitalisation and 241 did not, indicating that previous hospitalisation was not a risk factor for infection acquisition.

Among the 51 patients with a fatal outcome, 24 (47.1%) had a history of previous hospitalisation, whereas 27 (52.9%) did not. Statistical analysis did not demonstrate a significant association between prior hospitalisation and mortality ($p > 0.05$ for all applied tests – Pearson's chi-square and Fisher's exact test), and did not establish previous hospitalisation as an independent risk factor for fatal outcome. This result remained valid in the subgroup of elderly patients (>75 years), which constituted a considerable proportion of the study population.

Discussion

Previous hospitalisation has been reported as a significant risk factor both for the acquisition of nosocomial infections and for mortality (Zeng Q, 2014). In another study, it was identified as an independent risk factor for infection caused by ESBL-producing bacteria (Colodner, 2004). A large cohort study by Visade et al. in 2021 found that prior hospitalizations in elderly patients over 75 years of age were a significant predictor for rehospitalization and post-discharge mortality, with patients having more than one prior admission being at significantly higher risk of death within three months of discharge. (Visade F, 2021). A prospective investigation of 789 non-duplicate bacteraemia episodes over a one-year period demonstrated that prior hospitalisation had a substantial impact on the risk of bacteraemia due to resistant strains. The highest risk was observed in patients hospitalised 3–90 days before infection (14.6%), with risk progressively declining over time after discharge (OR 0.83 per month). In addition, prior carriage of antimicrobial-resistant bacteria and intensive care unit stay were identified as independent predictors of bacteraemia caused by resistant strains (Chen SY, 2008).

Conclusion

In our study, previous hospitalisation was not established as a risk factor for infection acquisition, nor as an independent risk factor for mortality.

- **Site of infection acquisition**

For the purposes of this study, *S. marcescens*-associated infections were defined as nosocomial when they developed ≥48 hours after admission, in the absence of evidence (from history, clinical status, or laboratory tests conducted prior to admission) that the infection was already in an incubation phase at the time of hospitalisation.

Our data demonstrated that the majority of *S. marcescens*-associated infections were hospital-acquired (97.5%), confirming hospitalisation as the most significant risk factor for infection caused by this pathogen ($p < 0.001$). Although 50 patients with nosocomial *S. marcescens* infections died (compared with only one patient with community-acquired infection), the difference was not statistically significant due to the very small sample of community-acquired cases.

Discussion

Multiple studies have identified hospitalisation as a major risk factor for nosocomial infections, particularly in the context of invasive procedures and intensive care. A 2023 study identified hospitalisation as the primary determinant significantly increasing the risk of nosocomial infection, especially among patients undergoing invasive interventions (Isigi S., 2023). According to ECDC data for Bulgaria, the most common Gram-negative pathogens causing nosocomial infections are *A. baumannii* and *K. pneumoniae* (15.1% each), followed by *P. aeruginosa* (11.1%) and *E. coli* (6.0%). The proportion of *S. marcescens*-associated nosocomial infections is relatively low—1.0% in Bulgaria and 1.4% at the European level (ECDC, 2024). Marked geographic variations are observed: <1% in Sweden and Croatia, approximately 1% in France, Italy, and Bulgaria, and up to 2.5% in Spain (ECDC, 2024). These findings underscore the need for harmonisation of prevention, control, and diagnostic standards across Europe in order to minimise the burden of *S. marcescens* infections.

Conclusion

This study demonstrated that the vast majority of *S. marcescens*-associated infections were nosocomial (98.8%), thereby identifying hospitalisation as the most significant risk factor for infection. Mortality occurred almost exclusively in nosocomial cases (50 vs. 1), but the difference did not reach statistical significance due to the limited number of community-acquired cases.

- **Comorbidities**

In this study, the most common comorbidities identified as significant risk factors for *S. marcescens*-associated infection were cardiovascular disease (n = 321, 65.8%),

endocrine disease (n = 154, 31.6%), and urological disease (n = 68, 13.9%). A statistically significant association was observed between the type of comorbidity and the risk of infection ($p < 0.001$), which is consistent with the finding that the highest proportion of *S. marcescens* infections occurred in patients hospitalised in therapeutic wards. No statistically significant association between comorbidity and mortality was identified ($p = 0.653$).

Discussion

Similar to our results, comorbidities such as cardiovascular disease, diabetes, and malignancy have been highlighted in previous studies. A 2023 investigation from Central America reported that the most common comorbidities among patients with confirmed *S. marcescens* infections were diabetes (74.3%) and hypertension (58.5%) (Mayorga DM, 2023). Another study identified malignant disease, cardiovascular disease, and diabetes as the most frequent comorbidities among patients with *S. marcescens*-associated bacteraemia, with sepsis rates of 58.7% and mortality rates of 34.8% (Jacot A, 2024).

Conclusion

Cardiovascular (65.8%), endocrine (31.6%), and urological (13.9%) comorbidities were confirmed as significant risk factors for acquiring *S. marcescens*-associated infection. No association was demonstrated between comorbidity and mortality.

- **Distribution of patients by hospital ward**

All 35 wards in which study patients were hospitalised were categorised into five main groups according to their profile: Intensive care units, Surgical wards, Therapeutic wards, Paediatric wards, and Other (ENT, psychiatry with day hospital, infectious diseases, and dermatology) (Figure 10).

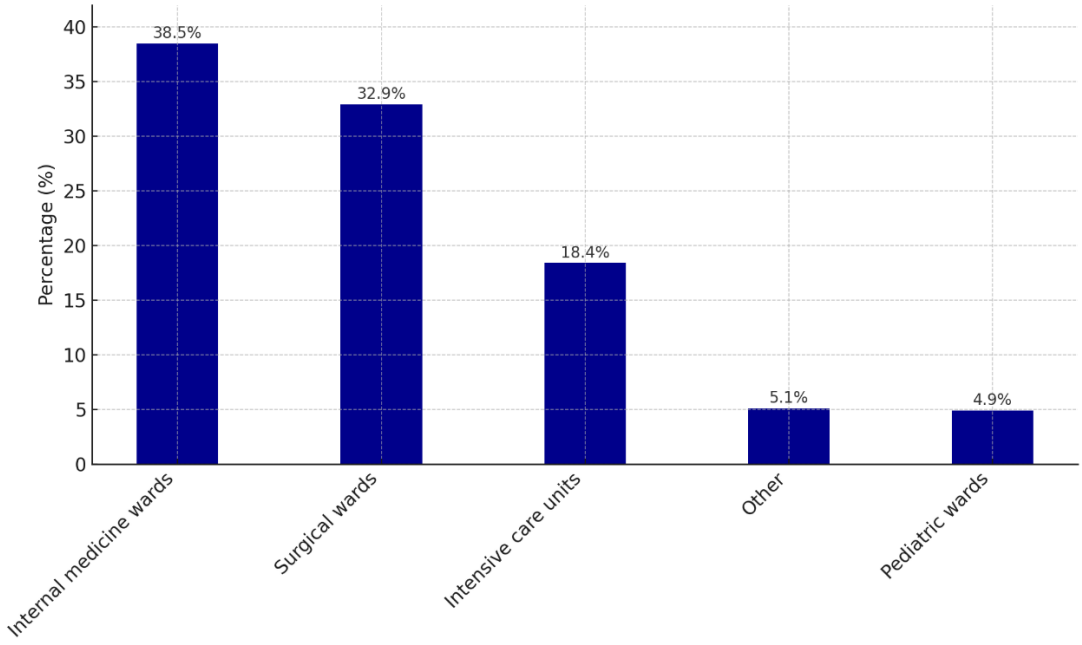


Figure 10. Distribution of isolates by hospital ward type, %

The highest proportion of patients with confirmed *S. marcescens*-associated infection was identified in therapeutic (internal medicine) wards (38.5%), followed by surgical wards (32.9%) and intensive care units (18.4%). The difference between wards was statistically significant ($p < 0.001$), indicating hospitalisation in therapeutic wards as a risk factor for acquiring *S. marcescens*-associated infection.

Discussion

The higher frequency of *S. marcescens* infections among patients in therapeutic wards may be explained by the fact that these patients often present with comorbidities and chronic or decompensated conditions (cardiovascular, pulmonary, renal, etc.), which increase the risk of rapid deterioration and the need for transfer to intensive care. This creates an epidemiological link between the two settings: the infection may develop or be documented as originating in the therapeutic ward, while in fact it may be attributable to subsequent intensive care unit stay. Therefore, intensive care admission should be considered an independent risk factor for infection acquisition**.**

Conclusion

The highest proportion of *S. marcescens*-associated infections was observed in therapeutic wards (38.5%), followed by surgical wards (32.9%) and intensive care units (18.4%). Hospitalisation in therapeutic wards was thus identified as a significant risk factor for infection acquisition.

- **Stay in intensive care unit / surgical treatment**

To assess the impact of surgical treatment and intensive care on mortality, patients were stratified into four groups: (1) those managed conservatively without surgery or intensive care (51.9%); (2) patients who underwent surgical intervention only (33.6%); (3) patients treated in the intensive care unit (ICU) (8.5%); and (4) patients receiving combined treatment involving both surgery and intensive care (5.9%).

In the present study, ICU admission was identified as a significant risk factor both for the acquisition of *S. marcescens*-associated infection and for adverse outcome. The highest mortality was observed among patients hospitalised in the ICU (50.0%) (Figure 11).

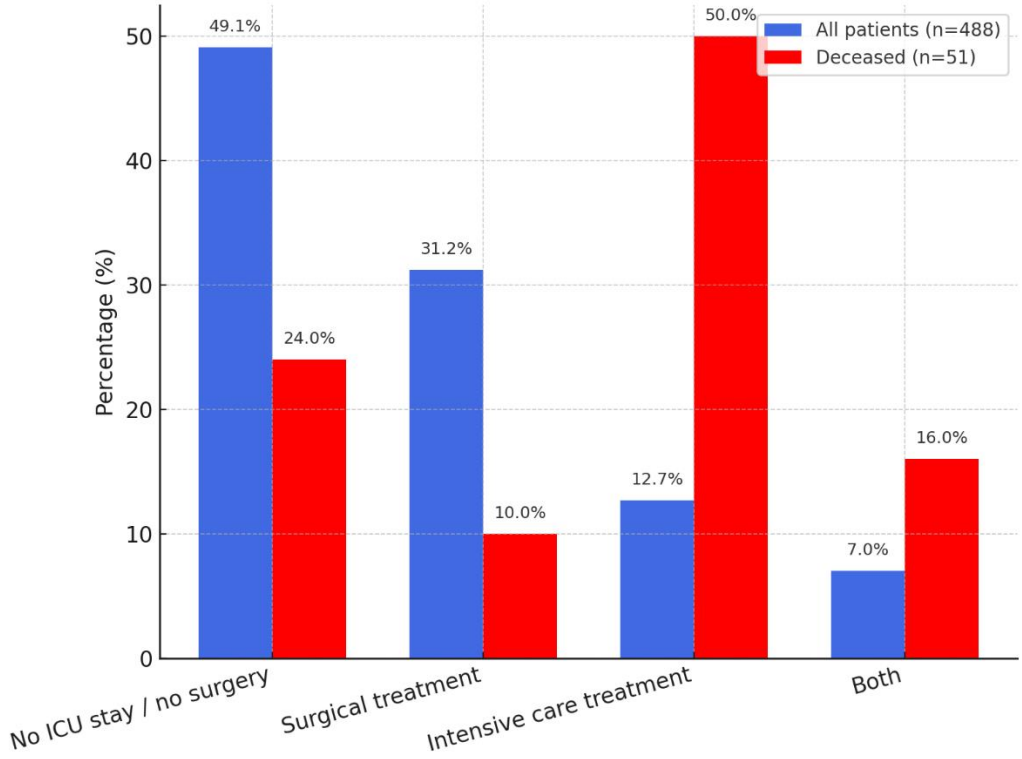


Figure 11. Distribution of all patients (n = 488) and deceased patients (n = 51) according to treatment modality (%)

Statistical analysis demonstrated a significant association between treatment category and mortality ($\chi^2 = 82.456$, df = 3, $p < 0.001$), confirming that treatment modality exerts a substantial influence on patient prognosis. The analysis of likelihood ratios (Likelihood Ratio = 62.429, $p < 0.001$) further supported this relationship. The additional test for linear association (Linear-by-Linear Association = 42.407, $p < 0.001$) indicated

that the probability of fatal outcome increases significantly with the transition from standard care to intensive care and surgical interventions.

Discussion

Across European Union countries, the overall proportion of *S. marcescens*-associated HAIs is 1.4%, with the highest prevalence reported from intensive care units (20.5%), followed by haematology (15.5%), nephrology (10.2%), surgery (7.3%), and paediatrics (2.4%) (ECDC, HAI-Net data 2020). Multiple studies have shown that the incidence of nosocomial infections in intensive care units is 5–10 times higher than in other hospital wards (Rejeb MB, 2016). This high prevalence substantially contributes to morbidity and mortality among hospitalised patients, increases economic burden, and leads to greater antibiotic use and longer hospital stays (Singer M, 2009).

Consistent with our findings, another study involving 355 patients identified ICU stay as an independent risk factor for mortality, both in patients with established infection at admission and in those without. The same study reported significantly longer hospital stays among patients with ICU-acquired infections (Ylipalosaari P, 2006). Further evidence indicates that ICU-acquired infection significantly increases the risk of sepsis-related death (van Vught, 2016; van Vught, 2017). Additional studies confirm that ICU patients are at markedly increased risk of infection due to frequent invasive procedures and their immunocompromised condition (Blot S, 2022).

Conclusion

ICU stay was identified as a strong predictor for both infection acquisition and adverse outcome.

- **Risk factors associated with medical interventions**

This study demonstrated that the majority of patients had an indwelling device (central venous catheter, peripheral venous catheter, urinary catheter, drain, cardiac valve, stoma, feeding tube) (n = 392; 80.3 %), followed by those undergoing invasive procedures (endoscopies, surgical interventions, transplantation, biopsy) (n = 78; 15.9 %). The least frequent risk factor was intubation/mechanical ventilation (n = 43; 8.8 %). The difference between groups was statistically significant, identifying the presence of an indwelling device as the predominant risk factor for infection acquisition ($p < 0.05$).

