MEDICAL UNIVERSITY
"PROF. PARASKEV STOYANOV" - VARNA
Faculty of Medicine
Department of Microbiology and Virology

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ANTIMICROBIAL RESISTANCE OF THE MOST COMMON CAUSATIVE AGENTS OF BACTEREMIA AND THE ASSOCIATED LETHALITY

S U M M A R Y

of dissertation
for the award of degree “Doctor of Science”

VARNA, 2023

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DEDICATION

I dedicate this work to my dear parents!

ABBREVIATIONS

OHDs - oncohematological diseases
bla - genes encoding β-lactamases
BCR - Blood Culture Rate
BSIs - Bloodstream Infections
BG - Bulgaria
CDC - Centre for Disease Prevention and Control
CLSI - Clinical and Laboratory Standards Institute
CRE - Carbapenem-Resistant *Enterobacteriaceae*
CR - Carbapenem-Resistant
CVC - Central Venous Catheter
CoNS - Coagulase-Negative Staphylococcus
DDDs - Defined Daily Doses
DALYs - Disability-Adjusted Life-Years (years of life lost due to disease and/or premature death)
EU - European Union
Efm - *Enterococcus faecium*
ESBL - Extended-Spectrum Beta-Lactamase
ESKAPE - ***E****. faecium,* ***S.*** *aureus,* ***K.*** *pneumoniae,* ***A.*** *baumannii,* ***P.*** *aeruginosa, Enterobacter* spp.
ESCAPE - ***E****. faecium,* ***S.*** *aureus,* ***C.*** *difficile,* ***A.*** *baumannii,* ***P.*** *aeruginosa,* ***E****nterobacteriacae*
ESKAPEEc - ***E****. faecium,* ***S.*** *aureus,* ***K.*** *pneumoniae,* ***A.*** *baumannii,* ***P.*** *aeruginosa, Enterobacter* spp., ***E****. coli*
EUCAST - European Committee on Antimicrobial Susceptibility Testing
ECDC - European Centre for Disease Prevention and Control
GVHD - Graft Versus Host Disease
HICs - High Income Countries
HLAR - High Level Aminoglycoside Resistance
ISs - Insertion Sequences
ICU - Intensive Care Unit
LCBI - Laboratory Confirmed Bloodstream Infections
MBI - LCBI - Mucosal Barrier Injury Laboratory Confirmed Bloodstream Infection
LICs - Low Income Countries
LMICs - Low- and Middle-Income Countries
MBLs - Metalo-beta lactamases
MLST - Multi Locus Sequence Typing
MRCoNS - Methicillin-Resistant Coagulase-Negative Staphylococci
MRSA - Methicillin-Resistant *Staphylococcus aureus*
MIC - Minimal Inhibitory Concentration
MDR - Multidrug Resistant
NA - not applicable
OXA - oxacillinase
PDR - Pan Drug Resistant
PCR - Polymerase Chain Reaction
ST - Sequence Type
TSM - trimethoprim/sulphomethoxazole
UH - University Hospital
VRE - Vancomycin-Resistant Enterococcus
VREfm - Vancomycin-Resistant *Enterococcus* faecium
VREf - Vancomycin-Resistant *Enterococcus faecalis*
XDR - Extensively Drug Resistant

INTRODUCTION

Despite the significant medical advances of recent decades, bloodstream infections continue to be a serious public health problem and a leading cause of morbidity and mortality. The incidence of these infections in population-based studies in Europe and North America ranges between 113 and 204 per 100,000 person-years *(Goto M, 2013),* and the mortality rate is estimated between 15% and 30% *(Hattori H, 2018).* A large study on global mortality associated with 33 clinically important bacterial pathogens conducted in 2019, covering 343 million individual records in 204 countries, reported a total of 13.7 million infection-related deaths. Of the 11 infectious syndromes studied, two were associated with over 2 million deaths each: lower respiratory tract infections with 4 million and bloodstream infections with 2.91 million deaths *(Ikuta K, 2022).* In addition, bloodstream infections have been identified as the leading infectious syndrome in two super-regions of the world, and *Staphylococcus aureus* as the global leading cause of fatal bloodstream infections, responsible for 299,000 deaths in 2019 *(Ikuta K, 2022).*

In 2008, Rice grouped the bacterial species *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp. into a group called **"ESKAPE"** as the most common causative agents of life-threatening healthcare-associated infections and for their ability to escape the effects of many antibiotics and for their multidrug resistance profile *(Rice L, 2008).* In 2009, a more appropriate acronym**, ESCARE,** was proposed, with "**C**" referring to *Clostridioides difficile,* an important nosocomial pathogen that can also readily acquire multidrug resistance, and "**E"** referring to *Enterobacteriacae*, referring to all Gram-negative enteric bacteria, incl. *E. coli, Klebsiella pneumoniae, Proteus* spp. and *Enterobacter* spp. *(Peterson L, 2009).* Because of their highly problematic resistance, some of these pathogens (vancomycin-resistant *Enterococcus faecium*, carbapenem-resistant *Pseudomonas aeruginosa, Acinetobacter baumannii* and *Enterobacteriacae*) are also included in the World Health Organization (WHO) list of microorganisms that are prioritized as "critical" for research and development of new, effective antibiotics *(WHO, 2017).*

A number of studies have demonstrated, in addition to an increasing burden of bacterial bloodstream infections worldwide, a steady trend towards a rapidly increasing incidence of infections caused by the ESKAPE group, representing between 50 and 70% of their aetiological spectrum, with these infections often associated with prolonged hospital stay, increased economic costs and worse outcomes due to inadequate and delayed antimicrobial therapy because of the antimicrobial resistance of their causative agents *(De Kraker M, 2013; Founou R, 2017; Marturano J, 2019; Pogue J, 2015; Stewardson A, 2016; Tacconelli E, 2019; Yang S, 2019; Zhen X, 2019).*

The medical advances and the new treatment options for a number of diseases are leading to a steadily growing group of immunocompromised patients (those with oncohematological diseases, transplant recipients, ICU patients, etc.). In recent years, there has been a clear global trend of increasing incidence of invasive, life-threatening infections caused by multidrug-resistant ESKAPE pathogens in this specific group as well, with these microorganisms being among the leading causes of mortality *(Gustinetti G, 2016; Martinez-Nadal G, 2020).*

Currently, antimicrobial resistance is one of the most important causes of death globally, associated with a disease burden higher than that of HIV or malaria *(Ikuta K, 2022),* and in 2050 it is estimated to be the number one cause of death, surpassing even the oncological diseases *(O'Neill J, 2016).* In 2019, the WHO announced "Antimicrobial Resistance" as one of the top ten public health threats, adding two more indicators to its 2019-2023 agenda: bloodstream infections caused by *E. coli* resistant to third-generation

cephalosporins and methicillin-resistant *S. aureus* (MRSA), and antibiotic consumption at the national level *(WHO, 2019).*

In the context of the above, bloodstream infections caused by resistant organisms represent a very topical medical problem. Surveillance and analysis of important microbiological and epidemiological aspects of these infections, regarding trends in the etiological spectrum and antimicrobial susceptibility of pathogens, risk factors and the lethality that accompanies them, provide important information to clinical specialists and infection control professionals for the design of adequate treatment algorithms and prevention and control programs at the local level. On the other hand, adequate surveillance at the local level contributes significantly to the expansion of data and scientific information on antimicrobial resistance at the national level and to the analysis of the information generated in order to assess the burden of antimicrobial resistance in different infections, in particular those of the blood. The generation of high quality and comparable data on these issues, allow their integration with data from European and Global Surveillance Networks and support policy making aimed at limiting and controlling the problem of Antimicrobial resistance at national and global level.

I. PURPOSE AND OBJECTIVES

The aim of this thesis is to perform a microbiological and epidemiological study on antimicrobial resistance of the most common causative agents of bacteremia over a 10-year period and the lethality associated with them.

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

1. To study and analyze the etiological spectrum of laboratory-confirmed bacterial bloodstream infections in hospitalized patients at St. Marina University Hospital in the period 2011-2020 (regardless of age, gender, diagnosis, clinic) and to investigate the resistance to antimicrobial drugs of the most common causative agents of bacteremia associated with these infections, and to assess the trend in the development of resistance over time.
2. To investigate the genetic mechanisms of resistance to third-generation cephalosporins and carbapenems in representative carbapenem-resistant *Klebsiella pneumoniae*, *Enterobacter cloacae* complex and *Acinetobacter baumannii* blood isolates and to perform epidemiological typing.
3. To study and analyze the etiologic spectrum of laboratory-confirmed bacterial bloodstream infections in hospitalized patients with oncohematological diseases (regardless of age, sex, diagnosis) between 2010 and 2020 and to investigate the antimicrobial drug resistance of the most common causative agents of bacteremia associated with these infections, and to assess the trend in resistance over time.
4. For all hospitalised patients with bloodstream infections caused by seven bacterial species between 2016 and 2020 (*S. aureus, S. pneumoniae, E. coli, K. pneumoniae, E. cloacae* complex, *A. baumannii - calcoaceticus* complex, *P. aeruginosa)* to extract, summarize and associate information related to 27 variables.
5. To calculate the 30-day lethality (overall and specific - by age group, sex, type of clinic, site of acquisition, microbial species, organism/antibiotic combination) in the group of all hospital-treated patients with laboratory-confirmed bloodstream infections caused by the 7 bacterial species between 2016 and 2020, and to identify risk factors that influence 30-day lethality.

II. MATERIALS AND METHODS

1. Study design

The present study has a retrospective descriptive design aimed to analyze the incidence of clinically significant bacteremias, their microbial causative agents and risk factors for death in bloodstream infections in patients treated at St. Marina Hospital, Varna over a 10-year period (2011-2020).

All clinically significant episodes of bacteraemia (associated with infection) documented in patients treated during the indicated period, irrespective of their age, sex, diagnosis and clinic, were included in the study. All clinically significant episodes of bacteraemia in hospital-treated patients with oncohematological diseases over an 11-year period (2010 to 2020), irrespective of their sex, age and diagnosis, were studied separately.

Episodes of fungaemia, episodes of bacteraemia reported as "contamination", duplicate bacterial isolates from the same patient, and single blood cultures positive for representatives of the normal skin microbial flora were excluded from the study. The Hospital and Laboratory Information Systems were used to collect information pertaining to evidence the episodes of clinically significant bacteremias in the period 2010-2020, their etiologic agents, and their susceptibility to antimicrobial drugs. All episodes of bacteraemia meeting the following definitions were included in the study:

- For episodes of bacteraemia associated with Gram-negative and Gram-positive bacteria other than resident skin microbiota (coagulase-negative staphylococci, corynebacteria, *Propionibacterium* spp., *Micrococcus* spp., *Bacillus* spp. (other than *B. anthracis*): any episode in which microbial isolation from 1 or more blood culture sets is present.
- For episodes of bacteraemia associated with members of the resident skin microbiota: any case of isolation from two or more blood culture sets of isolates of the same bacterial species within 48 hours, as well as an isolation of the same bacterial species from a central venous catheter and from a peripheral vessel simultaneously, in the presence of symptoms of infection (febrile (>38^0 C) / chills / hypotension).

The date of the first positive blood culture was taken as the date of the onset of bacteraemia and its diagnosis. A positive blood culture with the same organism (also demonstrating identical susceptibilty) within 30 days of the first positive blood culture in the respective patient was not included in the study. Polymicrobial bacteremia was reported when more than 1 microbial species was identified within an episode. When polymicrobial bacteremia was detected and one of the isolates was part of the normal skin microbiota, it was excluded from the study. When a bacterial species other than the species demonstrated in the first positive blood culture was identified in the blood culture, the second episode was reported as a new episode. A blood culture was interpreted as clinically not significant when any of the following microorganisms was identified in a single blood culture set obtained from a single patient on a single day: coagulase-negative staphylococci, *Corynebacterium* spp., *Propionibacterium* spp, *Micrococcus* spp, *Bacillus* spp (excluding *B. anthracis*). When more than one organism representatives of the normal skin microflora were isolated in a single blood culture, it was interpreted as contaminated and excluded from the study.

One set of blood cultures consisted of one blood sample inoculated into one aerobic and one anaerobic bottle containing broth culture medium.