Further analysis revealed that catheterisation was the principal risk factor for *S. marcescens*-associated urinary tract infection in 92.8% of cases. Sepsis secondary to

urinary tract infection developed in 11.1% of all bacteraemia episodes, while 6.7% followed a primary respiratory infection. Nearly one-third of bacteraemias (28.8%) were associated with the presence of a central venous catheter. The number of patients with ventilator-associated pneumonia (VAP) due to *S. marcescens* was too small to allow for robust statistical analysis.

Discussion

Data from ICUs across EU countries demonstrate that intubation is the principal risk factor for pneumonia in this patient population. Of 6,699 cases of pneumonia, 70.8% were intubation-related VAP episodes. Among ICU patients hospitalised for >2 days, 7.7% experienced at least one episode of pneumonia, with an average ICU pneumonia incidence of 7.3 episodes per 1,000 patient-days, ranging from 4.1 in ICUs with <30% of patients intubated, to 6.2 in ICUs with 30–59% intubated, and 8.5 in ICUs with >60% intubated patients (ECDC, HAI-Net data 2020). Device-adjusted incidence for VAP was 10.2 episodes per 1,000 ventilation-days, ranging from 3.4 in Austria to 16.6 in Italy (ECDC, HAI-Net patient-based data 2020).

According to recent ECDC data, ICU-acquired urinary tract infections occur in 3.2% of patients hospitalised for more than two days, with 95.2% of these cases associated with urinary catheter use. The average incidence was 3.0 UTI episodes per 1,000 patient-days (HAI-Net patient-based data 2020). Our findings are consistent with ECDC data indicating that nearly half of bloodstream infections are catheter-associated. Prolonged hospitalisation, antibiotic therapy, and invasive diagnostic and/or therapeutic interventions are recognised as major risk factors for catheter-associated bloodstream infections due to *Serratia* spp., which frequently occur secondary to primary urinary or respiratory tract infections (Tóth A, 2020; Cullen MM, 2005; Pursell KL, 2001; Mendes JC, 2022). The most frequently isolated microorganisms in bloodstream infections (including microbiologically confirmed catheter-associated cases) are coagulase-negative staphylococci, *Enterococcus* spp., *Klebsiella* spp., and *P. aeruginosa*, with *Serratia* spp. ranked ninth, followed by *Acinetobacter* spp. (ECDC, HAI-Net patient-based and unit-based data 2020).

Conclusion

The presence of an indwelling device was confirmed as the leading risk factor for *S. marcescens*-associated infections (80%), followed by invasive procedures (16%) and intubation/mechanical ventilation (7.9%). Catheterisation was associated with urinary

tract infection in 92.8% of cases, while nearly one-third of bacteraemias (28.8%) were linked to central venous catheter use.

- **Average hospital stay**

During the analysed period, the mean length of hospitalisation among patients with confirmed *S. marcescens*-associated infection was 11.9 days (SD ±12.2) (Figure 12). No statistically significant differences were observed with respect to sex or age (p = 0.649).

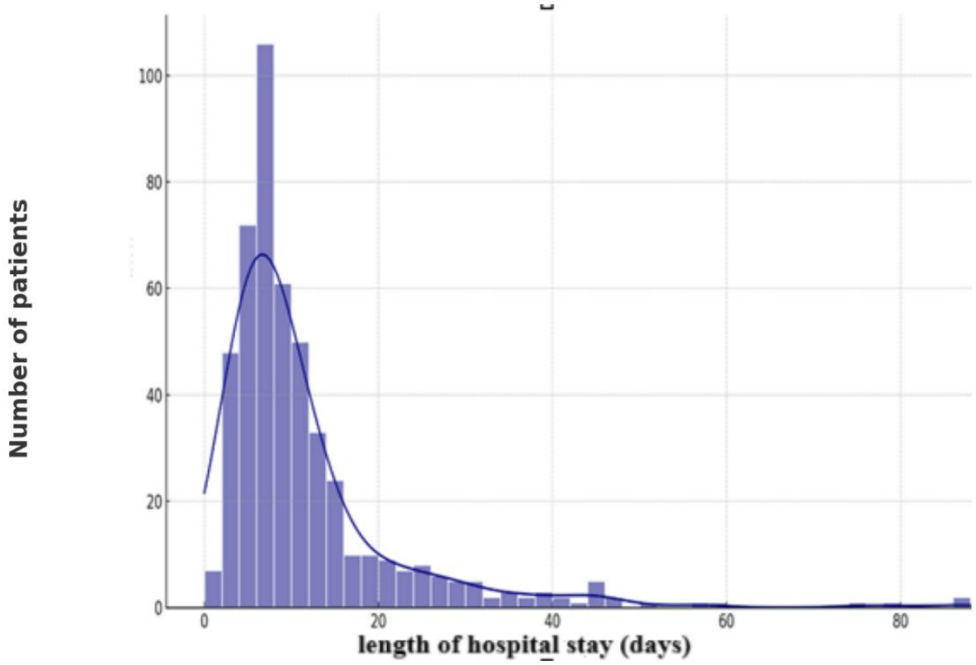
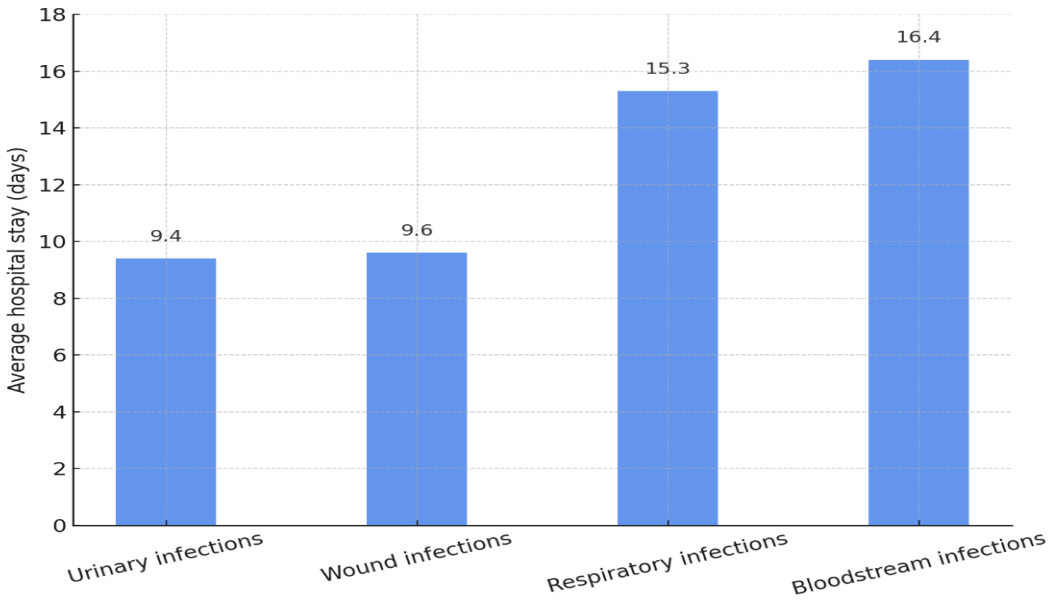


Figure 12. Distribution of hospital stay among patients with *S. marcescens*-associated infections

The distribution of hospital stay was asymmetrical, with the majority of patients experiencing a short hospitalisation (4–12 days), while only a small proportion had a prolonged stay (>18–20 days).

The mean length of hospital stay according to the type of *S. marcescens*-associated infection is presented in Figure 13.



Figype 13. Average Length of Hospital Stay according to the type of *S. marcescens*-associated infection.

In our study, bloodstream infections were identified as the primary cause of the longest hospital stays (mean 16.4 days), followed by respiratory and wound infections, whereas urinary tract infections were associated with the shortest mean stay (9.4 days). The difference between groups was statistically significant ($p < 0.001$), indicating that the type of infection serves as a predictor of prolonged hospitalisation, which in turn increases the risk of mortality.

Discussion

Our findings are consistent with previous reports demonstrating that bloodstream infections are associated with extended hospital stay and higher mortality risk (Barnett AG, 2013). A study from 2021 reported, however, that respiratory infections accounted for the longest hospitalisations, followed by bloodstream and wound infections (Del Giudice A, 2021). Despite such differences across studies, the prevailing evidence supports the view that prolonged hospital stay is strongly linked to an increased risk of acquiring nosocomial infections, which in turn elevates the risk of death (Jeon CY, 2012; Fierce Healthcare, 2025).

Conclusion

The average length of hospitalisation was 11.9 days, with no statistically significant differences by sex or age. The type of infection was identified as an independent predictor

of hospital stay duration, with the longest stays observed in cases of bacteraemia and respiratory infections, and the shortest in urinary tract infections.

4.2 Antibacterial resistance profiles of 488 S. marcescens isolates (2016–2023)

The resistance patterns of 488 *S. marcescens* isolates collected from patients between 2016 and 2023 are presented in table 3.

Antibiotic	2016-2023		2016		2017		2018		2019		2020		2021		2022		2023	
	Total number	n, R %	Total number	N, (r %)	Total number	N, (r %)	Total number	N, (r %)	Total number	N, (r %)	Total number	N, (r %)	Total number	N, (r %)	Total number	N, (r %)	Total number	N, (r %)
PIT	488	152 (31.2)	30	3 (10)	67	23 (34.3)	93	49 (52.7)	78	23 (37.2)	66	29 (29.3)	27	27 (29.6)	45	5 (11.1)	62	14 (22.3)
CRO	488	175 (35.9)	30	6 (20)	67	23 (34.3)	93	52 (56)	78	25 (32.1)	66	32 (48.5)	27	9 (33.3)	45	8 (17.8)	62	17 (27.4)
FEP	488	164 (33.6)	30	4 (13.3)	67	20 (30)	93	52 (56)	78	24 (30.8)	66	31 (47)	27	8 (29.6)	45	8 (17.8)	62	16 (25.8)
IPM	488	10 (2)	30	0	67	0	93	0	78	2 (2.6)	66	1 (1.5)	27	3 (11.1)	45	3 (6.7)	62	1 (1.6)
MEM	488	10 (2)	30	0	67	2 (3)	93	0	78	1 (1.3)	66	1 (1.5)	27	3 (11.1)	45	3 (6.7)	62	1 (1.6)
CIP	488	153 (31.3)	30	3 (10)	67	21 (31.3)	93	49 (52.7)	78	23 (29.5)	66	32 (48.5)	27	8 (29.6)	45	2 (4.5)	62	19 (30.6)
LVX	488	150 (31.8)	30	3 (10)	67	21 (31.3)	93	49 (52.7)	78	22 (28.2)	66	32 (48.5)	27	8 (29.6)	45	2 (4.5)	62	18 (29.1)
GEN	488	164 (33.6)	30	5 (16.7)	67	21 (31.3)	93	53 (57)	78	25 (32.1)	66	29 (43.9)	27	7 (25.9)	45	4 (9)	62	17 (27.4)
AK	488	108 (22.2)	30	3 (10)	67	24 (35.8)	93	29 (31.2)	78	11 (8.6)	66	19 (28.8)	27	7 (25.9)	45	4 (9)	62	17 (27.4)
SXT	488	163 (33.4)	30	6 (20)	67	22 (32.8)	93	54 (58)	78	20 (25.6)	66	29 (43.9)	27	7 (25.9)	45	6(13.3)	62	17 (27.4)

Table 3. Antimicrobial resistance of *S. marcescens* isolates in the period 2016–2023

The data illustrate the temporal dynamics of *S. marcescens* resistance to various antimicrobial agents over the study period. Notably, 2018 emerged as a critical year, with the highest levels of resistance observed for most of the tested antimicrobial agents.

The trends in resistance to β-lactam antibiotics throughout the studied period are depicted in figure 14.

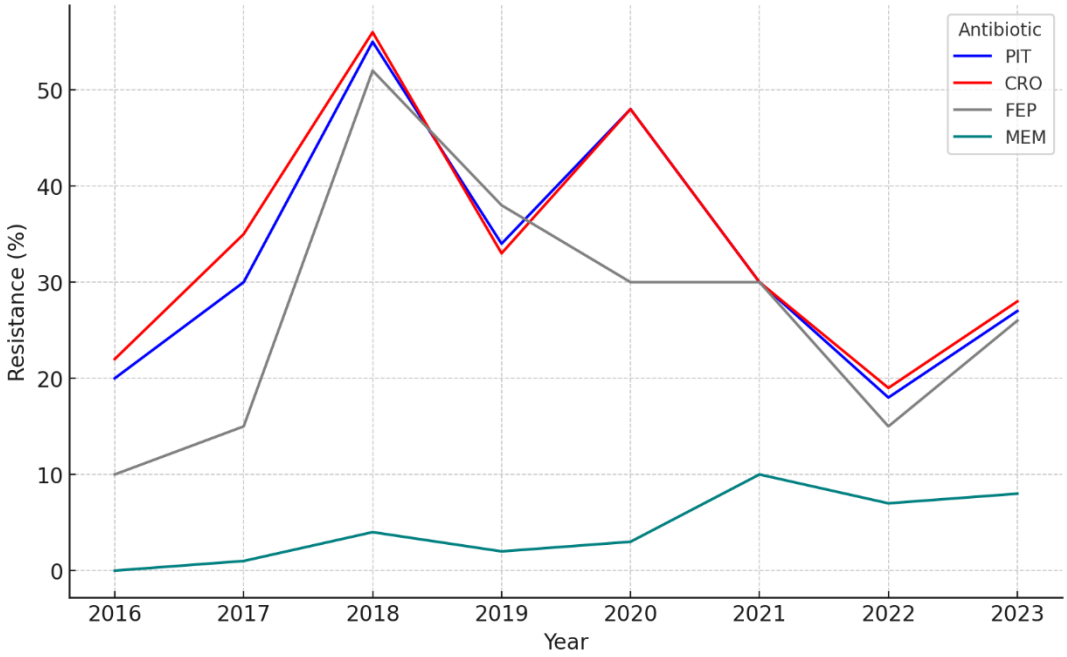


Figure 14. Resistance to β-lactam agents in the period 2016–2023 (%)

Resistance to third- and fourth-generation cephalosporins demonstrated consistently high levels, with peaks observed in 2018 and 2020, followed by a decline in 2022. No carbapenem-resistant isolates were identified prior to 2018; however, such cases began to emerge thereafter, reaching a maximum in 2021.

In our study, of the 51 recorded fatal cases, 20 (39.2%) were associated with ESBL-producing isolates, one of which was also carbapenem-resistant. To assess the relationship between ESBL production and mortality, a comparative analysis including all patients (n = 488) was performed (Table 8). The mortality rate among infections caused by ESBL-producing isolates was 12.1% (20/165), compared with 9.6% (31/323) for ESBL-negative isolates. No statistically significant association was identified between ESBL production and mortality ($\chi^2 = 0.50$, $p = 0.480$; Fisher's exact test $p = 0.435$). The calculated measures further confirmed the absence of a substantial association: odds ratio (OR) = 1.30 and relative risk (RR) = 1.26, 95% CI 0.74–2.15.

Table 8. Association between ESBL production and mortality in patients with *S. marcescens*-associated infections

Isolate characteristic	Deceased (n=50), n (%)	Survivors, n (%)	Total, n	Mortality (%)
ESBL- positive	20 (12.1%)	145 (87.9%)	165	12.1%
ESBL- negative	31 (9.6%)	292 (90.4%)	323	9.6%
Total	51 (10.4%)	437 (89.6%)	488	10.4%

Discussion

Our findings, showing that the majority of fatal cases were associated with *S. marcescens* isolates susceptible to the tested antibiotics, underscore the fact that antimicrobial resistance, although a critical determinant, is not the sole predictor of adverse clinical outcomes. This may be explained by the high virulence potential of certain *S. marcescens* strains, related to the expression of proteases, lipases, haemolysins, fimbriae, biofilm-forming capacity, and secretion systems that facilitate adhesion, invasion, and immune evasion. In addition, individual patient-related factors—including immune status, presence of severe comorbidities, age, prolonged hospitalisation, and the use of invasive devices—represent independent risk factors for mortality. Moreover, discrepancies between *in vitro* susceptibility and *in vivo* effectiveness (e.g., in the presence of biofilms or high bacterial load) may further worsen prognosis, irrespective of laboratory-confirmed sensitivity.

According to ECDC data from 2018, approximately 33,000 deaths occur annually in the European Union/European Economic Area (EU/EEA) as a result of infections caused by antibiotic-resistant bacteria (ECDC, 2018). A more recent report estimated that between 2016 and 2020, the number of infections due to antibiotic-resistant bacteria in the EU/EEA increased from ~685,000 in 2016 to ~866,000 in 2019, followed by a slight decline to ~841,000 in 2020. Associated deaths rose from ~30,700 in 2016 to ~38,700 in 2019, decreasing modestly to ~35,400 in 2020 (ECDC, 2021). In 2020, Bulgaria and Greece were among the countries with the highest resistance rates to third-generation cephalosporins in the EU (ECDC, 2021).

A large global analysis from 2019 on mortality attributable to 33 bacterial pathogens and 11 infectious syndromes (including bloodstream infections, BSI) reported 76,700 deaths worldwide due to *S. marcescens*-associated BSI, of which 33,023 were

caused by resistant isolates. In Bulgaria, 79 deaths attributable to *Serratia*-associated infections were recorded (GBD 2019 Antimicrobial Resistance Collaborators, 2022). Further analyses have shown that patients infected with resistant bacteria have a threefold higher likelihood of fatal outcome compared with those infected with susceptible strains (ECDC, 2016).

Conclusion

No statistically significant association was observed between ESBL production and mortality in *S. marcescens*-associated infections, with comparable lethality rates among ESBL-producing and ESBL-negative isolates.

- **Therapeutic approaches and antibiotics used**

Figure 15 illustrates the distribution of patients with confirmed *S. marcescens*-associated infection according to the applied therapeutic regimen (monotherapy, combination therapy, or absence of antibiotic treatment).

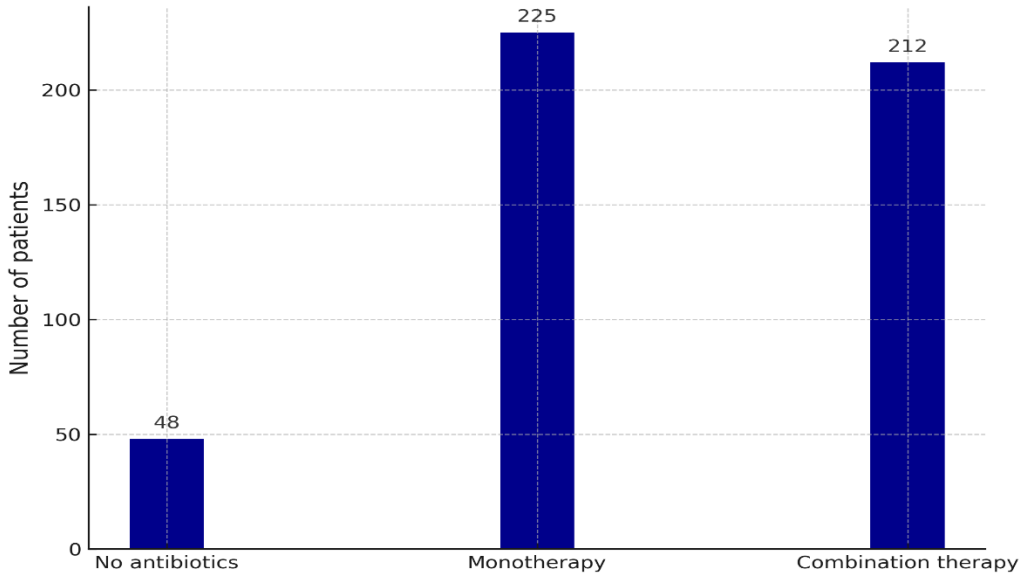


Figure 14. Distribution of patients according to the therapeutic approach used in *S. marcescens*-associated infection

Monotherapy was administered in 225 patients (46.1%), with the choice of antibiotics

applied presented in Figure 15.

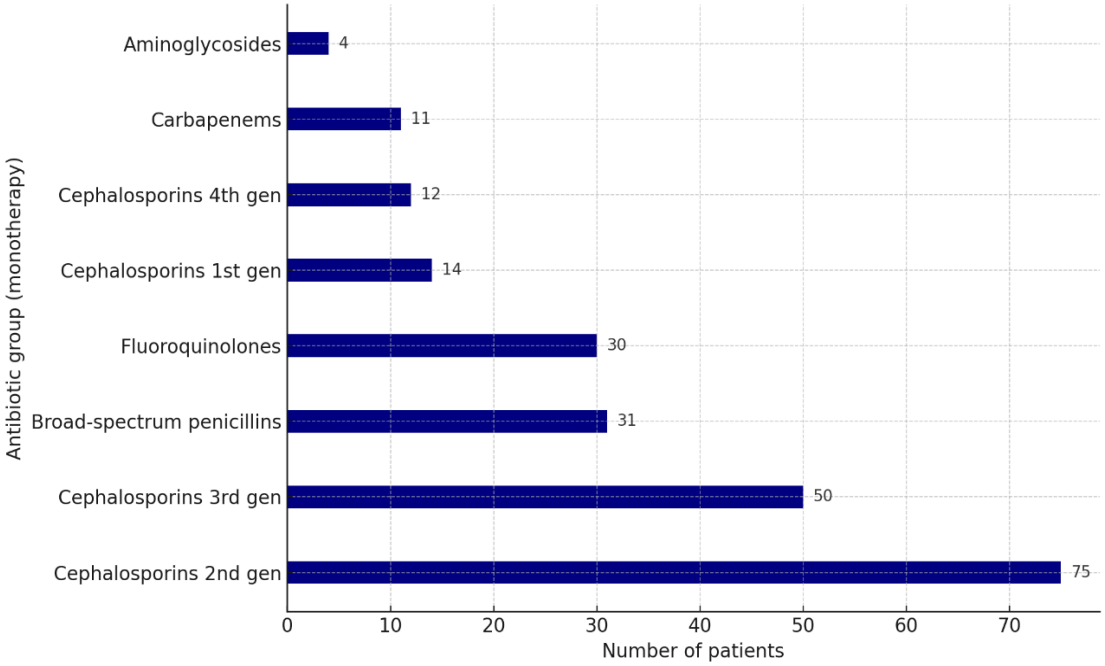


Figure 15. Distribution of antibiotic classes used in monotherapy

Given the central role of β-lactam antibiotics in therapy, a separate analysis of their use was performed, both within monotherapy and in combination therapeutic regimens (Figure 16).

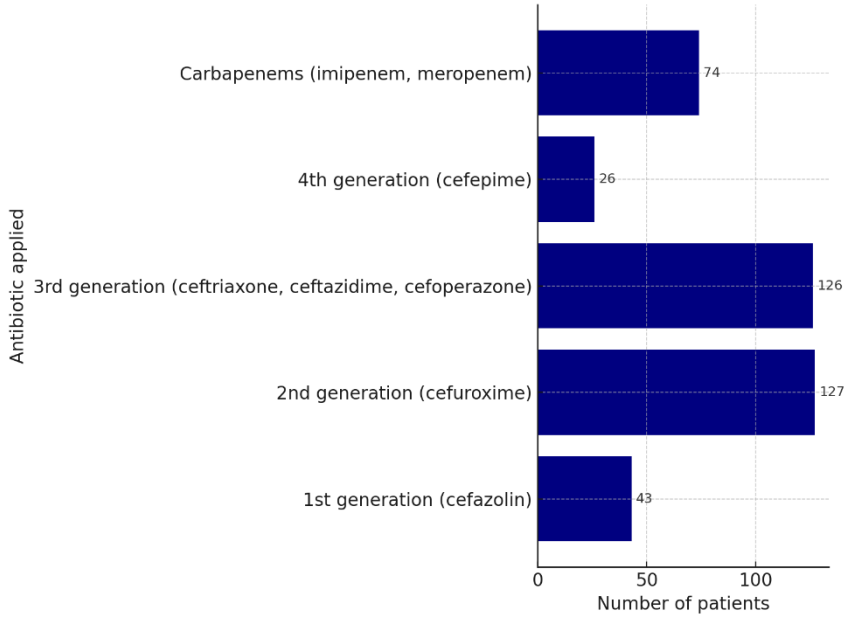


Figure 16. Distribution of β-lactam antibiotics by number of patients treated, both as monotherapy and as part of combination regimens

The clinical outcome associated with the most commonly administered antibiotic therapy combinations is presented in Figure 17.

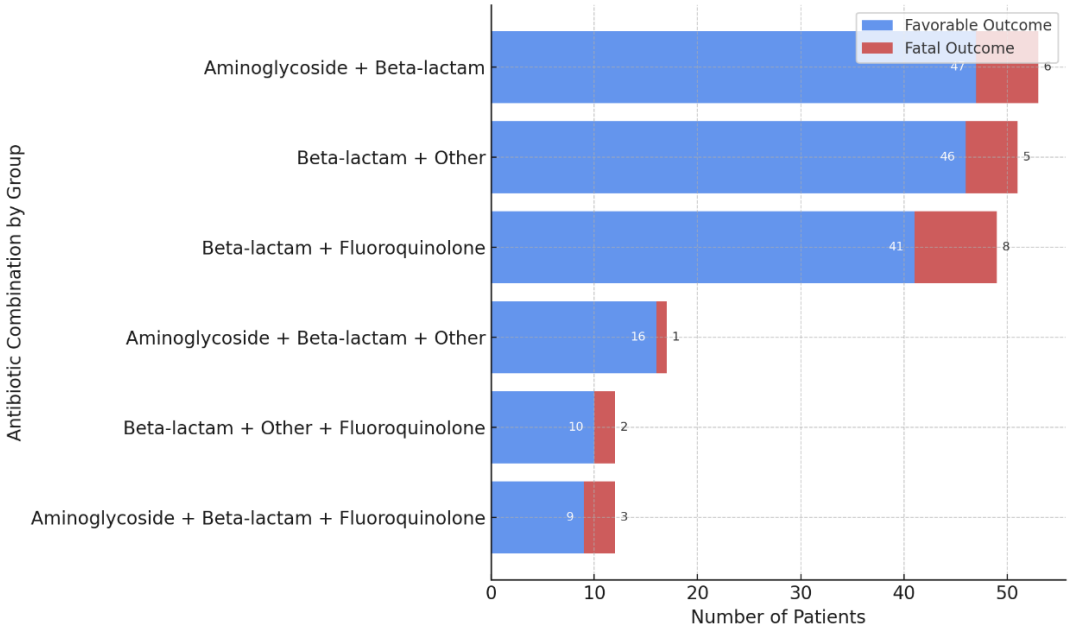


Figure 17. Distribution of patients according to antibiotic combinations used and treatment outcome

Figure 17 illustrates the distribution of the most frequently applied antibiotic combinations in hospitalised patients, together with the corresponding clinical outcomes. Only regimens associated with at least one fatal outcome were included (n = 27).

Table 7 presents the results of the logistic regression analysis assessing the association between the use of different antibiotics and the odds of 30-day mortality

Table 7. Results of logistic regression analysis evaluating the association between antibiotic therapy and mortality in patients with *Serratia marcescens* infections. *Note:* Odds ratios (OR), 95% confidence intervals (CI), and p-values are presented. A p-value < 0.05 was considered statistically significant.

Antibiotic	β-coefficient	95% CI	p
Amikacin	0.262	0.075 - 0.917	**0.036**
Gentamicin	0.064	0.014-0.288	**<0.001**
Levofloxacin	0.003	0.017-0.432	**0.003**
Trimethoprim/sulfamethoxazole	0.293	0.096-2.029	0.293

Piperacillin / tazobactam	0.063	0.34-1.096	0.063
Constant			<0.001

The use of amikacin, gentamicin, and levofloxacin was shown to reduce the likelihood of fatal outcome.

Conversely, in patients with infections caused by amikacin-resistant *S. marcescens*, the probability of adverse outcomes was higher, with the difference reaching statistical significance ($p = 0.004$).

Discussion

A noteworthy finding is that in 10.9% of cases, patient records lacked information on the type of antibiotic therapy administered. For some individuals, this may be attributed to short hospital stays, limiting the possibility of detailed documentation of treatment. In others, the primary reason for admission was the continuation of therapy for chronic non-infectious conditions (e.g., malignant diseases, central or peripheral nervous system disorders), where antibiotic therapy may not have constituted a central component of care. The higher proportion of unfavourable outcomes associated with certain therapeutic regimens cannot be interpreted as a direct causal effect of antibiotic treatment but more likely reflects the severity of the clinical cases in which broad-spectrum combinations were applied. Determining whether specific regimens exert a statistically significant impact on infection outcomes requires further multifactorial analyses, including assessment of disease severity, underlying risk factors, and timing of treatment initiation. Despite these limitations, our findings demonstrate favourable clinical effectiveness of combined β-lactam and aminoglycoside therapy, characterised by lower mortality rates and a predominance of positive clinical outcomes.