In 798 patients with laboratory (culture) confirmed bloodstream infections caused by *S. aureus, S. pneumoniae, E. coli, K. pneumoniae, E. cloacae* complex, *A. baumannii - calcoaceticus* complex and *P. aeruginosa* in the period 2016-2020, information on a total of 27 variables (demographic, clinical, microbiological - microbial species, antimicrobial resistance) was collected to assess 30-day lethality and risk factors. In order to summarize and associate data for each patient related to the 27 variables, a form was developed to collect

information from the hospital records of patients treated at St. Marina Hospital (Appendix 2 of the thesis).

In addition, the 30-day lethality associated with bloodstream infections caused by 7 antibiotic-resistant bacterial species *S. aureus, S. pneumoniae, E. coli, K. pneumoniae, E. cloacae* complex, *A. baumannii - calcoaceticus* complex, and *P. aeruginosa* (most importantly, MRSA, 3rd generation cephalosporin-resistant Gram-negative bacteria, carbapenem-resistant *P. aeruginosa, A. baumannii*, and *Enterobacteriacae*) was compared with 30-day lethality in the corresponding non-antibiotic-resistant infections to assess antimicrobial resistance as a risk factor for 30-day lethality.

The indications for inclusion of patients were:

1. Hospitalization at St. Marina University Hospital in the period 2016 - 2020.
2. A proven episode of clinically significant bacteremia caused by *S. aureus, S. pneumoniae, E. coli, K. pneumoniae, E. cloacae* complex, *A. baumannii - calcoaceticus* complex and *P. aeruginosa* in the period 2016-2020.
3. Information available for all 27 variables.

A total of 798 patients studied were stratified based on clinical outcome of hospitalization into two groups: a first group that survived for at least 30 days and a second group that died within 30 days of the time the positive blood culture was taken.

The study was approved by the Research Ethics Committee with protocol No92/02.04.2020.

Definitions:

- **Bacteremia:** the presence of bacteria in the blood.
- **Bacterial bloodstream infection:** isolation of a bacterial pathogen that is not part of the resident microflora from one or more blood cultures. In the case of skin commensals such as *CoNS*, *Corynebacterium* spp. (except *C. diphtheriae*), *Bacillus* spp. (except *B. anthracis*), *Micrococcus* spp. at least 2 consecutive positive blood cultures with the same microorganism taken at different times within 48h, and at least one of the following symptoms are required: fever, chills and hypotension.
- **Community-acquired bloodstream infection:** defined when the first proven positive blood culture was taken within <= 48 hours of hospitalisation.
- **Nosocomial bloodstream infection:** defined when the first proven positive blood culture is taken after 48 h of hospitalisation.
- **Laboratory-confirmed bloodstream infection:** the CDC/National Healthcare Safety Network (NHSN) criteria were used (*NHSN, 2023; https://www.cdc.gov/nhsn/psc/bsi/*)
- **Cumulative incidence of bloodstream infections/bacteraemias**: the indicator is calculated per 1000 hospitalisations for each year of the period 2011-2020.
- **Methicillin-resistant staphylococci:** cefoxitin/oxacillin-resistant staphylococci determined by the disk-diffusion method or by an automated system.
- **Isolates suspicious to be Extended spectrum beta-lactamases (ESBLs) producers**: isolates, resistant to ceftazidime/cefotaxime/ceftriaxone.
- **Carbapenem non-susceptible *Enterobacterales*, *A. baumannii* and *P. aeruginosa*:** isolates with intermediate susceptibility or resistance to one or more carbapenems according to CLSI used in the period 2010-2015 and EUCAST used in the period 2016-2020.
- **Multidrug resistance (MDR) in *P. aeruginosa*:** resistance to 3 or more of the following antimicrobial groups: third generation cephalosporins, piperacillin/tazobactam, carbapenems, fluoroquinolones and aminoglycosides *(Magiorakos A, 2012).*

- **MDR *A. baumannii*:** non-susceptibility to at least one agent in three or more of the following groups of antimicrobial drugs: aminoglycosides (gentamicin, amikacin, tobramycin), antipseudomonal carbapenems (imipenem, meropenem), antipseudomonal fluoroquinolones (ciprofloxacin, levofloxacin), penicillins + beta-lactamase inhibitor (sulbactam), folic acid synthesis inhibitors (trimethoprime/sulphomethoxazole), polymyxins (colistin, polymyxin B) and tetracyclines. **XDR *A. baumannii*:** non-susceptibility to at least one agent in all but 2 or 1 of the specified groups *(Magiorakos A, 2012)*.

2. Microbiological methods

2.1. Detection of microorganisms in blood

- Automated system for blood culture incubation (BACTEC, BD, USA).

2.2. Species identification

- Conventional biochemical methods: manual biochemical tests; Crystal (BD, USA); Phoenix (BD, USA)
- *gyr B* PCR for species identification of *A. baumannii (Higgins PG, 2007)*
- *hps60* sequencing for species identification of *E. cloacae* complex

2.3. Antimicrobial susceptibility testing

- Bauer-Kirby disk diffusion method (CLSI, EUCAST)
- automated system (Phoenix, BD, USA)
- microdilution method for determination of MIC (MIKROLATEST, Erba Lachema, Czech Republic)

2.4. Molecular genetic methods for detection of genes encoding ESBLs and carbapenemases (TEM, SHV, CTX-M, KPC, VIM, IMP, NDM, OXA-23/24/51/58/143/235)

- PCR *(Higgins P, 2010; Higgins P, 2013; Markovska R, 2008; Poirel L, 2006; Poirel L, 2011; Turton J, 2006; Woodford N, 2006)*
- DNA sequencing

2.5 Epidemiological typing

- ERIC-PCR (*Versalovic J, 1991)*
- RAPD PCR *(Grundmann H, 1997)*
- REP-PCR *(Healy M, 2005)*
- MLST *(Diancourt L, 2005)*

3. Statistical methods

All analyses in this study were performed with the statistical package IBM SPSS version 21.0.

In the descriptive analysis, qualitative variables are represented by absolute number and relative proportion, and quantitative variables by mean and standard deviation or median and interquartile range (IQR), depending on the type of distribution previously verified by Kolmogorov Smirnov test.

The cumulative incidence of bloodstream infections/bacteraemias in 1 year (per 1000 hospitalisations) was calculated as the number of newly diagnosed episodes of bacteraemia divided by the number of hospitalisations in one year x 1000.

Thirty-day lethality associated with a specific bloodstream infection was calculated as the number of patients with a bloodstream infection (from a given causative agent) who died within 30 days of diagnosis divided by the number of patients with infection from the same causative agent (presented as %). Thirty-day lethality in the entire study group of patients with bloodstream infections was calculated as the number of patients who died within 30 days of diagnosis (regardless of the specific causative agent) divided by the total number of patients with bloodstream infections (presented as %).

All frequency indices for lethality, cumulative incidence, morbidity are presented with 95% confidence intervals.

The chi-square test or Fisher's exact test was used to test hypotheses about the association between two qualitative variables (e.g., disease outcome and resistance). Spearman's rank correlation test was used when examining the relationship between two variables measured on the ordinal scale.

The time trend of antimicrobial resistance development was tested with Chi-squared test for trend.

Binary univariate and multivariable logistic regression were used to examine the effect of risk factors on the risk of dying.

All statistical tests are two-sided.

The results were reported as statistically significant at α=0.05 error level.

III. RESULTS AND DISCUSSION

1. Etiological spectrum of bacterial bloodstream infections in hospitalized patients over a 10-year period (2011-2020).

During the study period, 27 650 blood culture sets obtained from 15 602 hospitalized patients were examined in the Microbiology Laboratory of St. Marina University Hospital, Varna. A total of 2727 non-duplicate microbial isolates associated with clinically significant episodes of bacteremia and fungemia in 2715 patients (17.4%) were isolated and identified. The relative proportion of the positive blood cultures interpreted as clinically significant over the 10-year period averaged 9.9% (Table 7)[1]. The etiological spectrum of the bloodstream infections over the entire 10-year period is presented in Tables 8, 9 and 11.

The proportion of ESKAPEEc isolates (*E. faecium, S. aureus, K. pneumoniae, Acinetobacter* spp., *P. aeruginosa, Enterobacter* spp., *E. coli*) for the entire ten-year period was 66.8%. The proportion of isolates belonging to the eight bacterial species monitored by EARS Net (*S. aureus, S. pneumoniae, E. faecalis, E. faecium, E. coli, K. pneumoniae, Acinetobacter* spp., *P. aeruginosa*) averaged 64.7% over time from 2011 to 2020, with the proportion being lowest in 2012 (47.9%) and reaching 71.1% in 2020. The microbial species *S. aureus, E. faecalis, E. faecium, E. coli, K. pneumoniae, Acinetobacter* spp. and *P. aeruginosa* were among the most common bacterial species associated with bloodstream infections in all years studied.

Table 7: Total number of patients tested, proportion of positive blood cultures out of the total blood cultures, BCR and cumulative incidence of bloodstream infections between 2011 and 2020.

Year	Total number of patients tested, (n)	Patients with clinically significant microbial blood isolates, (%)	Positive blood cultures (%)*	Clinically significant positive blood cultures (%)	BCR**	Cumulative incidence of bloodstream infections
2011	1418	14.4	19.1	12.0	5.6	4.1/1000
2012	1424	15.8	20.0	13.6	5.3	4.4/1000
2013	1462	17.0	21.4	14.2	5.4	4.5/1000

[1] The numbers of the tables and figures in the SUMMARY correspond to those in the thesis.

2014	1549	20.1	20.6	14.2	7.0	5.6/1000
2015	1581	17.5	15.9	10.3	8.6	5.2/1000
2016	1541	19.7	20.2	11.4	8.1	5.7/1000
2017	1568	18.9	13.9	8.5	10.6	5.6/1000
2018	1536	18.6	13.4	8.1	10.7	5.1/1000
2019	1754	16.5	11.8	6.9	13.8	5.2/1000
2020	1769	15.4	13.6	7.6	13.9	5.9/1000
2011/2020	**15 602**	**17.4**	**16.0**	**9.9**	**-**	**-**

[*] including contaminated blood cultures;[**] BCR, Blood Culture Rate (number of blood culture sets/1000 bed-days).

Table 8: Spectrum of causative agents of laboratory-confirmed bloodstream infections in hospitalized patients over a 10-year period (2011-2020).

Microbial agents, 2011 - 2020г. n (%)	**2011** **n, (%)**	**2012** **n, (%)**	**2013** **n, (%)**	**2014** **n, (%)**	**2015** **n, (%)**	**2016** **n, (%)**	**2017** **n, (%)**	**2018** **n, (%)**	**2019** **n, (%)**	**2020** **n, (%)**	***p***
Gram negative bacteria 1605 (58.9)	117 (57.4)	146 (64.9)	147 (59.0)	195 (62.5)	175 (63.2)	192 (62.1)	172 (57.9)	164 (57.4)	157 (53.8)	140 (50.7)	**0.035**
Gram positive bacteria 972 (35.6)	68 (33.3)	64 (28.4)	89 (35.7)	83 (26.6)	84 (30.3)	104 (33.7)	117 (39.4)	111 (38.8)	126 (43.2)	126 (45.7)	**0.006**
Fungi 150 (5.5)	19 (9.3)	15 (6.7)	13 (5.2)	34 (10.9)	18 (6.5)	13 (4.2)	8 (2.7)	11 (3.8)	9 (3.0)	10 (3.6)	**0.009**
Total 2727 (100.0)	204 (100)	225 (100)	249 (100)	312 (100)	277 (100)	309 (100)	297 (100)	286 (100)	292 (100)	276 (100)	**-**

Table 9. Detailed etiological structure of laboratory-confirmed bloodstream infections in hospitalised patients over a 10-year period (2011-2020).

Etiological causative agent	**n, (%)**
Staphylococcus aureus	**462 (17.2)**
E. coli	**399 (14.6)**
Klebsiella pneumoniae	**329 (12.0)**
***Enterobacter cloacae* complex**	**218 (8.0)**
Enterococcus faecalis	**171 (6.3)**
***Acinetobacter baumannii - calcoaceticus* complex**	**171 (6.3)**
***Candida* spp.**	**150 (5.5)**
Pseudomonas aeruginosa	**120 (4.4)**
Streptococcus viridans	**86 (3.2)**
Enterococcus faecium	**72 (2.6)**
Coagulase negative staphylococci (CoNS)	**62 (2.3)**
Serratia marcescens	59 (2.2)
Citrobacter spp.	53 (1.9)
Enterobacter aerogenes	43 (1.6)
Klebsiella oxytoca	41 (1.5)
Proteus mirabilis	40 (1.47)
Streptococcus pneumoniae	35 (1.3)
Stenotrophomonas maltophilia	28 (1.0)
Streptococcus agalactiae	27 (0.99)
Pseudomonas spp. (other than *P. aeruginosa*)	23 (0.8)
Streptococcus bovis	22 (0.8)
Bacteroides spp.	17 (0.6)
Morganella morgannii	15 (0.55)
Salmonella spp.	13 (0.47)
Streptococcus pyogenes	12 (0.44)
Proteus vulgaris	8 (0.3)
Other*	51 (1.1)
Total clinically significant microorganisms from blood cultures	**2727 (100.0)**

*Bacterial species represented by <=5 isolates.