Available evidence regarding the effectiveness of combination therapy in *S. marcescens*-associated infections remains conflicting. A 2022 study on antimicrobial treatment of bacteraemia and endocarditis did not identify superiority of any particular combination (Slain D, 2022). In contrast, a 2023 investigation involving patients with endocarditis reported that the most frequent regimens included β-lactams combined with aminoglycosides or fluoroquinolones, with combination therapy associated with better outcomes compared with monotherapy (Shah S, 2023). Consistently, Patel emphasised that *S. marcescens* infections should be managed with an aminoglycoside combined with

an antipseudomonal β-lactam, as β-lactam monotherapy carries the risk of selecting resistant strains (Patel PK, 2024).

On the other hand, Albano et al. demonstrated a lack of synergism between aminoglycosides and β-lactams in NDM-producing *S. marcescens* strains, highlighting the therapeutic challenge posed by multidrug-resistant isolates (Albano RM, 2024). Furthermore, a 2023 systematic review concluded that although β-lactams are frequently combined with aminoglycosides in invasive infections, robust evidence supporting their superiority over monotherapy remains lacking (Zivkovic Zaric R, 2023).

The regression analysis in our study confirmed that the administration of amikacin, gentamicin, and levofloxacin was significantly associated with reduced risk of 30-day mortality, underscoring their potential role as protective factors in the therapeutic management of *S. marcescens* infections. Particularly noteworthy is the demonstrated relationship between amikacin resistance and adverse clinical outcomes. These results are consistent with existing literature, which indicates that amikacin retains high activity against *S. marcescens* and is frequently considered a preferred therapeutic option in severe infections, while resistance to this agent constitutes a negative prognostic marker (Ferreira RL, 2020; Cosimato I, 2024).

Conclusion

The use of amikacin, gentamicin, and levofloxacin emerged as an independent protective factor for reducing 30-day mortality. Conversely, resistance to amikacin was significantly associated with an increased risk of adverse outcome.

4.3. Microbiological studies on β-lactam antibiotic resistance

4.3.1. Determination of the antimicrobial susceptibility of 200 *S. marcescens* isolates

Resistance in the entire collection of clinical S. marcescens isolates (n = 200), presented in ascending order, is shown in figure 18.

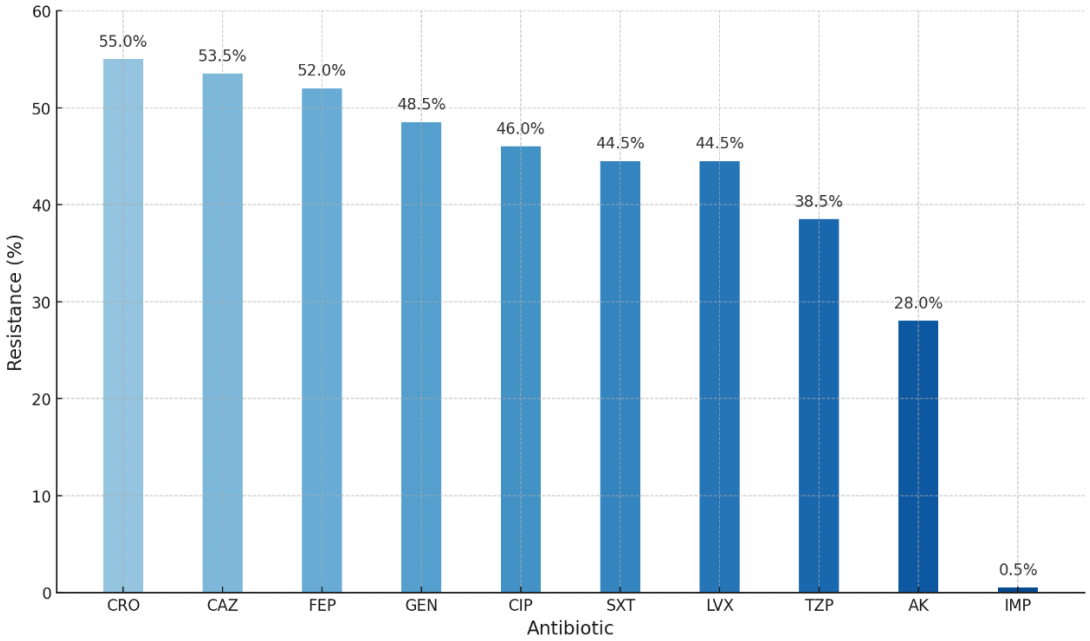


Figure 18. Antimicrobial resistance rates in the study group of *S. marcescens* isolates (n = 200)

The results demonstrated high levels of resistance among the 200 *S. marcescens* isolates in the study collection to the main antibiotic classes used in clinical practice, particularly β-lactams. The highest resistance rates were observed for third- and fourth-generation cephalosporins (ceftriaxone 55%, ceftazidime 53.5%, cefepime 52%), followed by gentamicin (48.5%), fluoroquinolones (ciprofloxacin 46%, levofloxacin 44.5%), trimethoprim/sulfamethoxazole (44.5%), and piperacillin/tazobactam (38.5%). The most active agents were imipenem (99% susceptibility), followed by amikacin (72%). Within the subgroup of carbapenem-resistant *S. marcescens*, amikacin was the only antibiotic demonstrating good in vitro activity.

Discussion

Comparable findings have been reported in the literature, although resistance rates vary widely across geographic regions and time periods. A 2019 study from Turkey analysing 158 clinical isolates reported resistance rates of 22.7% and 19.6% for ceftriaxone and ceftazidime, respectively, while resistance to cefotaxime and gentamicin was very low (0.6%) (Simsek M, 2019). In contrast, another study found resistance rates of 95.7% for amikacin, 93.5% for cefazolin, 94.1% for ampicillin/sulbactam, and lower rates for ciprofloxacin (8.5%), imipenem (11.1%), cefotetan (14.7%), and gentamicin (17.8%) (Bozkurt H, 2005). A Brazilian ICU-based study including 54 isolates from tracheal aspirates, blood, wound swabs, catheter tips, sputum, drains, and urine

demonstrated universal resistance to β-lactams, 92.6% resistance to tigecycline, and 96.3% intrinsic resistance to colistin, whereas susceptibility to fluoroquinolones (81.5%) and aminoglycosides (gentamicin 81.5%, amikacin 85.2%) remained high (Ferreira RL, 2020). Data from Central America revealed that over 80% of isolates produced ESBLs and more than 50% were multidrug-resistant (Mayorga DM, 2023). In earlier decades, resistance levels were substantially lower: a U.S. study reported 0% resistance to meropenem, 2.8% to imipenem, 8.3% to ceftazidime, 2.7% to gentamicin, and 16.9% to piperacillin/tazobactam (Jones RN, 1998). Similarly, a Japanese study of 114 cases of *S. marcescens* infections over two years demonstrated high resistance to piperacillin, third- and fourth-generation cephalosporins, newer fluoroquinolones, and aminoglycosides (Marumo K, 2000).

Conclusion

Susceptibility testing of the studied collection revealed high levels of resistance to third- and fourth-generation cephalosporins (ceftriaxone 55%, ceftazidime 53.5%, cefepime 52%), with additional resistance rates of 48.5% for gentamicin, 46% for ciprofloxacin, 44.5% for trimethoprim/sulfamethoxazole, 44.5% for levofloxacin, and 38.5% for piperacillin/tazobactam. Amikacin retained 72% activity, and imipenem remained the most effective agent (99% susceptibility). Among carbapenem-resistant isolates, only amikacin demonstrated reliable in vitro activity. Comparative analysis identified a statistically significant association between resistance to third-generation cephalosporins and resistance to other antibiotic classes.

- **Antibiotic resistance of *S. marcescens* isolates resistant to third-generation cephalosporins against other antibiotic classes**

In the subgroup of *S. marcescens* isolates resistant to ceftazidime and ceftriaxone (n = 108; 54.0%), the levels of resistance to other antibiotic agents are presented in ascending order in Figure 19.

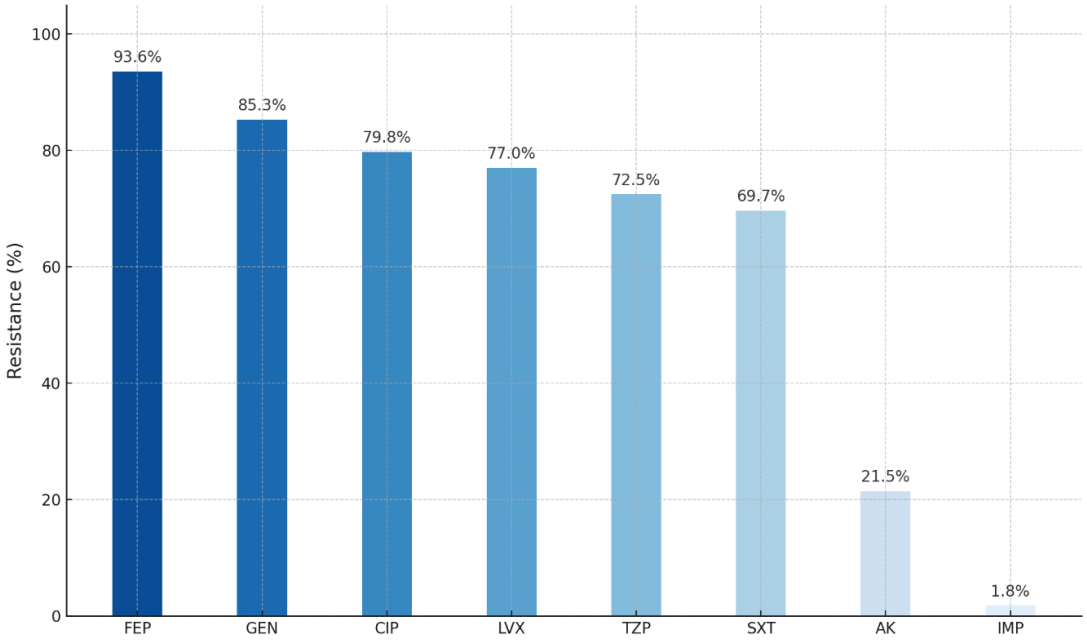


Figure 19. Antimicrobial resistance rates of 108 *S. marcescens* isolates resistant to third-generation cephalosporins (%)

A comparative analysis of resistance levels between third-generation cephalosporin-resistant and -susceptible isolates against other antibiotic classes is presented in Table 8.

Table 8. Comparison of resistance rates between third-generation cephalosporin-resistant and -susceptible isolates to non-β-lactam antibiotics

	CRO Resistant N=108	**CRO susceptible N=92**	**P - value**
AK resistant	44(40.7%)	**64 (59.3)**	**< 0.001**
AK susceptible	64 (59.3)	**77 (83.7)**	
GEN resistant	96 (88.9%)	4 (4.3%)	**< 0.001**
GEN susceptible	12 (11.1%)	88 (95.7%)	
LVX resistant	87 (80.6%)	21 (19.4%)	**< 0.001**
LVX susceptible	3 (3.3%)	89 (96.7%)	
SXT resistant	79 (73.1%)	21 (19.4%)	**< 0.001**
SXT susceptible	6 (6.5%)	86 (93.5%)	

The comparative analysis demonstrated a statistically significant association between resistance to third-generation cephalosporins and resistance to other antibiotic classes, underscoring the tendency toward multidrug resistance among *S. marcescens* isolates.

Discussion

The molecular mechanisms underlying this association involve the fact that plasmids carrying β-lactam resistance genes (IncFI, IncFII, IncA/C, IncM/L, and IncHI2) often harbor not only genes encoding β-lactamases but also genes mediating resistance to quinolones (*qnr, aac-(6')-Ib-cr, qepA, oqxAB*) and determinants associated with resistance to other antimicrobial agents such as aminoglycosides and trimethoprim/sulfamethoxazole. This explains the frequent co-occurrence of β-lactam resistance with resistance to other antibiotic groups and the multidrug-resistant (MDR) phenotype observed in these isolates (Ivanova D, 2008). Consequently, the clonal spread of MDR-ESBL-producing *S. marcescens* may substantially restrict therapeutic options. Furthermore, the intrinsic resistance of *Serratia* to colistin, a last-resort agent for MDR Gram-negative *Enterobacterales*, further limits treatment alternatives.

Conclusion

Isolates of *S. marcescens* resistant to third- and fourth-generation cephalosporins (n = 108) exhibited high rates of resistance to additional antibiotic classes: gentamicin (85.3%), ciprofloxacin (79.8%), levofloxacin (77.0%), piperacillin/tazobactam (72.5%), trimethoprim/sulfamethoxazole (69.7%), and amikacin (21.5%). The comparative analysis confirmed a statistically significant association between resistance to third-generation cephalosporins and resistance to other antibiotic classes, highlighting the pronounced trend toward multidrug resistance in *S. marcescens* isolates.

- **Antibiotic resistance according to the anatomical site of isolation**

The results of antimicrobial susceptibility testing, stratified by anatomical site of isolation, are presented in Figure 20.

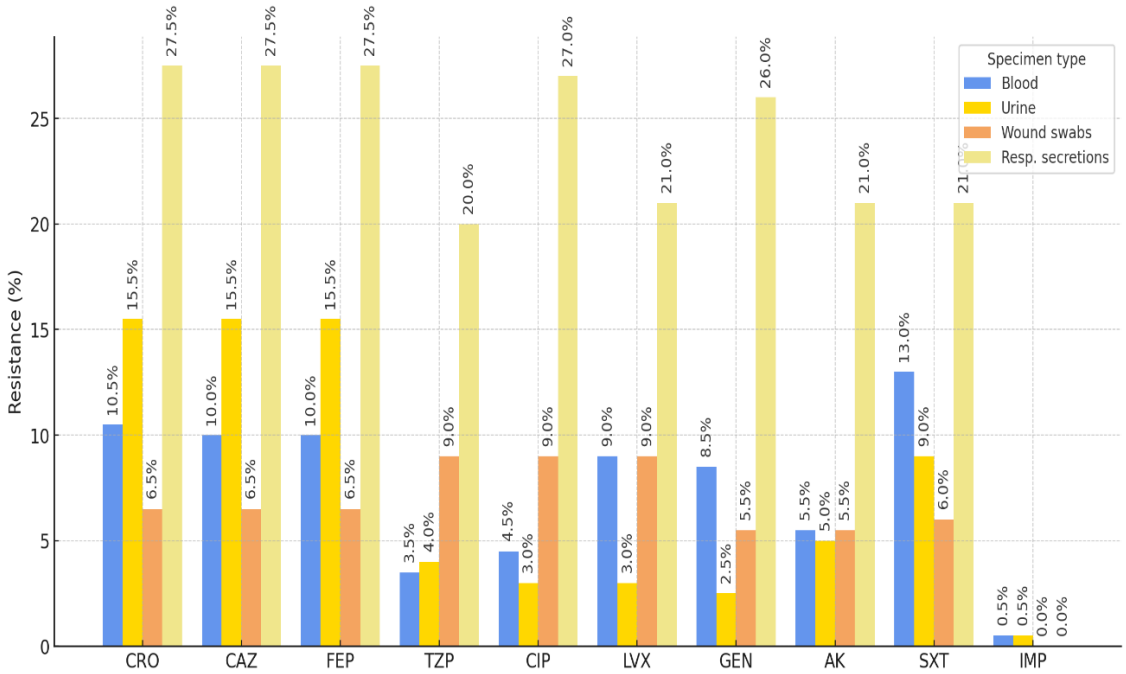


Figure 20. Antimicrobial resistance rates of 200 clinical *S. marcescens* isolates by anatomical site of isolation (%)

In the present study, antibiotic resistance among *S. marcescens* isolates was found to vary depending on the anatomical site of isolation. Isolates recovered from blood and urine demonstrated significantly higher resistance rates to all tested antimicrobial agents compared with those obtained from other clinical specimens. Within this group, the highest resistance was observed to third-generation cephalosporins—ceftriaxone and ceftazidime. Among blood isolates, resistance to these agents reached 10.5%, whereas for urine isolates it was 27.5%. In contrast, isolates obtained from wound and respiratory secretions exhibited markedly lower resistance rates to third- and fourth-generation cephalosporins, with only 5.5% demonstrating resistance to these agents. Across all four specimen types (blood, urine, wound, and respiratory isolates), the most active antibiotics were imipenem and meropenem, followed by amikacin.

Conclusion

S. marcescens isolates from urine, as well as invasive isolates from blood, exhibited significantly higher resistance to all tested antimicrobial agents compared with isolates from other clinical sources, with the highest resistance observed to third-generation cephalosporins. Regardless of the site of isolation, imipenem, meropenem, and amikacin retained the highest activity. The carbapenem-resistant *S. marcescens* isolate demonstrated preserved activity only against amikacin

4.4. Mechanisms of Resistance to β-lactam Antibiotics

4.4.1. Phenotypic Methods for the Detection of β-Lactamase Production

- **Double-Disk Synergy Test (DDST) for the Detection of Extended-Spectrum β-Lactamase (ESBL) Production**

For the purpose of ESBL screening, the double-disk synergy test (DDST) according to the method of Jarlier was applied to 108 clinical isolates previously identified as resistant to third-generation cephalosporins. A positive result was observed in 60 isolates, demonstrated by synergistic expansion of the inhibition zones between the disks containing a third-generation cephalosporin (CRO and/or CAZ) and the amoxicillin/clavulanic acid (AMC) disk. In 6 isolates, an antagonistic effect was noted, while in 17 isolates synergy was accompanied by antagonism, most likely attributable to induction of chromosomal AmpC enzymes by clavulanic acid. In 25 isolates, no interaction between the disks was detected.

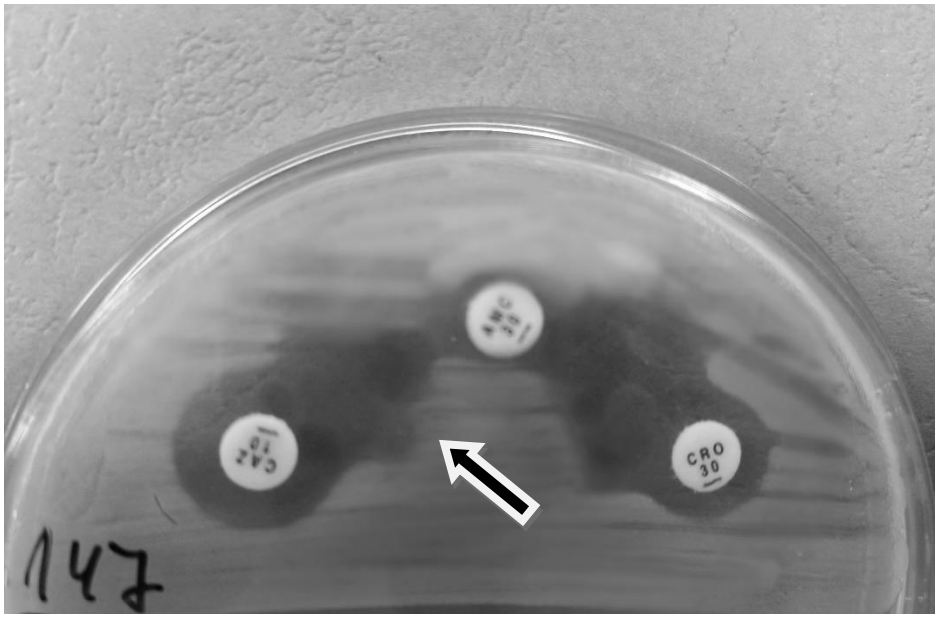


Figure 21. Double-Disk Synergy Test (DDST) for Detection of Extended-Spectrum β-Lactamase (ESBL) Production: Presence of a synergy zone between amoxicillin/clavulanic acid 20/10 (AMC 30) and ceftriaxone 30 µg (CRO 30) and

ceftazidime 30 µg (CAZ 30) disks.

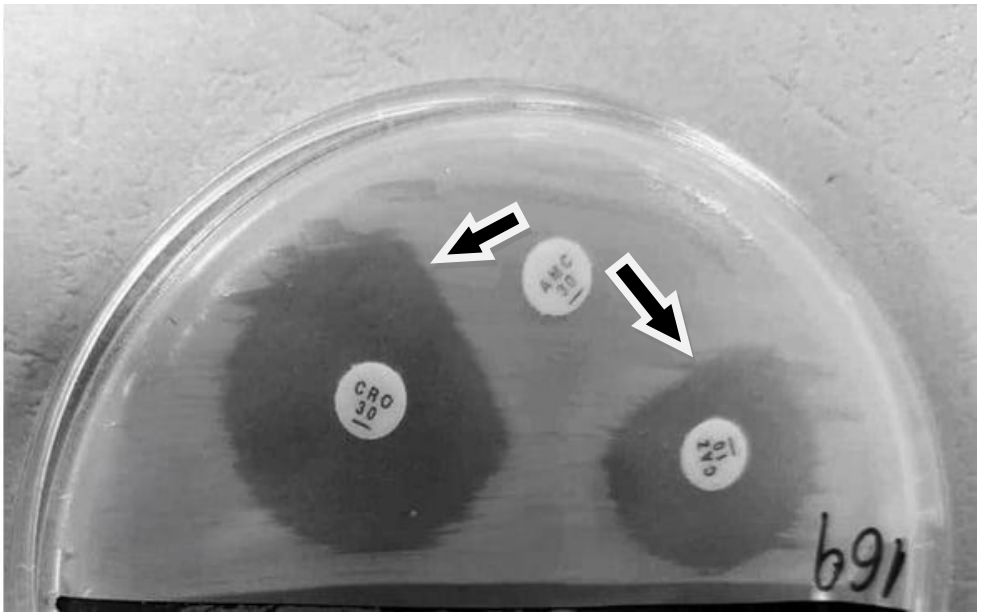


Figure 22. Double-Disk Synergy Test (DDST) for Detection of Extended-Spectrum β-Lactamase (ESBL) Production: Presence of an antagonism zone between amoxicillin/clavulanic acid 20/10 (AMC 30) and ceftriaxone 30 µg (CRO 30) and ceftazidime 30 µg (CAZ 30) disks.

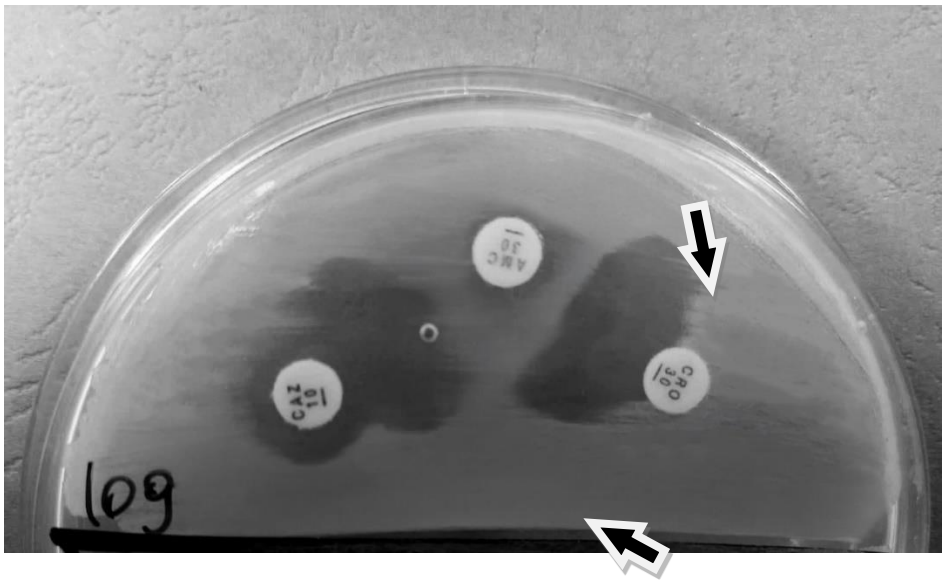


Figure 23. Double-Disk Synergy Test (DDST) for Detection of Extended-Spectrum β-Lactamase (ESBL) Production: Demonstration of synergy and antagonism zones between amoxicillin/clavulanic acid 20/10 (AMC 30) and ceftriaxone 30 µg (CRO 30) and ceftazidime 30 µg (CAZ 30) disks.

Subsequent PCR analyses identified a total of 95 isolates as ESBL producers. In comparison, the DDST showed positive results in 60 isolates, corresponding to a sensitivity of 62.3% (60/95).

- **Modified Hodge Test (MHT)**

The Modified Hodge Test was performed on the two carbapenem-resistant isolates (Figure 24). Both isolates showed positive results. Subsequent PCR assays confirmed these isolates as carbapenemase producers.

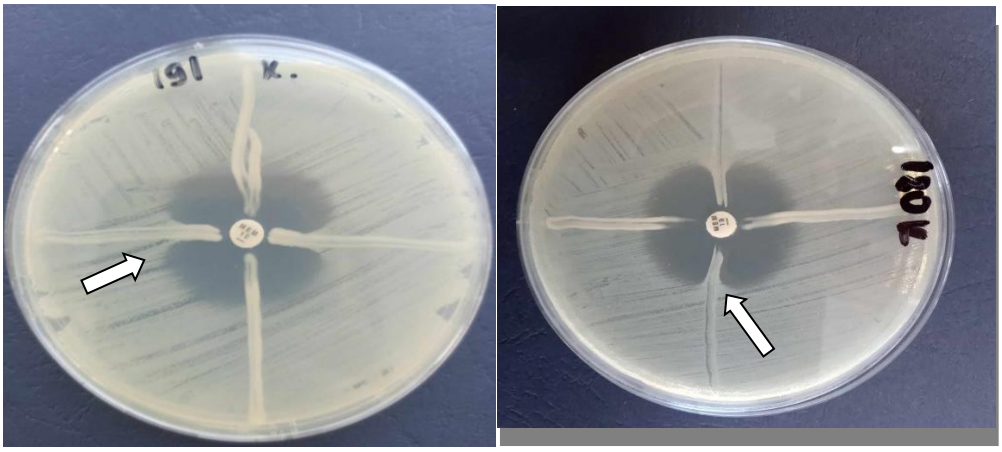


Figure 24. Modified Hodge Test

In one of the isolates, a synergy phenomenon was demonstrated between the ceftazidime/avibactam and meropenem disks. Subsequent PCR analysis confirmed the presence of a KPC carbapenemase (figure 25).

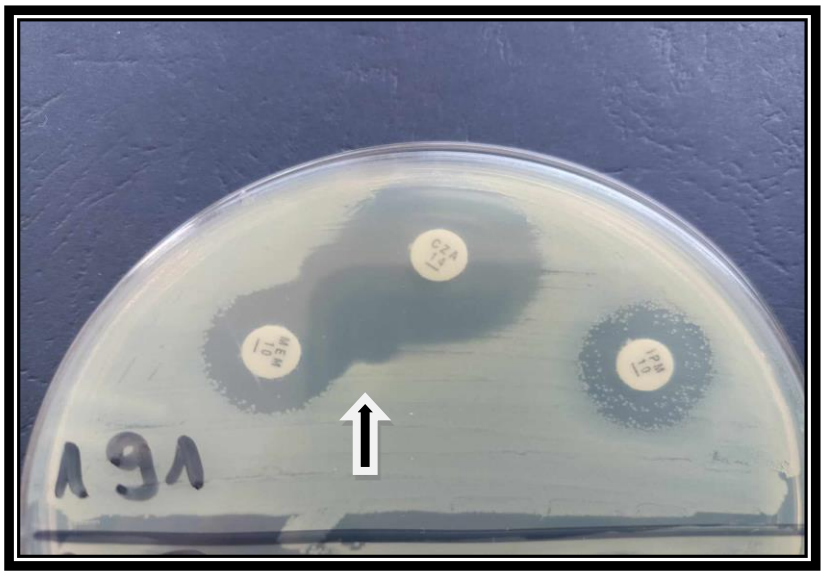


Figure 25. Synergy Phenomenon

- **Detection of Carbapenemases Using an Immunochromatographic Method**

The two carbapenem-resistant isolates were tested using a rapid immunochromatographic assay, with one isolate yielding a positive result (figure 26).