Table 11. Distribution by year of the 10 most frequently isolated microbial species from blood causing bacterial bloodstream infections during the period 2011-2020.

Bacterial species	**2011-2020 n (%)**	**2011 n (%)**	**2012 n (%)**	**2013 n (%)**	**2014 n (%)**	**2015 n (%)**	**2016 n (%)**	**2017 n (%)**	**2018 n (%)**	**2019 n (%)**	**2020 n (%)**	***p***
S. aureus	462 (17.2)	32 (15.7)	29 (12.9)	41 (16.5)	28 (9.0)	52 (18.8)	42 (13.6)	50 (16.8)	71 (24.8)	61 (20.9)	56 (20.3)	**0.048**
E. coli	399 (14.6)	35 (17.1)	29 (12.9)	24 (9.6)	30 (9.6)	38 (13.7)	51 (16.5)	33 (11.1)	50 (17.5)	62 (21.2)	47 (17.0)	0.145
K. pneumoniae	329 (12.0)	26 (12.7)	36 (16.0)	36 (14.4)	52 (16.7)	23 (8.3)	36 (11.7)	35 (11.8)	30 (10.5)	33 (11.3)	22 (8.0)	**0.035**
***E. cloacae* complex**	218 (8.0)	13 (6.4)	13 (5.8)	16 (6.4)	30 (9.6)	52 (18.8)	31 (10.0)	22 (7.4)	13 (4.5)	12 (4.1)	16 (5.8)	0.618
A. baumannii	171 (6.3)	12 (5.9)	17 (7.6)	22 (8.8)	30 (9.6)	15 (5.4)	19 (6.1)	19 (6.4)	13 (4.5)	8 (2.7)	16 (5.8)	0.067
E. faecalis	171 (6.3)	17 (8.3)	9 (4.0)	18 (7.2)	14 (4.5)	8 (2.9)	20 (6.5)	20 (6.7)	15 (5.2)	17 (5.8)	33 (12.0)	0.351
P. aeruginosa	120 (4.4)	7 (3.4)	13 (5.8)	16 (6.4)	8 (2.6)	15 (5.4)	14 (4.5)	19 (6.4)	11 (3.8)	8 (2.7)	9 (3.3)	0.381
S. viridans	86 (3.2)	2 (1.0)	5 (2.2)	5 (2.0)	12 (3.8)	4 (1.4)	14 (4.5)	9 (3.0)	9 (3.1)	13 (4.5)	13 (4.7)	**0.011**
E. faecium	72 (2.6)	5 (2.4)	5 (2.2)	10 (4.0)	7 (2.2)	2 (0.7)	3 (1.0)	14 (4.7)	6 (2.0)	9 (3.0)	11 (4.0)	0.486
CoNS	62 (2.3)	6 (2.9)	4 (1.8)	4 (1.6)	6 (1.9)	5 (1.8)	8 (2.6)	7 (2.4)	2 (0.7)	12 (4.1)	8 (2.9)	0.442
Other	637 (23.4)	49 (24.0)	65 (28.9)	57 (22.9)	95 (30.4)	63 (22.7)	71 (23.0)	69 (23.2)	66 (23.0)	57 (19.5)	45 (16.3)	-
Total	2727 (100.0)	204 (100.0)	225 (100.0)	249 (100.0)	312 (100.0)	277 (100.0)	309 (100.0)	297 (100.0)	286 (100.0)	292 (100.0)	276 (100.0)	-

The satisfactory level of detection of bloodstream infections depends very much on the frequency of blood culture testing. In the present study, covering an extended ten-year period, a 2.5-fold increase in the BCR indicator from 5.6 in 2011 to 13.9 in 2020 was demonstrated, which correlated with an increase in the "cumulative incidence of bloodstream infections" indicator over the years from 4.1 per 1000 hospitalizations in 2011 to 5.9 per 1000 hospitalizations in 2020. According to EARS Net data for 2020, in the European countries that participated in the surveillance of antimicrobial resistance of invasive bacterial isolates, the "BCR" indicator ranges very widely: from 3 for Ukraine, 5 for Georgia, 6 for Belarus and Kosovo, 10.4 for Bulgaria and 11 for Russia, between 17.2 - 37.9 for Hungary, Romania, Slovakia, Czech Republic, Estonia, Malta and Germany, between 54.4 - 109.5 for France, Iceland, Italy, Norway and Spain, to 202.4 for Denmark and 244.2 for Portugal *(ECDC, 2022).* However, despite the positive upward trend found in this study, the dramatic differences that exist in this indicator between Bulgaria and most European countries indicate the need for efforts to stimulate clinicians to test additional blood cultures in order to diagnose bloodstream infections more accurately and adequately.

In the present study, the proportion of true positive (clinically significant) blood cultures over the 10-year follow-up period was 9.9%. Organisms interpreted as contaminants were found in 6.1% of all blood cultures examined. This result significantly exceeds the generally accepted recommendation of a proportion of contaminated blood cultures of up to 3% *(Dargère, S 2018).* Insofar as the contaminating organisms are most often part of the normal skin microflora, in order to correctly interpret the microbiological result, the clinical microbiologist needs not only information on the patient's demographics but also complete clinical information, since the isolation of a microorganism from a blood culture in itself always raises the question of the need to start antimicrobial therapy.

Over the past two decades, the etiology of bloodstream infections has undergone significant changes, with relatively few studies evaluating large numbers of bloodstream infections in an unselected patient population. The present study demonstrates that over the entire ten-year follow-up period, Gram-negative bacteria (58.9%) were the more common etiologic agents of bloodstream infections in hospitalized patients, with the leading pathogens *E. coli, K. pneumoniae, E. cloacae, A. baumannii* and *P. aeruginosa*. However, when tracking the etiological spectrum over the years, there was a statistically significant trend of decreasing proportion of Gram negative bacteria from 57.4% in 2011 to 50.7% in 2020 (p=0.035). The proportion of Gram-positive bacteria for the entire ten-year period was 35.6%, with the leading microbial species being *S. aureus, E. faecalis, S. viridans, E. faecium* and *CoNS*. The study demonstrates a clear trend towards a sustained increase in the proportion of Gram positive bacteria from 33.3% in 2011 to 45.7% in 2020 (p=0.006). The ten most common pathogens found in this study (*S. aureus, E. coli, K. pneumoniae, E. cloacae, E. faecalis, A. baumannii - calcoaceticus complex, P. aeruginosa, S. viridans, E. faecium, CoNS*) accounted for 76.9% of all isolates, with a statistically significant increasing trend between 2011 and 2020 for *S. aureus* (p=0.048) and *S. viridans* (p=0.011), and a decreasing trend for *K. pneumoniae* (p=0.035). The most common bacterial species across the ten-year period, regardless of their Gram affiliation, was *S. aureus* (17.2%), followed by *E. coli* (14.6%), with both species frequently changing their position over the years. In five of the years, *S. aureus* occupied the first position (2013, 2015, 2017, 2018, 2020) and *E. coli* in 2011, 2016 and 2019, respectively. The bacterial species *K. pneumoniae*, occupying the third position in 5 of the 10 years, was first in the etiological spectrum in 2012 and 2014. Similarly, *E. cloacae*, traditionally occupying the 4th or 5th position, in 2014 and 2015 ranked second and first, respectively, together with *A. baumannii* and *S. aureus* in the respective year. In 2020, *E. faecalis* moved from its usual 4th or 5th position, to a more forward third position after *S. aureus* and *E. coli*, and in 2021 totally the isolates of *E. faecalis* and *E. faecium* occupy the first place in the aetiological spectrum of

bloodstream infections (data not shown), which is in line with what EARS Net reported for 2021 for the European Union *(ECDC, 2022; ECDC, 2022a).* With regard to S. *aureus*, a statistically significant increasing trend in the proportion of this bacterial species from 15.7% in 2011 to 20.3% in 2020 is found. For *E. coli*, the second most frequent species over the 10-year study period, we observe significant variation in the relative proportions without evidence of statistically significant differences between years ($p>0.05$): there is a downward trend from 17.1% to 13.7% between 2011 and 2015, then an upward trend to reach 2011 levels in 2020, passing through a peak of 21.2% in 2019.

The proportion of ESKAPEEc isolates in the total number of invasive isolates for the whole 10-year period was 66.8%, and that of the bacterial species monitored by EARS Net was 64.7%. The results obtained from the present study are consistent with the SENTRY Antimicrobial Resistance Program, which published its data pertaining to the species affiliation of 264 901 bacterial isolates obtained from patients with bloodstream infections over a 20-year period (1997-2016) reported from over 200 medical centers in 45 countries in Europe, the Americas and Asia-Pacific region *(Diekema D, 2019).* Similar to our findings, over the entire study period, *S. aureus* (20.7%) and *E. coli* (20.5%) were shown to be the most common etiological agents, collectively accounting for about 40% of all bloodstream infections, followed by *K. pneumoniae* (7.7%), *P. aeruginosa* (5.35) and *E. faecalis* (5.2%). In the present 10-year study, S. *pneumoniae* remained outside the top 10 most common pathogens, occupying the 17th position with 1.3%. The identification of serotypes 3 and 19A as the most common is consistent with the results of another surveillance system (EU-IBD), which reported for the period 2014 - 2018 serotypes 8, 3, 19A, 22F, 12F, 9N, 15A, 10A, 23B, 6C and 11A as the most common, responsible for 70% of all cases of invasive pneumococcal infections with a known serotype in 2018 *(ECDC, 2022d).* Although identifying *S. pneumoniae* among the 10 most frequent etiological agents, the SENTRY Antimicrobial Resistance Program also demonstrates a declining trend in the proportion of *S. pneumoniae* between 1997 and 2016 *(Diekema D, 2019)*. Consistent with this, other authors have reported the same trend after 2000 and an overall low relative proportion of *S. pneumoniae* bloodstream infections (Australia, Finland, Sweden, Germany, etc.), similar to what was found in the present study, which is largely a reflection of immunization and serotype coverage associated with available pneumococcal vaccines.

Contrary to our findings, the SENTRY Antimicrobial Resistance Program demonstrates an increase in the proportion of Gram-negative organisms among the top ten most common causative organisms (from 33.5% to 43.4%) between 1997 and 2016 *(Diekema D, 2019).* The dominance of *S. aureus* and *E. coli* as the most frequent etiological agents of bloodstream infections are confirmed by several large population-based and multicenter hospital-based studies conducted since 2010, although it should be emphasized that the existing information is extremely heterogeneous. A number of studies from regions such as Japan, Thailand, Australia, other countries in Asia, Africa reported high prevalence of *Burkholderia pseudomallei* (8.97%), *Salmonella non-typhi* spp. (67.7%), *Salmonella enterica* serovar Typhi (29.3%), mycobacteria (6.1%), confirming the importance of some geographical, climatic and socioeconomic characteristics of the respective region *(Douglas N, 2020; Marchello C, 2020; Rhodes J, 2019; Takeshita N, 2017*).

2. Antimicrobial resistance of the most common causative agents of bacteremia associated with bloodstream infections in hospitalised patients over a 10-year period (2011-2020).

2.1. Gram positive bacteria

Staphylococcus aureus

S. aureus is among the most common and important human pathogens, responsible for about 20-30% of bloodstream and surgical site infections and up to 50% of bone and joint infections. In the 10-year study period, susceptibility to methicillin, gentamicin, fluoroquinolones, macrolides, lincosamides, glycopeptides and linezolid was studied among a total of 462 blood isolates of *S. aureus*. Over the entire study period, the proportion of MRSA was 15.4%. Resistance to the respective antibiotic groups in decreasing order was as follows: macrolides, 20.6% > gentamicin, 16.5% > methicillin, lincosamides, 15.4% > fluoroquinolones, 12.3% > glycopeptides, linezolid, 0.2% (Table 12).