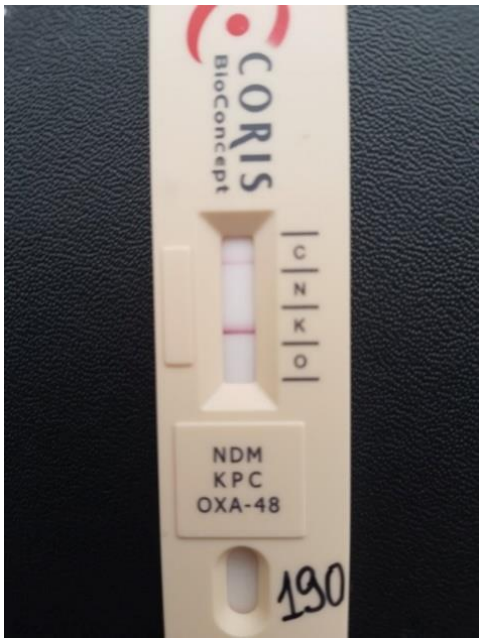


Figure 26. Detection of Class A β-Lactamases Using a Rapid Immunochromatographic Test

Discussion

In the present study, the sensitivity of the double-disk synergy test (DDST) in *S. marcescens* isolates was found to be lower compared with other members of the *Enterobacterales* order. This can be attributed to the fact that *S. marcescens* is a natural AmpC β-lactamase producer, and AmpC enzymes are not inhibited by clavulanic acid. As a result, the ESBL effect is often masked, leading to false-negative outcomes and thus reducing the diagnostic utility of this method in this species. These observations are consistent with previous reports, which emphasise the limited reliability of DDST in isolates harbouring AmpC β-lactamases (Jacoby, 2009). Nevertheless, DDST remains one of the most widely used phenotypic assays for rapid ESBL screening due to its simplicity and accessibility in routine microbiological practice (Jarlier V, 1988; EUCAST, 2017). A range of alternative methods for ESBL detection has been described (Polsfuss S, 2012; Fahim Q, 2017; Shaikh NK, 2016), which may complement or confirm phenotypic screening results. According to current EUCAST recommendations, susceptibility to cephalosporins in *Enterobacterales* should primarily be interpreted based on inhibition zone diameters, while phenotypic ESBL testing is now used predominantly for

epidemiological purposes (EUCAST, 2024). A synergistic effect was also observed between the discs loaded with ceftazidime/avibactam and meropenem in an isolate confirmed as a KPC-carbapenemase producer. This finding is consistent with the known activity of avibactam against class A enzymes, including KPC, which explains the observed synergism.

Various phenotypic methods are available for carbapenemase detection, yet none are universally reliable. The Modified Hodge Test (MHT), while straightforward to perform, frequently produces false-negative or weakly positive results, particularly for metallo-β-lactamases. As an alternative, immunochromatographic assays have been introduced in recent years, offering speed, ease of interpretation, and the ability to detect the most clinically relevant carbapenemase enzymes—KPC, NDM, and OXA-48 in the context of this study. These tests exhibit high specificity (>95%), although their sensitivity varies depending on the strain and enzyme type (Boutal H, 2018). In one study of 299 carbapenem-resistant clinical isolates, the NG-Test Carba 5 achieved an accuracy of 99.6–100% (Zhu Y, 2021). In the present investigation, a positive result was obtained for only one of the two carbapenem-resistant isolates. Although the small sample size precludes firm conclusions, this finding highlights the potential limitations of phenotypic assays and aligns with previous reports indicating that sensitivity may differ according to the specific carbapenemase produced. Despite these limitations, immunochromatographic tests remain valuable tools for rapid screening, enabling timely epidemiological interventions. However, for definitive confirmation and molecular characterisation of carbapenemase production, molecular methods continue to represent the gold standard.

Conclusion

The double-disk synergy test using third-generation cephalosporins and amoxicillin/clavulanic acid (20/10) demonstrated an expectedly reduced sensitivity of 62.3% for ESBL detection in *S. marcescens* isolates. In contrast, the Modified Hodge Test for carbapenemase detection exhibited excellent sensitivity (100%). The rapid immunochromatographic assay showed a positive result in only one of the two carbapenem-resistant isolates.

4.4.2. Molecular-Genetic Identification of ESBLs

4.4.2.1 PCR for Detection of β-Lactamase-Encoding Genes

To investigate the genetic mechanisms underlying β-lactam resistance, PCR was employed to detect the most commonly encountered genes encoding extended-spectrum β-lactamases of class A (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX,}$ *bla*$_{KPC}$), class B (bla$_{VIM}$, bla$_{NDM}$, bla$_{IMP}$) and class D (bla$_{OXA-48}$).

•PCR for Detection of Genes Encoding CTX-M, SHV, and TEM ESBLs

Multiplex PCR targeting *bla*$_{CTX-M}$ and *bla*$_{SHV}$ was performed on all 108 *S. marcescens* isolates. Amplification generated PCR products of 290 bp for *bla*$_{SHV}$ and 585 bp for *bla*$_{CTX-M}$, respectively (Figure 27). The *bla*$_{CTX-M}$ gene was detected in 71% of isolates (n = 77), while *bla*$_{SHV}$ was identified in a single isolate (0.9%).

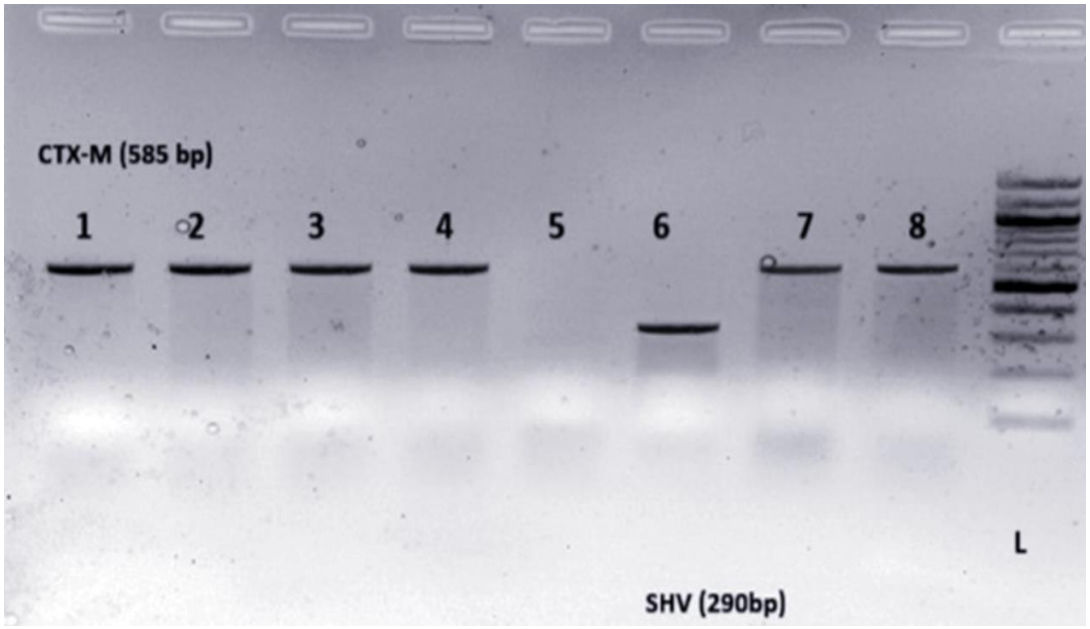


Figure 27. PCR Detection of Genes Encoding CTX-M and SHV ESBLs; L, 100 bp DNA Marker; *bla*$_{CTX-M}$ was detected in isolates **1, 2, 3, 4, 7, and 8** ***bla*$_{SHV}$** was detected in isolate **6**; No gene was detected in isolate **5**.

In the present study, the *bla*$_{SHV}$-positive isolate was confirmed as an ESBL producer. The strain was recovered from a 21-year-old patient with a history of multiple hospitalisations in both domestic and foreign healthcare settings, and with underlying comorbidities, including cerebral palsy. The isolate was obtained from a tracheal aspirate, in polymicrobial association with *S. aureus* (MSSA), during ongoing antibiotic therapy with amoxicillin/clavulanic acid. The clinical course was unfavourable, resulting in a fatal outcome.

For all 108 *S. marcescens* isolates, singleplex PCR was performed for the detection of *bla*$_{TEM}$, generating products of 1075 bp. The *bla*TEM gene was detected in 56 isolates (52%).

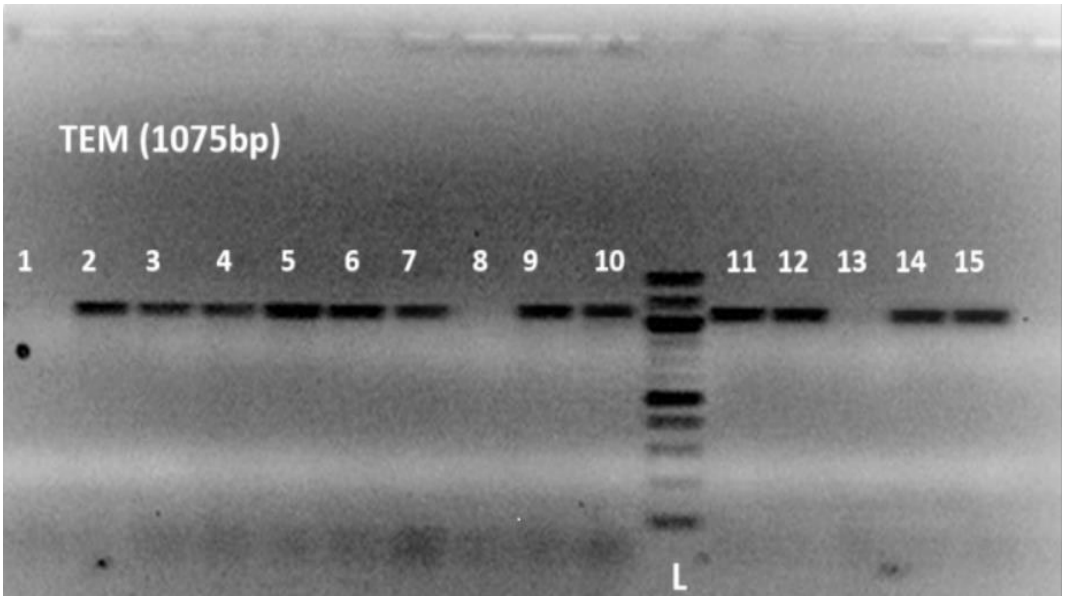


Figure 28. PCR detection of *bla*$_{TEM}$; L, 100 bp DNA ladder; isolates 1, 8, and 13 – no gene detected; isolates 2–7, 9–12, 14, and 15 – detection of *bla*$_{TEM}$. No *bla*$_{TEM}$ gene was detected in isolates 1, 8, and 13. Positive detection of *bla*$_{TEM}$ was observed in isolates 2–7, 9–12, 14, and 15.

- **Detection of genes encoding AmpC enzymes. One of the carbapenem-resistant isolates tested positive for the presence of *bla*$_{CMY-2}$ using the real-time PCR assay MM GRAM NEG RES (Alifax S.r.l, Italy).**

Detection of class A (KPC) and class B carbapenemase genes (*bla*$_{NDM}$, *bla*$_{VIM}$, *bla*$_{IMP}$) Both carbapenem-resistant isolates were tested using the real-time PCR assay MM GRAM NEG RES (Alifax S.r.l, Italy). One isolate was confirmed to carry *bla*$_{VIM}$, while the other tested positive for *bla*$_{KPC}$. The results are shown in Figure 29 and Figure 30.

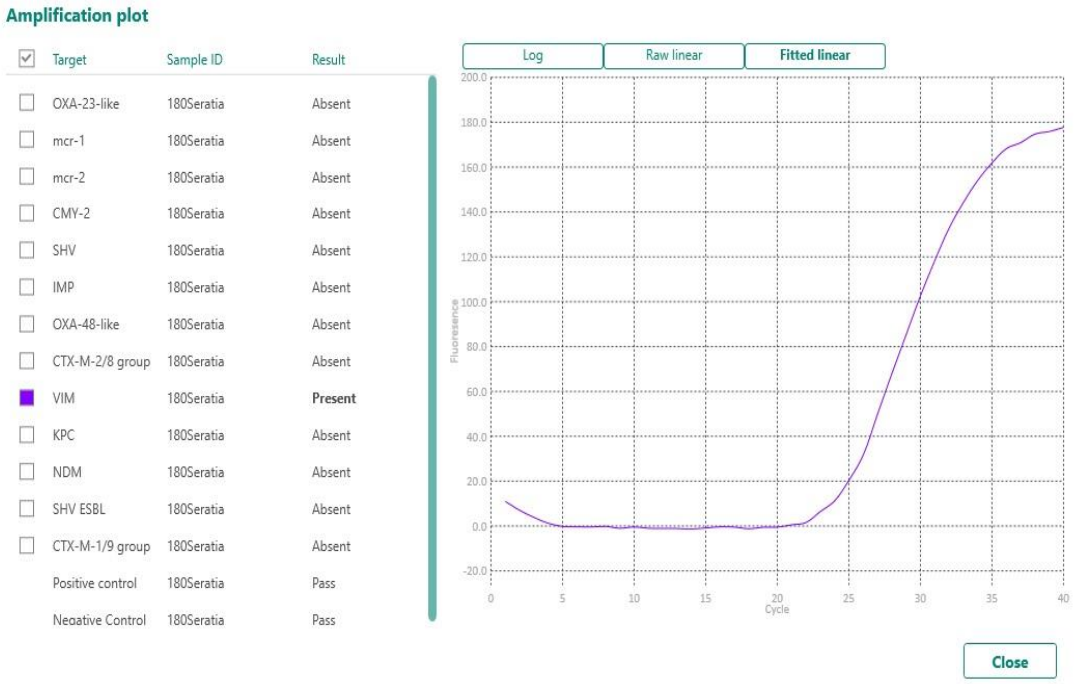


Figure 29. Detection of *bla*$_{VIM}$ чрез real-time PCR MM GRAM NEG RES

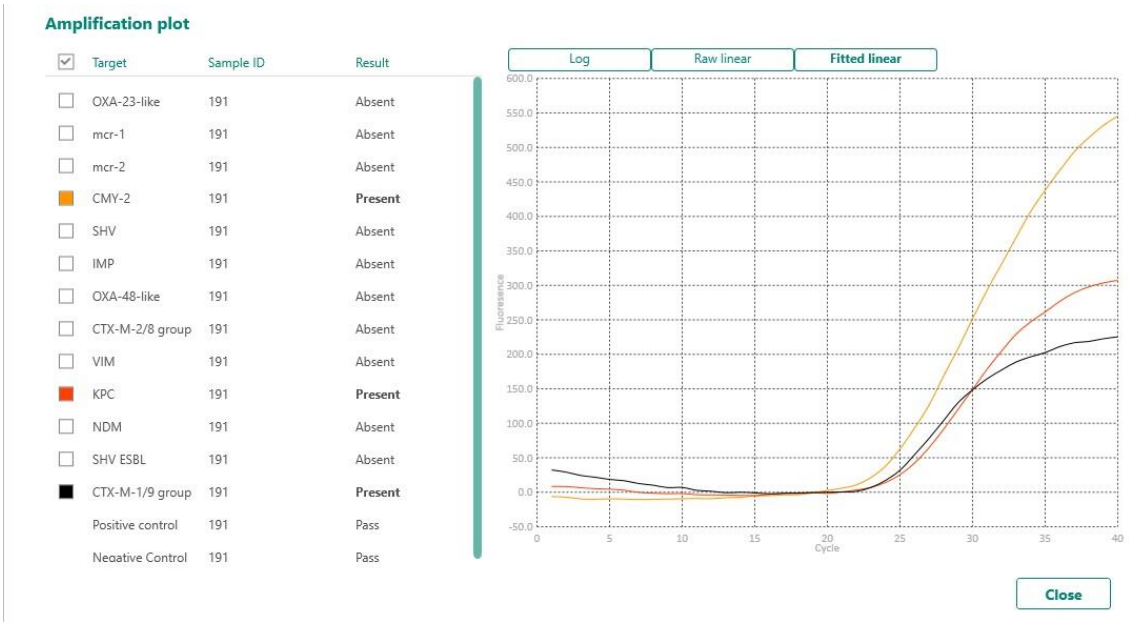


Figure 30. Detection of *bla*$_{CMY-2}$, *bla*$_{KPC}$, and *bla*$_{CTX-M}$ Genes by Real-Time PCR (MM GRAM NEG RES Panel)

The isolate identified in our study represents the first documented Bulgarian *S. marcescens* isolate confirmed as a producer of KPC carbapenemase. It was recovered from a blood culture obtained from a patient admitted with COVID-19 infection, who also had multiple comorbidities, including diabetes mellitus, ischemic heart disease, and an aortic aneurysm, as well as a history of prior hospitalization in the same hospital.