Among the most problematic and monitored resistance specific to *S. aureus* is methicillin resistance (MRSA). This type of resistance determines non-susceptibility to almost the entire spectrum of beta-lactam antibiotics, and many MRSA isolates are simultaneously resistant to other antibiotic groups, making treatment significantly more difficult. For the ten-year follow-up period, the relative proportion of MRSA blood isolates was found to be close to and slightly lower than the European average in the period 2011-2020 (15.5-18.8%). In this time interval, a statistically significant trend of decreasing MRSA prevalence is demonstrated for the European Union *(ECDC, 2012; ECDC, 2022)*, which is also confirmed as a trend by some multicentre and single-centre studies in different European and Asian countries. EARS Net data for 2020 show that Cyprus and Romania have the highest MRSA rates (49.1% and 47.3% respectively), while the lowest rates are reported in the Netherlands, Denmark and Norway (1.4 - 1.7%) (*ECDC, 2022*). In Bulgaria for the period 2011 - 2020, the relative proportion of MRSA shows a steady downward trend from 22.4% in 2011 to 11.8% in 2020 *(ECDC, 2012; ECDC, 2017; ECDC, 2022)*. In the present single-centre study, there was significant variation in the proportion of MRSA across years (between 32.1% in 2014 to 12.5% in 2020) with no evidence of a statistically significant downward trend (p=0.973). However, at the end of the study period (2020) the proportion of MRSA (12.5%) is lower than the European average (16.7%) and very close to the Bulgarian average (11.8%) for the same year *(ECDC, 2022)*.

Tracked over time, the activity of glycopeptide antibiotics and linezolid remained stable, with these two antibiotic groups demonstrating the highest activity (99.8%) among all 462 isolates. Only one *S. aureus* isolate resistant to both vancomycin and linezolid was identified over the entire 10-year period (2020). In this sense, in cases of MRSA bloodstream infections, vancomycin or linezolid remain the drugs of choice for treatment.

In the present study, a statistically significant trend was found across years for increasing resistance of *S. aureus* to gentamicin (from 6.3% in 2011 to 14.3% in 2020) (p=0.045), macrolides (from 9.4% to 32.1%) (p=0.001) and clindamycin (from 6.3% to 28.6%) (p=0.001).

Table 12. Antimicrobial resistance of *S. aureus* isolated from blood cultures of patients with laboratory-confirmed bloodstream infections between 2011 and 2020.

AB Group	**2011 - 2020**		**2011**		**2012**		**2013**		**2014**		**2015**		**2016**		**2017**		**2018**		**2019**		**2020**		***p***
	n	% R	n	% R	n	% R	n	% R	n	% R	n	% R	n	% R	n	% R	n	% R	n	% R	n	% R	
MRSA	462	15.4	32	9.4	29	10.3	41	19.5	28	32.1	52	15.4	42	16.7	50	8.0	71	14.0	61	19.7	56	12.5	0.973
gentamicin	462	16.5	32	6.3	29	13.8	41	4.9	28	14.3	52	21.2	42	19.0	50	22.0	71	18.3	61	21.3	56	14.3	**0.045**
ciprofloxacin	462	12.3	32	9.4	29	13.8	41	17.0	28	17.9	52	21.2	42	9.5	50	12.0	71	7.0	61	11.5	56	8.9	0.251
macrolides	462	20.6	32	9.4	29	10.3	41	7.3	28	17.9	52	23.0	42	23.8	50	16.0	71	23.9	61	26.2	56	32.1	**0.001**
clindamycin	462	15.4	32	6.3	29	3.4	41	2.4	28	10.7	52	19.2	42	9.5	50	14.0	71	19.7	61	21.3	56	28.6	**0.001**
glycopeptides	462	0.2	32	0.0	29	0.0	41	0.0	28	0.0	52	0.0	42	0.0	50	0.0	71	0.0	61	0.0	56	1.8	0.122
oxazolidinones	462	0.2	32	0.0	29	0.0	41	0.0	28	0.0	52	0.0	42	0.0	50	0.0	71	0.0	61	0.0	56	1.8	0.122

***Enterococcus* spp.**

The enterococci are Gram-positive bacteria that are found as part of the normal microflora of the gastrointestinal tract in humans and animals, but have the potential to cause invasive infections under conditions of dysbiosis. These microorganisms possess mechanisms that allow them to colonise the hospital environment and persist there for long periods, facilitating their transmission through multiple cross-contamination routes, including foreign bodies. *E. faecalis* and *E. faecium* are the most commonly isolated species associated with nosocomial outbreaks of bloodstream infections, urinary tract infections and endocarditis. An important feature of enterococci is, in addition to their innate resistance to cephalosporins, their acquired high-level resistance to several groups of antibacterial agents: glycopeptides, aminopenicillins and aminoglycosides. Currently, the most problematic is glycopeptide resistance mediated by *van* genes. Often vancomycin-resistant enterococci demonstrate a multidrug-resistant phenotype.

In the 10-year study period, the susceptibility to aminopenicillins, aminoglycosides (HLAR), ciprofloxacin, glycopeptides and linezolid was studied among a total of 171 *E. faecalis* and 72 *E. faecium* isolates. Over the entire period, the proportion of vancomycin-resistant *E. faecium* (VREfm) was 4.2%, and these isolates demonstrated also resistance to teicoplanin, aminopenicillins, HLARs, and fluoroquinolones, but had preserved susceptibility to linezolid. No vancomycin- and linezolid-resistant *E. faecalis* were found.

Resistance to the respective antibiotic groups in decreasing order for *E. faecalis* is as follows: HLAR, 48.5% > ciprofloxacin, 35.7% > аминопеницилини, 9.4%. Resistance levels for *E. faecium* were respectively: aminopenicillins, 95.3% > HLAR, 90.3% > ciprofloxacin, 84.7% > glycopeptides, 4.2%. No linezolid-resistant *E. faecium* were detected (Figure 4, Table 14).

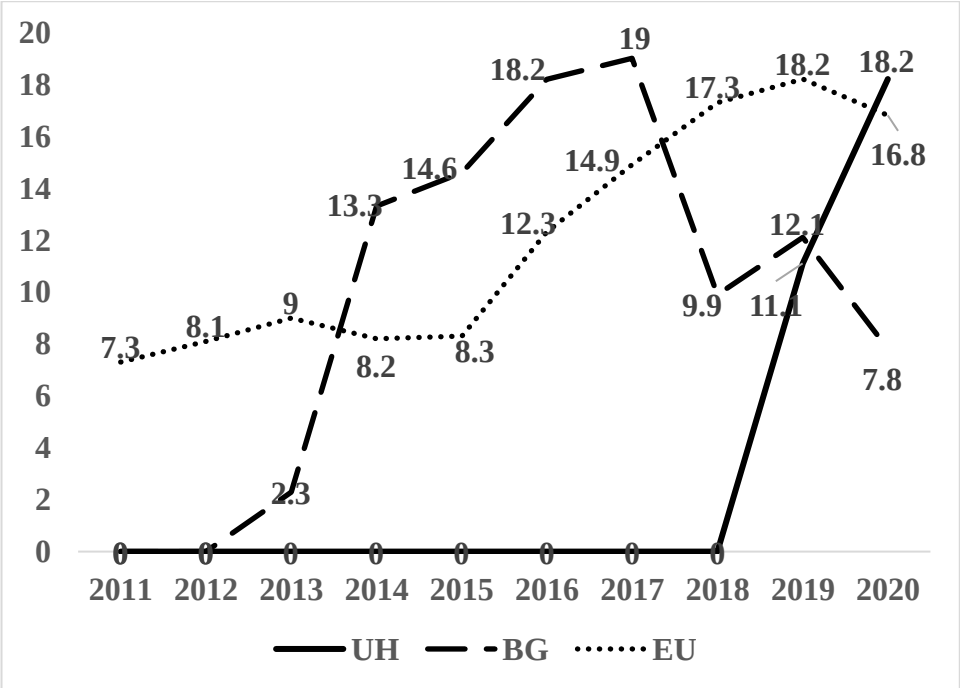

Figure 4. Relative proportion of vancomycin-resistant E. *faecium* isolated from blood cultures between 2011 and 2020. (source for EU and BG: https://atlas.ecdc.europa.eu/; https://ecdc.europa.eu).

Table 13. Antimicrobial resistance of *E. faecalis* isolated from blood cultures of patients with laboratory-confirmed bloodstream infections in the period 2011-2020.

AB Group	**2011 - 2020**		**2011**		**2012**		**2013**		**2014**		**2015**		**2016**		**2017**		**2018**		**2019**		**2020**		***p***
	N	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	
aminopenicillins	**171**	9.4	**17**	0.0	**9**	11.1	**18**	0.0	**14**	0.0	**8**	0.0	**20**	5.0	**20**	0.0	**15**	0.0	**17**	0.0	**33**	0.0	0.544
HLAR	**171**	48.5	**17**	17.6	**9**	33.3	**18**	33.3	**14**	50.0	**8**	75.0	20	85.0	**20**	90.0	**15**	46.7	**17**	29.4	**33**	33.3	0.532
glycopeptides	**171**	0.0	**17**	0.0	**9**	0.0	**18**	0.0	**14**	0.0	**8**	0.0	20	0.0	**20**	0.0	**15**	0.0	**17**	0.0	**33**	0.0	NA
linezolid	**171**	0.0	**17**	0.0	**9**	0.0	**18**	0.0	**14**	0.0	**8**	0.0	20	0.0	**20**	0.0	**15**	0.0	**17**	0.0	**33**	0.0	NA
ciprofloxacin	**171**	35.7	**17**	23.5	**9**	33.3	**18**	22.2	**14**	0.0	**8**	37.5	20	65.0	**20**	45.0	**15**	13.3	**17**	41.2	**33**	48.5	0.268

Table 14. Antimicrobial resistance of *E. faecium* isolated from blood cultures of patients with laboratory-confirmed bloodstream infections in the period 2011-2020.

	2011 - 2020		**2011**		**2012**		**2013**		**2014**		**2015**		**2016**		**2017**		**2018**		**2019**		**2020**		***p***
AB Group	**N**	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	
aminopenicillins	**72**	95.3	**5**	100	**5**	60.0	**10**	100	**7**	100	**2**	100	**3**	100	**14**	100	**6**	100	**9**	88.9	**11**	100	0.414
HLAR	**72**	90.3	**5**	100	**5**	80.0	**10**	100	**7**	100	**2**	100	**3**	66.6	**14**	100	**6**	83.3	**9**	77.8	**11**	81.8	0.208
glycopeptides	**72**	4.2	**5**	0.0	**5**	0.0	**10**	0.0	**7**	0.0	**2**	0.0	**3**	0.0	**14**	0.0	**6**	0.0	**9**	11.1	**11**	18.2	**0.026**
linezolid	**72**	0.0	**5**	0.0	**5**	0.0	**10**	0.0	**7**	0.0	**2**	0.0	**3**	0.0	**14**	0.0	**6**	0.0	**9**	0.0	**11**	0.0	NA
ciprofloxacin	**72**	84.7	**5**	100	**5**	100	**10**	100	**7**	85.7	**2**	100	**3**	0.0	**14**	71.4	**6**	100	**9**	88.9	**11**	81.8	0.524

A statistically significant trend for an increase in resistance over the years among *Enterococcus* spp. isolates was demonstrated only for the glycopeptide group of antibiotics among representatives of *E. faecium*: from 0% in 2011-2018 to 11.1% in 2019 and 18.2% in 2020. This result confirms the particularly unfavourable trend reported by EARS Net for Europe between 2011 and 2020 of an increase in the proportion of vancomycin-resistant *E. faecium* from an average of 7.3% in 2011 to 16.8% in 2020 *(ECDC, 2012; ECDC, 2022)*, with Lithuania (66.4%), Malta (55%) and Cyprus (51.2%) being the most affected. For Bulgaria, the proportion of VRE in 2021 is 10.1%, with an increasing trend in this type of resistance also evidenced between 2011 and 2020 (from 0.0% in 2011 to 7.8% in 2020), with a peak in the period 2014-2017 (13.3%-19%) *(ECDC, 2018; ECDC, 2020).*

A study on epidemiological trends and risk factors associated with vancomycin resistance of enterococci in patients with bloodstream infections in Europe (30 countries in total) between 2012 and 2018 also confirmed as statistically significant the trend of a steadily increasing proportion of VREfm from 8.1% in 2012 to 19.0% in 2018. The authors report that this is a trend observed in all 4 European regions, with significant regional differences: a higher proportion of VREfm in Eastern and Northern Europe (32% and 28.4%) compared to that in Southern and Western Europe (15.3% and 18.5%), which is in contrast to the typical North/West-South/East gradient specific for many other bacterial pathogens. There is also significant intra-regional heterogeneity at the country level in the 4 European regions, probably related to specific local factors *(Ayobami O, 2020a).*

The growing problem of an increasing proportion of VREfm causing bloodstream infections is also confirmed by independent studies conducted in individual European countries (Germany, Spain, Italy, Slovakia, Norway) and Turkey. It should be noted that despite the increased proportion of VREfm for Europe (20% in 2021), it remains lower than reported data for the USA (66%) and Australia (45%) *(Armin S, 2019; Coombs G, 2020; Jabbari S, 2019; Mendes R, 2018; Moghimbeigi A, 2018).*

In the present study, all VREfm isolates were also teicoplanin-resistant, suggesting that resistance in these isolates is likely mediated by the VanA gene. Co-resistance in VREfm to all antibiotic groups tested, excluding linezolid, demonstrates the extremely limited therapeutic options for VREfm-associated bloodstream infections. The preserved activity of linezolid in such cases makes it the drug of last choice for therapy, although there are already reports of emergence in Europe of linezolid resistance in enterococci, including in VREfm.