The *bla*$_{VIM}$ -harbouring isolate was recovered from a urine sample of a patient with bladder carcinoma and additional comorbidities. During treatment, the patient received antibiotic therapy with cefuroxime, amikacin, and metronidazole, and was discharged in improved condition.

- **Detection of class D β-lactamase genes (OXA-48)**

No isolates carrying the *bla*$_{OXA-48}$ gene were detected in the studied collection.

Table 10. Frequency of detected resistance genes among *S. marcescens* isolates (n, %).

Gene	Number of *S. marcescens* Isolates
bla$_{CTX-M}$	36 (33.3%)
bla$_{TEM}$	19 (17.6%)
bla$_{CTX-M}$ + *bla*$_{TEM}$	37 (34.3%)
bla$_{SHV}$	1 (0.9%)
bla$_{CTX-M}$ + *bla*$_{VIM}$	1 (0.9%)
bla$_{CTX-M}$+*bla*$_{KPC}$+ *bla*$_{CMY}$	1 (0.9%)

Two of the isolates carrying *bla*$_{TEM}$ displayed resistance to third-generation cephalosporins while remaining susceptible to fourth-generation agents.

Discussion

More than 400 distinct ESBL enzymes have been described worldwide, distributed across diverse members of the Enterobacterales (Lawrence J, 2024). Multidrug-resistant *S. marcescens* strains have been linked to more invasive infections and are frequently associated with rapid nosocomial dissemination (Vilacoba E, 2014; Álvarez VE, 2020; Bielli A, 2020).

Chromosomally mediated β-lactamases are widespread among Gram-negative bacteria. The first plasmid-mediated β-lactamase, TEM-1, was reported in the 1960s (Datta, 1965). Initially identified in an *E. coli* isolate obtained from the blood of a patient in Greece named Temoniera—hence the acronym TEM—this enzyme rapidly disseminated via plasmids and transposons to multiple bacterial species (Medeiros, 1984). Within just a few years of its discovery, TEM-1 had spread globally and is now found in various Enterobacterales, as well as in *P. aeruginosa*, *Haemophilus influenzae*, and *Neisseria gonorrhoeae*. In the present study, two isolates carrying only *bla*$_{TEM}$ genes

exhibited resistance to third-generation cephalosporins but remained susceptible to cefepime, while the remaining isolates were resistant to both third- and fourth-generation agents. This phenotype is most likely attributable to the production of extended-spectrum β-lactamases (ESBLs) of the TEM type, such as TEM-3, TEM-10, or TEM-52. Unlike the classical TEM-1 and TEM-2 enzymes, which do not hydrolyse third-generation cephalosporins, these variants possess an extended substrate profile. Cefepime, however, often retains activity due to its stability against hydrolysis by some ESBLs and its superior penetration through the Gram-negative outer membrane, including that of *S. marcescens*. Additional mechanisms, such as porin loss or efflux activity, may also explain differential resistance to third- versus fourth-generation cephalosporins.

Another important plasmid-mediated β-lactamase is SHV-1 (sulfhydryl variable), initially identified in *K. pneumoniae* and *E. coli* (Wyres KL, 2018). By the late 1990s, European surveys of ESBLs reported mainly SHV variants (SHV-2 and SHV-5), predominantly in *Klebsiella spp.* (Livermore, 2007). Subsequent studies have described *S. marcescens* isolates carrying $bla_{\text{SHV-12}}$ (Crivaro V, 2007; Ivanova D, 2008; Musafer HK, 2024; Pérez-Viso B, 2024), and the detection of $bla_{\text{SHV-134}}$ in carbapenem-resistant strains (Bolourchi N, 2022).

CTX-M enzymes, rarely reported until the mid-1990s, have since emerged as the most widespread ESBLs globally. Initially linked to outbreaks of *S. Typhimurium* producing CTX-M-4 and CTX-M-5 in several European countries, CTX-M variants are now the dominant ESBLs in clinical Enterobacterales isolates (Livermore, 2007). More than 130 CTX-M enzymes have been characterised, grouped into seven clusters, with CTX-M-15 and CTX-M-14 being the most prevalent, followed by CTX-M-2, CTX-M-3, and CTX-M-1 (Zhao WH, 2013; Pitout JD, 2010).

In a 2011 study conducted at University Hospital “Sveta Marina” -Varna, CTX-M-3 was identified as the dominant enzyme, present in 83% of *S. marcescens* isolates—a finding confirmed by the current investigation (Markovska RD, 2014). Other European studies similarly report high rates of CTX-M production among *S. marcescens*, often alongside TEM variants (Mlynarczyk A, 2009). In a recent study involving 322 clinical isolates, 166 (51.6%) were identified as ESBL-positive using phenotypic methods. The prevalence of ESBL production among *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *Acinetobacter* spp., *P. mirabilis*, *Enterobacter* spp., *Citrobacter* spp., and *S. marcescens* was 55.3%, 63.4%, 17.8%, 57.1%, 33.3%, 28.5%, 38.4%, and 4%, respectively. Of the

322 isolates, 144 were tested for the presence of *bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$ and *bla*$_{\text{SHV}}$ genes. PCR analysis confirmed that 85 (59%) harbored at least one of these genes. The prevalence of *bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$, and *bla*$_{\text{SHV}}$ genes was 60%, 57.6%, and 38.3%, respectively. Among the tested antibiotics, meropenem and amikacin exhibited the highest susceptibility rates (83.1% and 82.5%, respectively), whereas amoxicillin (3.1%) and cephalexin (13.9%) were the least effective. ESBL-producing isolates demonstrated high levels of resistance to cefotaxime, ceftriaxone, and ceftazidime (79.5%, 78.9%, and 79.5%, respectively) (El Aila NA, 2023).

In 2022, a multidrug-resistant *S. marcescens* strain (YL4) was isolated in China, harboring three chromosomally located antibiotic resistance genes: *bla*$_{\text{SRT-1}}$, associated with resistance to β-lactam antibiotics (aminopenicillins); *aac(6')-Ic*, conferring resistance to aminoglycosides; and *tet*(41), responsible for tetracycline resistance, as well as *mcr-9*, mediating colistin resistance. In addition to these chromosomal determinants, the strain carried two plasmids (pYL4.1 [321,744 bp] and pYL4.2 [46,771 bp]) that encoded further resistance genes. Plasmid pYL4.1 harbored *bla*$_{\text{IMP}}$ (encoding a metallo-β-lactamase), mcr-9 (an additional copy of the colistin resistance gene), two copies of *bla*$_{\text{TEM-1B}}$, *aac(3)-IId*, *aadA2*, *dfrA12*, *sul*1 (conferring resistance to trimethoprim and sulfonamides), and qacEΔ1 (associated with resistance to cationic antiseptics). Plasmid pYL4.2 carried *bla*$_{\text{TEM-1B}}$, *qnrB6* (fluoroquinolone resistance), *aac(6')-Ib-cr* (conferring modification-mediated resistance to both aminoglycosides and ciprofloxacin), as well as *sul1* and *qacEΔ1*, identical to those identified in pYL4.1. The combination of chromosomal and plasmid-mediated resistance determinants, encompassing β-lactams, aminoglycosides, tetracyclines, fluoroquinolones, sulfonamides, colistin, and antiseptics, classifies this strain as an extensively drug-resistant (XDR) organism (Zhong Y, 2022).

It is hypothesised that mcr-9 was integrated into *S. marcescens* YL4 through plasmid-mediated transfer — a mechanism that represents a major challenge, as it may potentially facilitate the dissemination of colistin resistance among bacteria that do not inherently possess this trait. Horizontal gene transfer of resistance determinants has been documented in previous studies (Liu Z, 2021). A similar mechanism was described in 2016, when the *mcr-1* gene was first identified in *E. coli* isolates from China (Liu YY, 2016). Following its discovery, numerous reports documented the emergence of *mcr-1* to *mcr-10* across diverse bacterial species and geographic regions (Ling Z, 2020; Sun J, 2018; Wang C, 2020). To date, *mcr-9* has been detected in 40 countries spanning six

continents, underscoring the urgent need for enhanced epidemiological surveillance and control of plasmid-mediated colistin resistance (Liu Z, 2021).

In 2022, four carbapenem-resistant *S. marcescens* isolates were found to harbour the *bla*$_{\text{OXA-48a}}$ gene, located on an *mcr*-plasmid (Bolourchi N, 2022). The IncL-pOXA-48a plasmid had previously been identified among carbapenem-resistant *S. marcescens* strains in Spain (Perez-Viso B, 2021). During 2016–2017, a total of 32 OXA-48-producing *Enterobacterales* were reported in Israel, of which 81% were *S. marcescens* (Regev-Yochay G, 2018).

Numerous cases of KPC-producing *S. marcescens* have also been documented in the literature (Tsakris A, 2010; Cai JC, 2008). A study from 2020 reported that all isolates expressed KPC carbapenemase (*bla*$_{\text{KPC}}$) in combination with *bla*$_{\text{TEM}}$ genes, while 14.8% also carried *bla*$_{\text{OXA-1}}$, and 16.7% carried genes from the *bla*$_{\text{CTX-M-1}}$ group. The efflux-related genes *SdeB/HasF* and *SdeY/HasF* were identified in 88.9% of isolates, whereas *aac(6')-Ib-cr*, a gene conferring resistance to aminoglycosides and fluoroquinolones, was detected in 24.1%. In the same cohort, no variants of *bla*$_{\text{SHV}}$, *bla*$_{\text{IMP}}$, *bla*$_{\text{OXA-48}}$, blaNDM, *bla*$_{\text{VIM}}$, or *mcr-1* were detected (Ferreira RL, 2020). In Brazil, an investigation of 10 *S. marcescens* isolates demonstrated that all carried the *bla*$_{\text{KPC-2}}$ gene, with infections caused by these strains being associated with high mortality among affected patients (Cruz TTPS, 2021). Importantly, KPC enzymes frequently coexist with other β-lactamases, including ESBLs and AmpC enzymes, thereby contributing to complex resistance phenotypes.

A study involving 58 patients with infection or colonisation by KPC-producing *Klebsiella pneumoniae* reported that 36% of cases were admitted from nursing homes (Chen LF, 2012). The mean time to isolation of these microorganisms was 1.5 days after hospital admission, suggesting that infections may be community-acquired and transmitted person-to-person. Community spread of KPC-producing *K. pneumoniae* has also been described in a patient who likely acquired the isolate from his wife, who had been previously hospitalised (Gottesman T, 2008). Moreover, a case of community-acquired infection caused by a KPC-2-producing *S. marcescens* has been documented (Margate E, 2015). Notably, the isolate identified in our study represents the first Bulgarian *S. marcescens* strain confirmed as a KPC carbapenemase producer.

In 2020, an epidemic outbreak in a long-term care facility in the United States was attributed to *S. marcescens* carrying the *bla*$_{\text{KPC-3}}$ gene. Whole-genome sequencing and

plasmid profiling revealed the involvement of three distinct *S. marcescens* clonal lineages and two plasmids harbouring bla_{KPC-3} (Jimenez A, 2020). More recently, in 2023, two *S. marcescens* isolates producing KPC-2 were identified in China from patients with bacteraemia. Genomic analysis demonstrated the presence of IncR-type plasmids carrying the bla_{KPC-2} gene (Jia J, 2023).

Class B metallo-β-lactamases (MBLs), including IMP, VIM, and NDM, represent some of the most prevalent and clinically relevant carbapenemases, owing to their ability to hydrolyse a broad spectrum of β-lactam antibiotics (with the exception of aztreonam). These enzymes are globally distributed, with the highest prevalence reported in Southeast Asia and various European countries.

VIM variants are more frequently encountered among Gram-negative non-fermenting bacteria, whereas within Enterobacterales they are most commonly identified in *K. pneumoniae*, *E. coli*, and *E. cloacae*. A global survey of VIM dissemination among *Enterobacterales* revealed the following geographic distribution: VIM-1 is widespread worldwide; VIM-2 has been reported in Mexico and Spain; and VIM-4 has been documented in Europe, including in a single *S. marcescens* isolate from the Czech Republic. In addition, VIM-5 and VIM-31 appear restricted to Turkey; VIM-19, -26, -27, and -33 to Greece; VIM-23 to Mexico; and VIM-29 to Saudi Arabia and the United Kingdom (Matsumura Y, 2017). Nosocomial outbreaks caused by VIM-producing *S. marcescens* are rarely described in the literature, with most reports originating from neonatal intensive care units (Ghaith DM, 2018). The first documented Gram-negative pathogen producing VIM was *P. aeruginosa*, which remains the most frequently reported microorganism with this resistance mechanism, followed by *K. pneumoniae* and *A. baumannii*. The first case of a VIM-producing *S. marcescens* responsible for a hospital outbreak was reported in Argentina (Nastro M, 2013), and several other outbreaks have since been described across different regions. In 2018, an outbreak in an adult intensive care unit caused by VIM-producing *S. marcescens* isolates was associated with a mortality rate of 50% (Iovene MR, 2019). In 2020, a *S. marcescens* isolate producing VIM-4 was recovered from a patient following cardiac surgery, with the sample obtained from an infected area surrounding a pacemaker implantation site (Akos T, 2020). More recently, in 2024, an outbreak in a burn unit involving various VIM-producing carbapenem-resistant bacteria, including *S. marcescens*, was reported. Several isolates

exhibited rapid development of resistance to cefiderocol, underscoring the risk of therapeutic failure even with the use of novel antimicrobial agents (Freiberg JA, 2024).