In the present study, among the entire collection of 72 isolates of *E. faecium*, we found highly reduced activity of all antibiotic groups (over 80%), except for glycopeptides and linezolid, which remain the drugs of choice for the treatment of bloodstream infections caused by *E. faecium.*

While VREfm are reported from different regions of the world, *E. faecalis* remains a significantly less affected species in terms of resistance to glycopeptide antibiotics. No blood isolates of *E. faecalis* resistant to vancomycin and teicoplanin were found. This result confirms what Ayobami et al. reported about a low mean proportion of vancomycin-resistant *E. faecalis* (VREf) associated bloodstream infections in Europe between 2012 and 2018 (1.1%), but with clear regional differences: a higher proportion of VREf in Northern and Eastern Europe (2.2% and 2.3%) compared to Western and Southern Europe (0.21% and 1.0%) *(Ayobami O, 2020a)*. National data for Bulgaria between 2011 and 2020 show a VREf proportion below 1% in 2011 to 0% in 2020, but with a peak of 3% in 2013 *(https://atlas.ecdc.europa.eu/).*

In the present study, in the group of *E. faecalis* isolates were found high levels of quinolone resistance and HLAR (over 30% and over 48%, respectively), with preserved aminopenicillin activity (less than 10% resistance) ($p>0.05$). The European mean HLAR in blood isolates of *E. faecalis* shows a statistically significant decreasing trend between 2011 and 2020 (from 33.7% to 29%), ranging widely from 6.7% in Sweden to 55.2% in Poland. For the

same period in Bulgaria, the proportion of HLAR *E. faecalis* increases from 30.6% in 2011 to 47.9% in 2021, which is in line with the results of this study *(ECDC, 2022a).*

2.2. Gram negative bacteria

E. coli

E. coli is a Gram-negative bacterial species representative of the *Enterobacteriaceae* family. It is part of the normal intestinal flora but also a causative agent of intestinal and extra-intestinal infections, including severe invasive infections such as bloodstream infections, sepsis and meningitis *(Bonten M, 2020).* Among the most problematic resistance in this bacterial species is resistance to third-generation cephalosporins and carbapenems, and the underlying genetic base of this resistance is the enzymatic mechanism, mediated by production of various β-lactamases (ESBLs; AmpC β-lactamases, carbapenemases) *(Bush K, 2020).*

In the 10-year study period, susceptibility to aminopenicillins, third-generation cephalosporins, carbapenems, aminoglycosides and fluoroquinolones was studied among a total of 399 blood isolates *of E. coli*. Resistance to the respective antibiotic groups in decreasing order over the entire period was as follows: ampicillin, 63.2% > ciprofloxacin, 33.6% > third-generation cephalosporins, 28.8% > gentamicin, 21.3% > amikacin, 3.3% > meropenem, 0.3% (Table 15, Figure 6).

The relative proportion of 3-rd generation cephalosporin-resistant blood isolates of *E. coli* in this study is significantly higher than the European average between 2011 and 2020 (between 9.6% - 15.1% - 14.9%) *(ECDC, 2017; ECDC, 2022).* Of note is the positive trend in the European Union in the time interval between 2016 and 2021 for a statistically significant decrease in the proportion of these isolates, with the average proportion reaching 14.9% and 13.8% in 2020 and 2021 *(ECDC, 2022).* In 2021, the largest number of countries in the EU is with resistance rates between 5 and 10%, with the lowest reported for Norway (5.5%) and the highest for Bulgaria (37.3%). For our country, the levels of this resistance in the period 2011-2020 are between 23% and 41.4% and are the highest in the EU. Followed over a longer time interval (2002 - 2020), the trend in Europe in terms of resistance to 3rd generation cephalosporins in blood isolates *E. coli* is increasing, particularly demonstrably until 2012 and less pronounced after that period *(https://ecdc.europa.eu)*. Similar trends of increasing proportion of 3-rd generation cephalosporin-resistant blood isolates of *E. coli* are reported in recent studies by European authors and international networks. For 2019, the Global Surveillance System for Antimicrobial resistance and Use reports a higher average proportion of bloodstream infections caused by *E. coli* resistant to 3-rd generation cephalosporins (36.6%), but also reports significant differences in the resistance rates between LMICs (58.3%) and HICs (17.53%) *(GLASS, 2021)*.

Among the studied 399 blood isolates of *E. coli*, only one carbapenem-resistant isolate (0.3%) was identified, making the carbapenem group with the highest and preserved activity against *E. coli* at present.

Table 15. Antimicrobial resistance of *E. coli* isolated from blood cultures of patients with laboratory-confirmed bloodstream infections in the period 2011-2020.

	2011-2020		2011		2012		2013		2014		2015		2016		2017		2018		2019		2020		
AB Group	**n**	**% R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	***p***
aminopenicillins	399	63.2	35	68.6	29	69.0	24	75.0	30	80.0	38	60.5	51	56.9	33	60.6	50	62.0	62	51.6	47	65.9	0.1
3rd generation cephalosporins	399	28.8	35	34.3	29	20.7	24	20.8	30	43.3	38	34.2	51	23.5	33	39.4	50	24.0	62	22.6	47	31.9	0.9
carbapenems (meropenem)	399	0.3	35	0.0	29	0.0	24	0.0	30	0.0	38	0.0	51	0.0	33	3.0	50	0.0	62	0.0	47	0.0	0.6
gentamicin	399	21.3	35	22.8	29	20.7	24	29.2	30	26.7	38	26.3	51	19.6	33	30.3	50	20.0	62	16.1	47	12.8	0.9
amikacin	399	3.3	35	0.0	29	0.0	24	4.2	30	3.3	38	7.9	51	5.9	33	0.0	50	4.0	62	0.0	47	6.4	0.4
fluoroquinolones	399	33.6	35	40.0	29	31.0	24	29.2	30	40.0	38	26.3	51	37.3	33	39.4	50	20.0	62	38.7	47	34.0	0.8

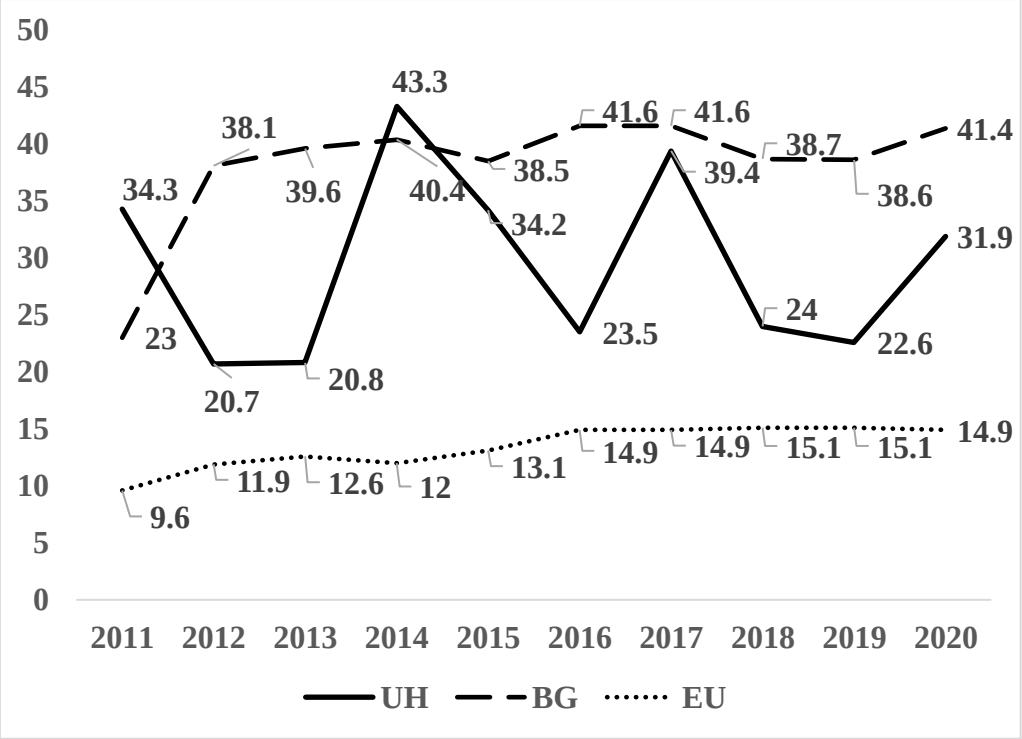

Figure 6: Relative proportion of 3-rd generation cephalosporin-resistant *E. coli* isolated from blood cultures between 2011 and 2020 (%). (source for EU and BG: https://atlas.ecdc.europa.eu/; https://ecdc.europa.eu)

Although remaining low in Europe, there is a significant increasing trend in the proportion of carbapenem-resistant *E. coli* in the last 5-year period (2017-2021), with the highest proportion in Greece (1.1%) and Cyprus (1%), followed by Italy, Romania and Bulgaria with 0.4% each. This is the only type of resistance in *E. coli* in the EU, which shows a statistically significant increasing trend between 2017 and 2021 (*ECDC, 2022a).*

The present study also found a high proportion of aminopenicillin-resistant *E. coli* (63.2% on average) - from 68.6% in 2011 to 65.9% in 2020. These results are consistent with the national data for Bulgaria, ranging from 60.5% in 2011 to 66.7% in 2020, with a significant downward trend in the second five-year period. This is also the general European trend, although it is in the lower range: from 57.6% in 2011 to 54.6% in 2020 *(ECDC, 2012; ECDC, 2017; ECDC, 2022).*

After aminopenicillins, in the group of isolates of *E. coli*, fluoroquinolone susceptibility is the most significantly affected - over 30% resistance. Although there has been a decline in the proportion of fluoroquinolone-resistant isolates, from 40% in 2011 to 34%, it is not statistically significant. In the same period, the levels of average fluoroquinolone resistance in this bacterial species for Europe were lower - between 22% and 23.8% with fluctuations from year to year, and for Bulgaria they were close to those we found - between 30% and 42.9% *(ECDC, 2012; ECDC, 2022).*

The average resistance to gentamicin in *E. coli* over the ten-year period is 21.3%, and although statistically not significant, there is evidence of a decline from 22.8% in 2011 to 12.8% in 2020. A possible explanation for this trend is the restricted consumption of gentamicin in our hospital during this period.

After carbapenems, amikacin is the antibacterial agent with the highest activity and the lowest average resistance rates (3.3%), despite the trend of increasing over the years - from 0% in 2011 to 6.4% in 2020 ($p>0.05$).

Klebsiella pneumoniae

K. pneumoniae is a Gram-negative opportunistic pathogen. Among members of the *Enterobacteriaceae* family, this bacterial species is most commonly associated with nosocomial infections, having the ability to spread rapidly and affect patients *(Di Franco S, 2021)*. However, the most problematic aspect of this bacterial species is related to the production of several types of beta-lactamases (ESBLs and carbapenemases) mediating resistance to beta-lactam antibiotics. The documented high resistance to strategic beta-lactam antibiotics in *K. pneumoniae*, is also associated with the dissemination of global, high-risk clones carrying mobile elements (plasmids, integrons) that mediate, on the one hand, the global spreading of ESBLs and/or carbapenemases, and, on the other hand, the emergence of resistance to different antibiotic groups (fluoroquinolones, aminoglycosides, tetracyclines, trimethoprim/sulfamethoxazole, etc.), so determing the isolates as multiple- and even pan-drug resistant.