Conclusion

The principal mechanism of resistance to third-generation cephalosporins among the studied *S. marcescens* isolates was ESBL production, predominantly CTX-M (71%), followed by TEM (51%) and SHV (0.9%). One carbapenem-resistant isolate harboured bla_{KPC}—the first reported case in Bulgaria—along with bla_{CTX-M}, bla_{TEM}, and bla_{CMY-2}. A second carbapenem-resistant isolate carried bla_{VIM}. No isolates carrying bla_{OXA-48} were detected.

4.5. Investigation of hospital transmission of *S. marcescens* using RAPD and ERIC-PCR

In our study, the results obtained with RAPD, RAPD-2, and RAPD-3 proved unsatisfactory, as they did not provide sufficient discriminatory power among the analyzed strains. Comparisons were also made using RAPD-4, RAPD-5, and RAPD-6. However, for a large proportion of isolates, only 1–2 bands were generated, which precluded reliable comparison and differentiation of strains. This substantially limits the applicability of RAPD for robust epidemiological typing (figure 31).

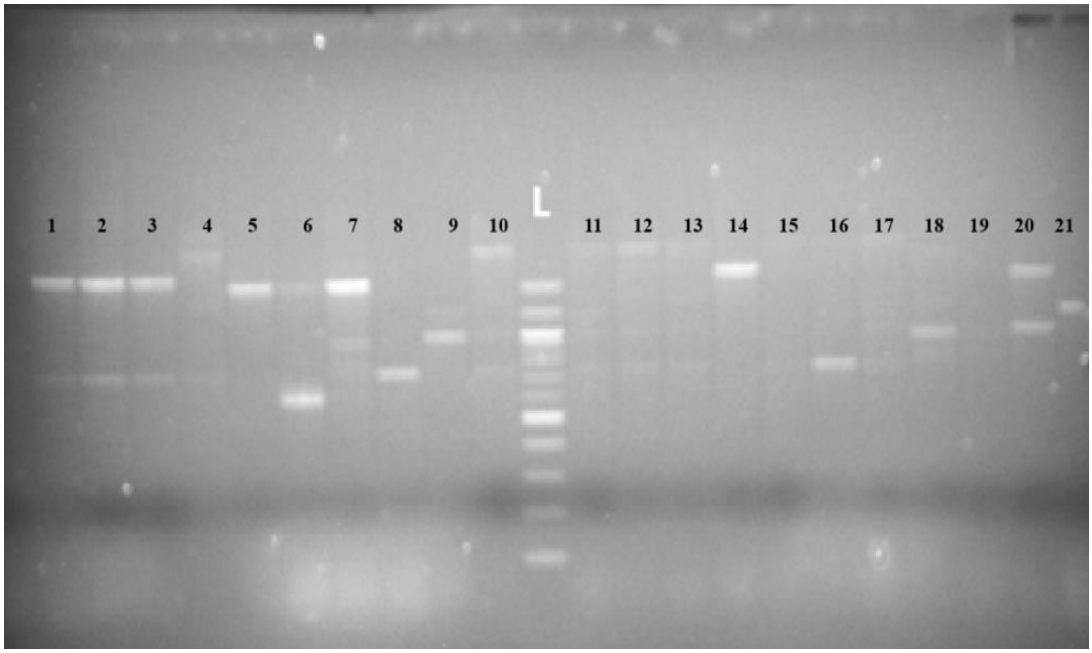


Figure 31. RAPD5-PCR of *S. marcescens* isolates using primers 5'-ACGCGCAAC-3'; L, 100 bp DNA marker

For the purposes of epidemiological typing, the ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus PCR) method was also tested. However, it did not provide satisfactory discrimination among the isolates. The obtained results confirm the limited applicability of this method for differentiating epidemic *S. marcescens* strains.

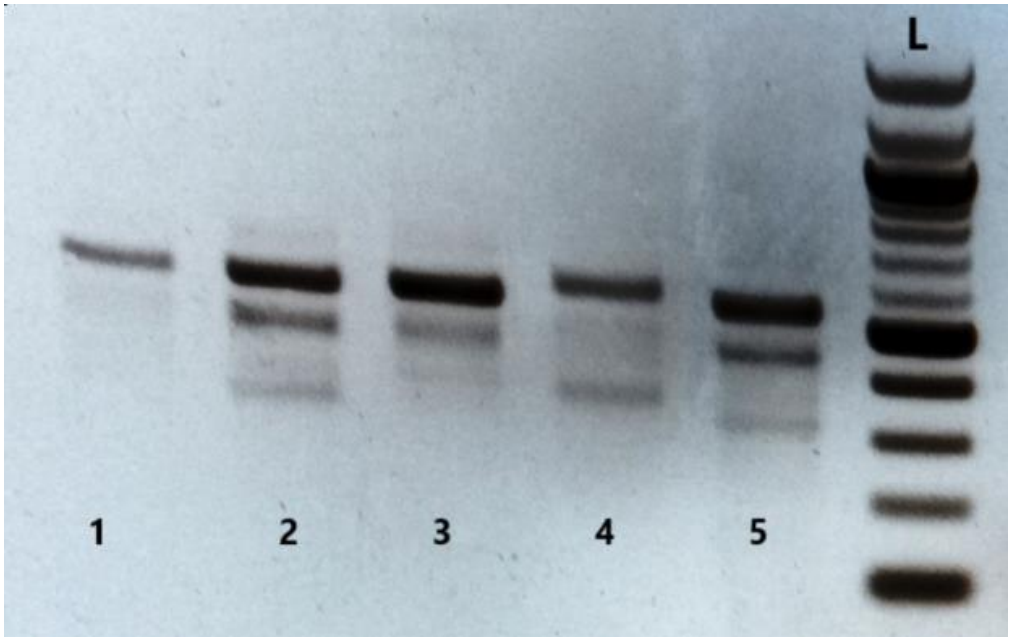


Figure 32. ERIC-PCR; L, 100 bp DNA marker

Discussion

Globally, whole-genome sequencing (WGS) has emerged as one of the most sensitive and reliable methods for epidemiological investigations, enabling detailed strain characterization and the tracking of transmission mechanisms (Rossen JWA, 2019). Despite its advantages, WGS remains considerably more costly, requires specialized equipment, and demands advanced bioinformatics expertise, limiting its routine applicability. More accessible molecular methods for the typing of *S. marcescens* include RAPD-PCR (Random Amplified Polymorphic DNA) and PFGE (Pulsed-Field Gel Electrophoresis).

In Bulgaria, Ivanova et al. applied RAPD-PCR for the epidemiological typing of 11 clinical *S. marcescens* isolates. The patterns generated with primer RAPD-2 demonstrated a high degree of genetic homogeneity—seven isolates were indistinguishable, and the remaining four differed by only a single band, suggesting a close genetic relationship. In the same study, the use of RAPD-4 showed indistinguishable profiles across all isolates (Ivanova D, 2008).

The discriminatory power of molecular typing methods such as RAPD-PCR and ERIC-PCR is influenced by primer selection, laboratory conditions, and the genomic characteristics of the studied bacterial species. In *S. marcescens*, these methods often exhibit lower discriminatory capacity compared to other Enterobacterales, such as *E. coli* and *K. pneumoniae*. One key reason is the limited and uneven distribution of repetitive elements within the *S. marcescens* genome, including ERIC sequences—essential for the efficacy of ERIC-PCR. Similarly, in RAPD-PCR, the low genetic variability within conserved genomic regions frequently results in highly similar electrophoretic profiles, even among epidemiologically unrelated strains. Furthermore, the relative genomic stability of *S. marcescens*—manifested by a lower prevalence of mobile genetic elements such as integrons, IS elements, and plasmids—further reduces PCR profile diversity (Poirel L, 2017; van Belkum A, 2007). Consequently, even the use of multiple primers fails to provide robust strain differentiation, limiting the utility of these techniques for routine epidemiological surveillance.

Conclusion

The results of the present study demonstrate that RAPD-PCR and ERIC-PCR possess limited discriminatory capacity for the epidemiological typing of *S. marcescens*. The intrinsic genomic stability and low variability of the species constrain the effectiveness of these methods in routine practice, underscoring the need for the implementation of high-resolution approaches such as PFGE or WGS.

5. Conclusions

The analysis of the results obtained in the present study, in comparison with data from the literature, allows us to draw the following conclusions:

1. The majority of *Serratia marcescens*-associated infections are nosocomial (97.5%), which identifies hospitalization as the most significant risk factor for developing an infection caused by this pathogen.
2. The highest mortality was observed among patients with neurological diseases (28.6%) and malignant conditions (17.1%). The difference between groups and clinical outcomes was statistically significant, indicating that neurological and malignant diseases are leading diagnoses associated with fatal outcomes,

3. The highest incidence was recorded in the 70–79-year age group, and the lowest in the 20–29-year group, identifying age as a significant risk factor for *S. marcescens* infection.
4. Cardiovascular (65.8%), endocrinological (31.6%), and urological (13.9%) comorbidities were shown to be significant additional risk factors for acquiring *S. marcescens*-associated infection, but not for mortality.
5. Stay in the intensive care unit was established as a major predictor both for infection occurrence and for fatal outcome.
6. The presence of a foreign body was identified as the leading risk factor for *S. marcescens*-associated infections (80.0%), followed by invasive procedures (16.0%) and intubation/mechanical ventilation (7.9%).
7. The type of infection was identified as an independent prognostic factor for the duration of hospital stay, with bloodstream infections showing the longest hospitalization and urinary infections the shortest.
8. The administration of amikacin, gentamicin, and levofloxacin was found to be an independent protective factor for reducing 30-day mortality. Resistance to amikacin was associated with a statistically significant increase in the risk of fatal outcome.
9. No statistically significant association was found between colistin consumption and the incidence of *S. marcescens*-associated infections. Despite the lack of proven correlation, the parallel increasing trend of both variables in the last years of the study (2021–2023) suggests a potential relationship requiring further monitoring and long-term analysis.
10. The main mechanism of resistance to third-generation cephalosporins in the studied *S. marcescens* isolates was associated with ESBL production, with CTX-M β-lactamases being the most prevalent (71.0%), followed by TEM (52.0%) and SHV (0.9%). The main mechanisms of carbapenem resistance were the production of KPC and VIM carbapenemases.
11. RAPD-PCR and ERIC-PCR demonstrated limited discriminatory power for the epidemiological typing of *S. marcescens*.

6. Statement of the Contributions of the Dissertation

Original Contributions

1. An in-depth analysis of the main epidemiological aspects of *Serratia marcescens*-associated infections among hospitalized patients over an eight-year period was conducted, representing the first study of its kind in Bulgaria. The main risk factors for infection acquisition and mortality were identified.
2. The resistance of clinical *S. marcescens* isolates from a large hospital facility to a wide range of antibacterial agents was tested. The obtained data enable the development of an algorithm for the therapeutic management of infections caused by *S. marcescens*.
3. A detailed study of the mechanisms of resistance to β-lactam antibacterial agents, including carbapenems, was carried out in a collection of clinical *S. marcescens* isolates, contributing to the accumulation of scientific knowledge at the local, national, and European levels.

Confirmatory Contributions

1. The molecular genetic mechanisms of β-lactam resistance in clinical *S. marcescens* isolates were confirmed, establishing the predominant role of CTX-M and TEM β-lactamases.
2. Carbapenem resistance within the studied collection was demonstrated to result from the production of KPC and VIM carbapenemase.
3. The most significant risk factors for the different types of infections and for 30-day mortality were confirmed.

Scientific and Applied Contributions

1. The sensitivity of phenotypic tests for the detection of ESBLs in *Serratia marcescens* isolates resistant to third-generation cephalosporins was evaluated.
2. A protocol was developed for DNA extraction from DNase-producing bacteria, such as *S. marcescens*.
3. The effectiveness of rapid molecular genetic methods for the detection of β-lactamases was presented and evaluated.
4. The effectiveness of rapid immunochromatographic tests for the detection of carbapenemases in clinical *S. marcescens* isolates was presented and evaluated.

Scientific Publications and Presentations Related to the Dissertation

Publications in Scientific Journals:

1. Niyazi D, **Radeva S**, Bozhkova M, Savova D, Stoeva T. A cheap and simple method for DNA isolation from endonuclease-producing *Serratia marcescens*. *Iran J Public Health*. 2023 May;52(5):1089–91. doi:10.18502/ijph.v52i5.12731
2. **Radeva S,** Bozhkova M, Niyazi D. History and presence of *Serratia marcescens*. *Acta Microbiol Bulg*. 2023;39(3):233–8. doi:10.59393/amb23390303
3. **Radeva S**, Niyazi D, Bozhkova M, Stoeva T. Antimicrobial resistance of *Serratia marcescens* causing bloodstream infections in a large university hospital in Bulgaria: an 8-year analysis (2016–2023). *Acta Microbiol Hung*. 2024;71(3):191–6. doi:10.1556/030.2024.02330
4. **Radeva S**, Niyazi D, Bozhkova M, Stoeva T. Wound infections caused by *Serratia marcescens*: a microbiological survey. *Scr Sci Med*. 2024;55(2). doi:10.14748/ssm.v55i2.9855

Presentations at Scientific Forums:

1. Radeva S., Bozhkova M., Niyazi D., Savova D., Stoeva T. *Resistance Rates to Third-Generation Cephalosporins and Carbapenems in Serratia marcescens Isolates Obtained from Various Clinical Samples from Two Bulgarian Hospitals*. Medical Sciences Forum. 2022; 12(1):32. https://doi.org/10.3390/eca2022-12693
2. Radeva S., Bozhkova M. *Clinical Significance and Mechanisms of Resistance of Serratia marcescens*. 11th South-East European Conference and 31st Annual Assembly of IMAB, online presentation, 28–31 October, 2021.