In the 10-year study period, susceptibility to 3-rd generation cephalosporins, carbapenems, aminoglycosides, and fluoroquinolones was studied among a total of 331 *K. pneumoniae* blood isolates. Resistance to the respective antibiotic groups is presented in Table 16 and Figures 11 and 12. The resistance in the carbapenem-resistant group of isolates (11.8%) to major antimicrobial groups was as follows: amoxicillin/clavulanic acid, 100%; ceftriaxone, ceftazidime, 100%; piperacillin/tazobactam, 92.3%; tobramycin, 89.7%; gentamicin, 76.9%; amikacin, 17.9%; ciprofloxacin, 100%; levofloxacin, 97.4%; trimethoprime/sulphomethoxazole, 51.3%; colistin, 6%.

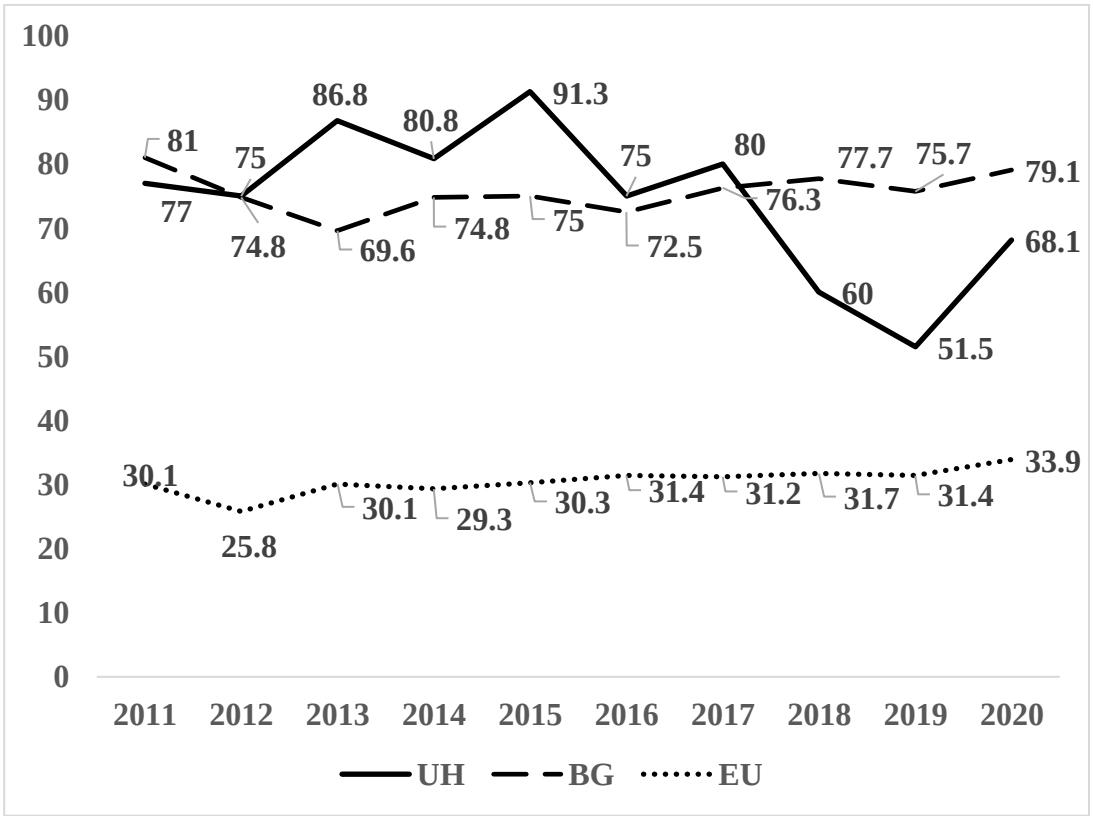

Figure 11. Comparative representation of the relative proportion of 3-rd generation cephalosporin-resistant *K. pneumoniae* isolated from blood cultures between 2011 and 2020. (%) (source for EU and BG: https://atlas.ecdc.europa.eu/; https://ecdc.europa.eu)

Table 16. Antimicrobial drug resistance of isolates of *K. pneumoniae* from blood of patients with laboratory-confirmed bloodstream infections in the period 2011-2020**.**

	2011-2020		**2011**		**2012**		**2013**		**2014**		**2015**		**2016**		**2017**		**2018**		**2019**		**2020**		
AB Group	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	***p***
3rd generation cephalosporins	331	74.9	26	77.0	36	75.0	38	86.8	52	80.8	23	91.3	36	75.0	35	80.0	30	60.0	33	51.5	22	68.1	0.063
carbapenems (meropenem)	331	11.8	26	0.0	36	2.8	38	13.2	52	32.7	23	8.7	36	8.3	35	2.9	30	3.3	33	18.2	22	13.6	0.554
gentamicin	331	58.6	26	73.0	36	55.5	38	71.0	52	77.0	23	60.8	36	52.8	35	62.9	30	43.3	33	33.3	22	40.9	**0.006**
amikacin	331	13.9	26	15.4	36	19.4	38	13.2	52	9.6	23	21.7	36	2.8	35	17.1	30	20.0	33	6.0	22	22.7	0.940
Fluoroquinolones	331	61.0	26	61.5	36	50.0	38	76.3	52	55.	23	69.6	36	63.9	35	68.6	30	60.0	33	45.5	22	63.6	0.736

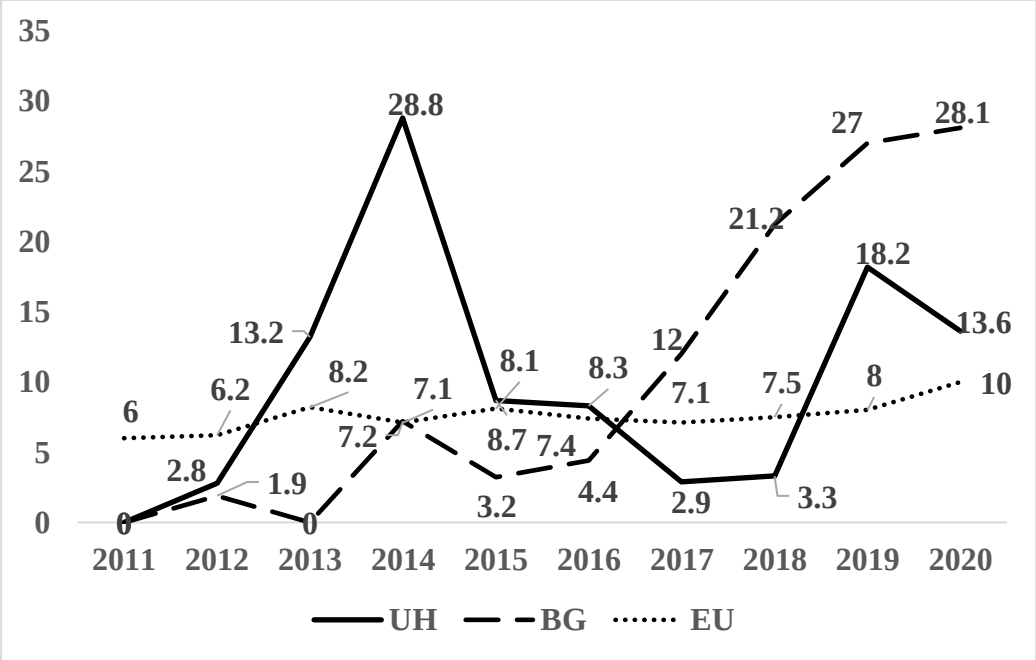

Figure 12. Comparative representation of the relative proportion of carbapenem-resistant *K. pneumoniae* isolated from blood cultures between 2011 and 2020 (%). (source for EU and BG: https://atlas.ecdc.europa.eu/; https://ecdc.europa.eu).

Genetic mechanisms of resistance to third-generation cephalosporins and carbapenems in carbapenem-resistant K. pneumoniae isolated from blood

In order to establish the genetic mechanisms of resistance to β-lactam antibiotics in the group of most problematic, carbapenem-resistant *K. pneumoniae*, PCR and nucleotide sequencing were used to detect the most common genes encoding ESBLs (CTX-M, SHV and TEM ESBLs), AmpC enzymes (DHA, FOX, EBC, MOX, ACC, CMY) and carbapenemases from classes A, B and D. A total of 29 (74.4%) of all 39 carbapenem-resistant (CR) *K. pneumoniae* isolated between January 2011 and December 2020 were studied. In 93.1% (n=27) $bla_{\text{CTX-M-15}}$ was detected. In two CR isolates, no genes encoding ESBLs from class A were identified. $bla_{\text{KPC-2}}$ was identified in 27 CR isolates (93.1%), of which 25 were also positive for $bla_{\text{CTX-M-15}}$ (92.6%). The gene encoding NDM-1 metallo-carbapenemase was detected in only two CR isolates (6.9%), simultaneously carrying both $bla_{\text{CTX-M-15}}$ and $bla_{\text{CMY-4}}$ (Table 17).

Epidemiological typing of carbapenem-resistant K. pneumoniae isolates

The genetic relationship between 28 CR *K. pneumoniae* isolates whose β-lactamase production was investigated was determined by ERIC PCR and MLST. For comparison, 20 carbapenem-susceptible *K. pneumoniae* blood isolates, isolated during the same period, as well as one isolate from the hands of medical personnel were also included in the epidemiological analysis. The study identified eleven different ERIC types (A, B, C, D, E, F, G, H, I, J, K), all corresponding very well to 11 MLST types - ST15, ST76, ST11, ST340, ST1350, ST151, ST902, ST70, ST359, ST37 and ST35, respectively (Table 17). The epidemiological typing demonstrated 3 major MDR clones of *K. pneumoniae* among all 49 isolates: ST15 (59.2%), ST76 (10.2%), and ST11 (8.2%). ST15 was the dominant clone represented by 29 isolates obtained from patients hospitalized in 12 hospital clinics and by one isolate from the hands of the medical staff in the ICU.

Table 17. Distribution of 49 isolates of *K. pneumoniae* obtained from blood cultures according to MLST type, year of isolation, clinic, and carriage of genes encoding ESBLs and carbapenemases.

MLST (n)	ESBL(n)/carbapenemase(n)	Year of isolation (n)	Clinic (n)
ST15 (n=29)**	*bla*$_{\text{CTX-M-15}}$ (n=12)	2014 $_{n=2,}$ 2016$_{n=3}$ 2017$_{n=7}$	ICU$_{n=1}$, INU$_{n=2}$, Neurology$_{n=1}$, IPU$_{n=1}$, Hemodialysis$_{n=1}$, Pediatrics$_{n=1}$, Hematology$_{n=2}$, Gastroenterology$_{n=1}$, Internal Medicine Clinic$_{n=1}$, Endocrinology$_{n=1}$
	bla$_{\text{CTX-M-15}}$ + **blaKPC-2 (n=17)***	2013$_{n=5,}$ 2014$_{n=9}$ 2015$_{n=1,}$ 2016$_{n=2}$	ICU$_{n=5}$, INU$_{n=5}$, Neurology$_{n=3}$, IPU$_{n=1}$, Cardiac Surgery$_{n=1}$, ICcU$_{n=1,}$ Internal Medicine Clinic$_{n=1}$
ST76 (n=5)	*bla*$_{\text{CTX-M-15}}$ + ***bla*$_{\text{KPC-2}}$ (n=5)***	2014$_{n=4,}$ 2015$_{n=1}$	ICcU$_{n=1}$, INU$_{n=1}$, ICU$_{n=1}$, Neurology$_{n=1}$, IPU$_{n=1}$
ST11 (n=4)	*bla*$_{\text{CTX-M-15}}$ + *bla*$_{\text{CMY-4}}$ + ***bla*$_{\text{NDM-1}}$ (n=2)***	2016$_{n=1}$, 2017$_{n=1}$	Hematology$_{n=1}$, ICcU$_{n=1}$
	bla$_{\text{CTX-M-15}}$ (n=2)	2016$_{n=1}$, 2017$_{n=1}$	Hemodialysis$_{n=1}$, Oncology$_{n=1}$
ST340 (n=2)	*bla*$_{\text{CTX-M-15}}$ (n=2)	2017$_{n=2}$	ICU$_{n=2}$
ST1350 (n=2)	***bla*$_{\text{KPC-2}}$ (n=2)***	2014$_{n=2}$	ICcU$_{n=1}$, INU$_{n=1}$
ST151 (n=2)	*bla*$_{\text{CTX-M-15}}$ + ***bla*$_{\text{KPC-2}}$ (n=2)***	2014$_{n=2}$	INU$_{n=2}$
ST902 (n=1)	*bla*$_{\text{CTX-M-15}}$ (n=1)	2014 $_{n=1}$	Nephrology$_{n=1}$
ST70 (n=1)	*bla*$_{\text{CTX-M-3}}$ (n=1)	2014 $_{n=1}$	Pediatrics$_{n=1}$
ST359 (n=1)	*bla*$_{\text{CTX-M-15}}$ + *bla*$_{\text{SHV-12}}$ (n=1)	2014 $_{n=1}$	Cardiac Surgery$_{n=1}$
ST37 (n=1)	*bla*$_{\text{CTX-M-3}}$ (n=1)	2016 $_{n=1}$	Pediatrics$_{n=1}$
ST35 (n=1)	*bla*$_{\text{CTX-M-15}}$ (n=1)	2016 $_{n=1}$	Haematology$_{n=1}$

Abbreviations: *CR isolates ,**included one isolate from the hands of medical personnel; **ICcU**, Intensive Cardiology Unit; **INU**, Intensive Neurology Unit; **ICU**, Intensive Care Unit; **IPU**, Intensive Pediatric Unit.

A higher proportion of ST15 isolates were obtained from patients hospitalized in the Neurological Intensive Care Unit (INU) (n=7) between November 2013 and May 2017; in the Neurology Clinic (n=4) predominantly in December 2017 (presented as a cluster) and as a single isolate in June 2017. ST15 was also identified in the ICU (n=6) between January-May 2013 and March-May 2015, and in the Pediatric Intensive Care Unit (IPU) (n=2) in January and June 2014. Presented as single isolates, ST15 was identified in 8 other clinics of the hospital between April 2014 and December 2017. The highest incidence of isolates was found in 2014 and 2017 and the lowest in 2015 (1 isolate in February 2015 in the Cardio-Surgery Clinic). All 29 ST15 *K. pneumoniae* isolates were positive for *bla*$_{\text{CTX-M-15}}$, and seventeen were also KPC-2 producers (58.6%) (Table 17).

ST76 was presented as a cluster of five KPC-2 and CTX-M-15 producing *K. pneumoniae* isolates from blood of patients hospitalized in INU, ICU, IPU and Neurology clinic between December 2014 and January 2015. The first ST76 CR KPC-2 producing isolate was identified earlier in March 2014 in the Intensive Cardiology Unit (ICcU).

ST11 was found in 4 MDR isolates obtained from blood of patients hospitalized in the Clinic of Hematology, Hemodialysis, Oncology and ICcU in 2016 (March, June) and 2017 (January, December). No clusters of cases were demonstrated. ST11 was associated with CTX-M-15 production in all isolates and with NDM-1 metallo-carbapenemase production in two CR *K. pneumoniae* isolated in March 2016 and December 2017, respectively.

Two CR K. *pneumoniae* isolated between November and December 2014 from blood of patients hospitalized in ICU co-producing KPC-2 carbapenemase and CTX-M-15 ESBL were assigned to ST151 type.

The ST1350 type represented by two CR KPC-2 producing *K. pneumoniae* isolates, was identified in November 2014 in the INU and ICcU.

Types ST35, ST37, ST70, ST359 and ST902 were only demonstrated in single isolates and interpreted as sporadic. ST37 and ST70 were associated with isolates producing only CTX-M-3 ESBL, and ST35, ST359 and ST902 with CTX-M-15 ESBL.

Overall, CR isolates (n=28) were represented by 5 sequence types: ST15 (60.7%), ST76 (17.9%), ST11 (7.1%) ST1350 (7.1%), ST151 (7.1%). The distribution of isolates according to the type of clinic they originated from, MLST type and their β-lactamase production are shown in Table 17.

The present study demonstrates a very high level of resistance to third-generation cephalosporins (74.9%) in the collection of 331 invasive isolates *of K. pneumoniae* over the ten-year study period, significantly exceeding the proportion of the resistant to the same antibiotic group *E. coli* (28.8%) and higher than among *Enterobacter* spp. isolates (68.5%) (see page 34). Of all antibiotics monitored, third-generation cephalosporins were the group with the most dramatically reduced activity against *K. pneumoniae*. Very high levels of resistance were also reported to fluoroquinolones and gentamicin, although the latter showed a positive, statistically significant trend for decreasing resistance from 73% in 2011 to 40.9% in 2020. Although with resistance levels above 10%, meropenem (11.8%) and amikacin (13.9%) are the antimicrobials with the highest activity against *K. pneumoniae*. It should be noted that among all antibiotic groups monitored, the greatest fluctuations in resistance over the years were observed for carbapenems: if in 2011 this resistance was practically 0%, in 2012 only one resistant isolate was detected, but a peak was observed in 2014 (32.7%), followed by a significant decline to 2.9% in 2017 and an upsurge again in 2019-2020 (18.2 - 13.6%). The group of carbapenem-resistant isolates in this study demonstrated very high levels of resistance to all tested antimicrobials except amikacin and colistin, which appear to be the drugs of choice for the treatment of carbapenem-resistant *K. pneumoniae* associated infections.

The results obtained in this study are consistent with those reported by EARS Net for the period 2011-2020: the average resistance of invasive isolates of *K. pneumoniae* for Europe

shows an increasing trend to third-generation cephalosporins (30% - 33.9%) as well as to fluoroquinolones (25% - 33.8%) and carbapenems (6% - 10%) *(ECDC, 2015; ECDC, 2018; ECDC, 2022).* In Bulgaria, similar trends are found, but at significantly higher levels than the European average: third-generation cephalosporins, 81%-79.1% (highest in Europe); fluoroquinolones, 51%-67.1%; and carbapenems, from <1 in 2011 to 28.1% in 2020, and for fluoroquinolones and carbapenems this increasing trend is statistically significant *(ECDC, 2015; ECDC, 2018; ECDC, 2022).* Similar to what is found in this study, a statistically significant decrease in resistance to aminoglycosides is also found in Europe between 2016 and 2020, from 24.4% in 2016 to 23.7% in 2020 *(ECDC, 2022).* Among all antibiotics, a statistically significant trend towards an increase between 2011 and 2020 for Europe is only demonstrated for the group of carbapenems, from 6% in 2011 to 10% in 2020. Moreover, for laboratories that fall into the group of "persistent reporters" to EARS Net in the period 2017 - 2021, the annual change in the proportion of carbapenem-resistant *K. pneumoniae* is dramatic (0%, 8%, 31% and 20% for 2018 - 2021) *(ECDC, 2022a).* A characteristic of all antibiotic groups monitored is the wide variation in resistance levels between European countries. For example, in terms of resistance to third-generation cephalosporins: although the highest average resistance rate for 2021 is that to third-generation cephalosporins (34.3%), it varies between 3.4% for Iceland, 5.1% in Denmark, 7% Sweden to 81.4% for Bulgaria *(ECDC, 2022a).* Similarly for the carbapenem resistance in 2021: 0% resistance is demonstrated in Iceland and the Netherlands to 46.3% in Bulgaria, 54.5% in Romania and 73.7% for Greece. In 12 countries, carbapenem resistance levels are below 1%. Also regarding this type of resistance, countries particularly affected include those in southern and south-eastern Europe (Greece, 73.7%; Romania, 54.5%; Bulgaria, 46.3%; Croatia, 32.9%; Italy, 26.7%, Cyprus, 26.2%) *(ECDC, 2022a).* These variations in resistance levels are confirmed by various multicentre studies on the antimicrobial profile of the main aetiological agents of bloodstream infections *(De Socio G, 2019; Holmbom M, 2020; Pérez-Crespo PM, 202; Schöneweck F, 2021).*

The trends of increasing proportion of third-generation cephalosporin and carbapenem-resistant *K. pneumoniae* from blood (over 30% and over 3%, respectively) between 1997 and 2016 is confirmed by the SENTRY program *(Diekema DJ, 2019)* The same program also reported a higher incidence of invasive ESBL-producing *Klebsiella* spp. and CRE in Europe compared to North America (24.1% and 10.9% vs. 12.5% and 2.3%) *(Pfaller M, 2020)*. Data from SENTRY for a later period (2013 - 2019) show higher levels of resistance among over 6800 *K. pneumoniae* blood isolates to ciprofloxacin, 30.7%; gentamicin, 19.0%; 3rd generation cephalosporins, 29.7 - 30.6% and carbapenems, 8% - 8.4%; amikacin resistance was 8.7% *(Di Franco S, 2021).* Higher rates of resistance to third-generation cephalosporins in 2019 in *K. pneumoniae* (40-50%) and low to intermediate levels of resistance to carbapenems in the same bacterial species associated with bloodstream infections are reported by GLASS *(GLASS 2021).*

One of the objectives of this study was to identify *bla* genes mediating resistance to beta-lactam antibiotics (third-generation cephalosporins and carbapenems) in carbapenem-resistant isolates of *K. pneumoniae* and their association with certain ST types.

CTX-M-15 beta-lactamase was identified as the predominant ESBL, both in the group of carbapenem-resistant isolates and among carbapenem-susceptible *K. pneumoniae* included for comparative epidemiological analysis. Of the carbapenem- susceptible isolates, only two produced CTX-M-3 ESBL. Co-production of CTX-M-15 and KPC-2 carbapenemase was detected in 92.6% of CR *K. pneumoniae*. Globally, *bla*$_{\text{CTX-M-15}}$ has been shown to be the most common gene associated with resistance to third-generation cephalosporins in clinically significant Gram-negative bacteria, particularly *E. coli* and *K. pneumoniae*. Consistent with the present study, widespread dissemination of *bla*$_{\text{CTX-M-15}}$ has been demonstrated in the United

Kingdom, the Netherlands, Germany, Greece, the Czech Republic, France and Denmark (*Bevan E, 2017).* Results from SENTRY 2016 demonstrate that 60.3% of *K. pneumoniae* in the USA carry *bla*$_{\text{CTX-M}}$, with 52.7% being CTX-M-15 producers *(Mendes R, 2019).* A study in Bulgaria from 2017 on *K. pneumoniae* isolates collected from 6 hospitals identified CTX-M-15 and CTX-M-3 producers in 87% and 9%, respectively, as well as SHV-12 and SHV-2 producing *K. pneumoniae*, but only in 2% and CTX-M-14 in 1% *(Markovska R, 2017).* *A* high relative proportion of ESBLs producing invasive isolates of *K. pneumoniae* associated with bloodstream infections and prevalence of CTX-M ESBLs are reported in similar studies by authors in Italy (32.6%), Russia (60.8%), USA (51.8%), Korea (52.9%) and China (27.5%) *(Xiao S, 2017).* In the present study, the plasmid-encoded AmpC cephalosporinase - CMY-4 was identified in two carbapenem-resistant isolates of *K. pneumoniae*, both co-producing CTX-M-15 ESBL and NDM-1 metallo-carbapenemase. A recent study on fecal carriage of high-risk *Enterobacterales* clones among patients hospitalized in 6 hospitals between 2017 and 2019 in Bulgaria demonstrated a diversity of ESBLs; among *K. pneumoniae* isolates, CTX-M-3 was the dominant ESBL (41%), followed by CTX-M-15 (33%) and CTX-M-14 ESBLs (1.4%), and the identified carbapenemases were NDM-1 (14.8%) and KPC-2 (1.4%), with NDM-1 isolates being co-producers of CTX-M-15/-3 ESBLs and/or CMY-4 β-lactamases *(Markovska R, 2022).*

International high-risk *K. pneumoniae* clones are among the most common and clinically important hospital pathogens. *Bla* genes, usually with plasmid localization, are frequently associated with "successful" *K. pneumoniae* ST types. In the present work, all carbapenem-resistant isolates studied (with two exceptions) were KPC-2 producers, with the first KPC-2 producer from blood identified in April 2012. *bla*$_{\text{KPC}}$ is currently endemic in many countries worldwide: in Europe, especially in Austria, Germany, Russia and the United Kingdom; in Latin America, incl. in Asia, China, Japan, Taiwan, as well as in Israel and the USA. The dominant ST among the 49 isolates of *K. pneumoniae* in this study was ST15, and this type was found in various hospital clinics during the study period. Similar to ST258 and ST11, ST15 *K. pneumoniae* is also known as a "successful" international clone. It often carries *bla*$_{\text{CTX-15}}$, but also KPC, NDM, VIM and OXA-48 genes. This is consistent with the results of the present study, which demonstrate that all ST15 isolates carry *bla*$_{\text{CTX-M-15}}$. In this aspect, the high proportion of CTX-M-15 ESBLs and their widespread dissemination are mostly associated with the epidemic ST15 international clone, which also co-produces KPC-2 carbapenemase. In this study, *bla*$_{\text{CTX-M-15}}$ dissemination was also mediated by other, less widespread ST types: ST11, ST340, ST76, ST902, ST359, ST35. A study in Bulgaria on clinical isolates of ESBL-producing *K. pneumoniae* also found that CTX-M-15 isolates were predominantly ST15 (34.1%) and to a lesser extent CC17 (ST16, ST17, ST336), while CTX-M-3 producing *K. pneumoniae* are mainly ST29, ST70, ST432, ST542 and ST15 *(Markovska R, 2017).* In the present study, *bla*$_{\text{CTX-M-3}}$ and *bla*$_{\text{CTX-M-12}}$ genes, demonstrated in only two and one isolates respectively, were associated with ST70, ST37 and ST359, the latter reported as a widespread MDR clone associated with CTX-M-15 and KPC-2 production *(Aires-de-Sousa M, 2020; Markovska R, 2017; Woodford N, 2011).*

The dominant ST15 clone in this study, first demonstrated in early 2013, persisted between 2014 and 2017 and was found in 12 hospital clinics, including 4 intensive care units. ST15 was dominated as a cluster of bloodstream infections in INU and Neurology Clinic in December 2014, Hemodialysis, ICcU and INU in July 2016, respectively. As single isolates, ST15 *K. pneumoniae* was documented in 8 other clinics throughout the period, but mainly in 2014 and 2017. The detection of isolates with identical ST types obtained from patients hospitalized in different clinics of the hospital and the temporal distribution of cases over the 5 years of follow-up is an indication of intrahospital intermittent cross-transmission from a human source or from a source in the environment. Apparently, this epidemic lineage of *K. pneumoniae*, represented

by ST15 isolates, the majority of which are carbapenem-resistant and *bla*$_{KPC-2}$ positive, appears to be well adapted for prolonged transmission in the hospital setting, as confirmed by the long period of time in which it is documented and persists. The identification of *K. pneumoniae* ST15 from the hands of medical staff in one of the hospital's clinics demonstrates that medical staff are an important factor for nosocomial dissemination of the pathogen. Nosocomial acquisition and dissemination by the hands of medical personnel is one possible explanation for the nosocomial epidemic process associated with this particular ST type. Microbiological examination failed to identify *K. pneumoniae* from objects in the hospital environment.

The present study demonstrates ST15 as the dominant type associated with carbapenem-resistant *K. pneumoniae*, and KPC-2 carbapenemase as the most common carbapenemase mediating carbapenem resistance. Consistent with multiple similar studies showing *K. pneumoniae* ST15 widespread in Europe and associated with different carbapenemases (KPC-2, OXA-48, VIM-1) *(Rodrigues C, 2014)*, the results of the present study demonstrate a high relative proportion of ST15 (60.7%) in the group of carbapenem-non-susceptible invasive isolates studied, followed by ST76 (17.9%) and ST11, ST 151 and ST1350 (7.1% each). ST76, a variant of *K. pneumoniae* ST495, was identified in Greece in 2009-2010 and associated with KPC-2 production *(Giakkoupi P, 2011).* In 2018, Gong *et al.* reported a hospital outbreak (including cases of bloodstream infections) caused by *bla*$_{KPC-2}$ positive *K. pneumoniae* ST76 in China (*Gong X, 2018).* In 2014, Zhu also reported a hospital outbreak among newborns in Shanghai caused by carbapenem-resistant *K. pneumoniae* ST76 but producing NDM-1 carbapenemase (*Zhu J, 2016)*.

In the present study, *K. pneumoniae* ST11 type is the third most frequently demonstrated ST, being associated with *bla*$_{CTX-M-15}$ and *bla*$_{NDM-1}$ genes. This type was identified in May and June 2016, and later in January and December 2017, affecting patients in four different clinics of the hospital. *K. pneumoniae* ST11 is known as one of the important pathogenic clones of *K. pneumoniae*, widely distributed in countries in Asia (especially China), Latin America, the USA and Europe. The ST11 clone is also associated with the production of various carbapenemases (NDM-1, NDM-5, KPC-2, OXA-48) in both clinical and faecal isolates. This sequence type is associated with a variety of nosocomial infections, such as urinary tract infections, bloodstream infections, lower respiratory tract infections, etc. In recent studies from China, ST11 has been identified as the dominant ST among KPC-producing *K. pneumoniae* associated with bloodstream infections *(Fu P, 2019; Xiao S, 2017).* In a similar study on the distribution of different MLSTs in bacterial species of the *Enterobactericeae* family, Teo at al. reported MDR (including colistin-resistant) *K. pneumoniae* ST11 and ST20 as the dominant types *(Teo J, 2019).* Similar to our results, ST11 *bla*$_{NDM-1}$ */bla*$_{CTX-M-15/3}$/*bla*$_{CMY-4}$ positive isolates have also been identified in the Czech Republic *(Studentova V, 2015).*

In the present work, the dominant *bla*$_{KPC-2}$ gene is associated with four different types: ST15, ST1350, ST151 and ST76. *bla*$_{KPC-2}$ transmission is known to be associated predominantly with IncFIIAs plasmids. In this sense, dissemination of *bla*$_{KPC-2}$ containing plasmid between different clones of *K. pneumoniae* and introduction of new CR ST types with epidemic potential may explain the development of the epidemic process in the hospital. The high selective pressure in Bulgarian hospitals contributes significantly to the further expansion of new clones. Among all European countries monitored, the consumption of strategic antibiotics in the hospital sector in Bulgaria is the highest *(ECDC, 2022b).* The present study identified only two carbapenem-resistant NDM-1-producing *K. pneumoniae*, both from ST11. *bla*$_{NDM-1}$ gene has been demonstrated in isolates of *K. pneumoniae* from Africa, Europe, Australia, USA and Asia, specifically Turkey, Algeria, France, Italy, Greece, New Zealand, Mexico and China *(Gong X, 2018; Markovska R, 2022; Pitout J, 2015; Savov E, 2018; Todorova B, 2016).* A major reason for this is the rapid dissemination of ST11, ST15, ST70, ST258 and ST1883, all associated with the NDM-1 metallo-carbapenemase.

***Enterobacter* spp.**

Representatives of the genus *Enterobacter* are facultative anaerobic Gram-negative bacteria, which refer to 22 species. They are found both in environmental factors and as part of the normal intestinal flora of animals and humans. Currently, *Enterobacter aerogenes* and the species belonging to the *E. cloacae* complex are accepted as classical opportunistic pathogens, causing mostly nosocomial infections (bloodstream infections, pneumonias, urinary tract infections, post-operative peritonitis, meningitis), including nosocomial outbreaks, mainly affecting immunocompromised patients. Nowadays, most *Enterobacter* spp. isolates demonstrate resistance to beta-lactam antibiotics (including third-generation cephalosporins and carbapenems), quinolones and aminoglycosides. In the family *Enterobacteriaceae*, after *E. coli* and *K. pneumoniae, E. cloacae* complex are the third most common enteric bacteria resistant to 3rd generation cephalosporins, and this resistance is mostly associated with overexpression of chromosomally encoded AmpC cephalosporinase, ESBLs of the TEM, SHV, CTX-M and VEB classes, as well as with membrane-associated mechanisms and efflux pumps. In recent years, there have been increasing reports for emergence and increasing the proportion of clinical CR isolates of *E. aerogenes* and *E. cloacae* complex in Europe, Asia and America, associated with the production of carbapenemases of different classes.

In the 10-year period of the present study, the susceptibility to third-generation cephalosporins, carbapenems, aminoglycosides, and fluoroquinolones was studied among a total of 260 *Enterobacter* spp. blood isolates (Table 18).

Table 18. Antimicrobial resistance of *Enterobacter* spp. (*E. cloacae*, n=218*; E. aerogenes*, n=39*; E. agglomerans*, n=3) isolated from blood cultures of patients with laboratory-confirmed bloodstream infections between 2011 and 2020.

	2011-2020		**2011**		**2012**		**2013**		**2014**		**2015**		**2016**		**2017**		**2018**		**2019**		**2020**		
AB Group	**N**	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	**N**	**%R**	**p**
3rd generation cephalosporins	260	68.5	18	72.2	20	50.0	16	87.5	39	41.0	54	85.1	33	48.5	28	85.7	19	68.4	16	56.3	17	52.9	0.709
carbapenems (meropenem)	260	0.4	18	0.0	20	0.0	16	0.0	39	0.0	54	0.0	33	0.0	28	3.6	19	0.0	16	0.0	17	0.0	0.312
gentamicin	260	61.5	18	61.1	20	45.0	16	81.3	39	33.3	54	77.7	33	42.4	28	85.7	19	52.6	16	43.8	17	47.0	0.667
amikacin	260	5.0	18	22.2	20	5.0	16	0.0	39	5.1	54	0.0	33	0.0	28	14.3	19	0.0	16	12.5	17	0.0	0.434
fluoroquinolones	260	45.8	18	16.7	20	10.0	16	50.0	39	25.6	54	66.6	33	48.5	28	67.9	19	52.6	16	43.8	17	47.0	0.072

***Genetic mechanisms of resistance in carbapenem-resistant Enterobacter* spp.**

Among all 260 invasive *Enterobacter* spp. isolates tested, only one isolate, identified as *Enterobacter asburiae,* was resistant to carbapenems ($MIC_{imipenem,\ meropenem}$>8mg/L) and multidrug resistant (resistant to all cephalosporins, piperacillin/tazobactam, all aminoglycosides, ciprofloxacin and colistin). Susceptibility was found only to levofloxacin (MIC=0.25mg/L), fosfomycin (MIC<16mg/L), nitrofurantoin (MIC<16mg/L) and tigecycline (MIC=0.38mg/L). The isolate was identified as a producer of NDM-1 metallo-carbapenemase, CTX-M-3 ESBL and ACT-68 AmpC and assigned to *E. cloacae* MLST type ST23.

Over the 10-year study period, a high level of resistance to third-generation cephalosporins was demonstrated in *Enterobacter* spp. (68.5%), very similar to that found for *K. pneumoniae* and significantly exceeding the proportion resistant to the same antibiotic group *E. coli*. Very high rates of resistance were also reported to gentamicin (61.5%) and fluoroquinolones (45.8%). Carbapenems (0.4% resistance) and amikacin (5%) proved to be the antimicrobials with the highest activity. Lower levels of resistance in *Enterobacter* spp. isolated from blood of patients with bloodstream infections were reported by S. De Franco and M. A. Pfaller based on results from the SENTRY Antimicrobial Surveillance Program: resistance to ceftriaxone, 26.1 - 31.6%; to ciprofloxacin, 14.3%; gentamicin, 8.3%, but similar levels to those we found to meropenem (0.4 - 0.7%) and amikacin (2.1%) *(Di Franco S, 2021; Pfaller M, 2020).* Higher levels of carbapenem resistance in *Enterobacter* spp. (9.47%) were reported in a large Chinese study from 2012-2017 *(Yang S, 2019).* The authors reported resistance to third-generation cephalosporins around 40%, to gentamicin 20-25%, ciprofloxacin and amikacin less than 20%, with no significant changes in resistance levels over the years. Also authors from China, report for the period 2010-2019 a significant trend of decreasing proportion of invasive isolates of *E. cloacae* resistant to third-generation cephalosporins (from 53.8% to 34.3%), to gentamicin (from 38.5% to 17.1%), cefepime (38.5% to 15.7%) and piperacillin/tazobactam (from 23.1% to 8.6%). Although without a significant trend, meropenem (7.7% to 10%) and amikacin (7.7% to 5.7%) were among the most active antibiotics over the 10-year period *(Liu C, 2022)*.

In contrast to our findings, in a Spanish study, J. Robleto reported an increase (albeit statistically not significant) in the proportion of ceftazidime (from 58.5% to 78%), cefepime (from 75.5% to 82.9%) and imipenem-susceptible *E. cloacae* (from 88.7 to 89.2%) from blood also isolated between 2010 and 2019. The proportion of carbapenem-resistant isolates was higher than our findings and ranged between 10.8 and 11.3% for imipenem and between 7.3% and 9.6% for meropenem *(Robledo J, 2022).* A high relative proportion of resistant *Enterobacter* spp. to different antibiotics was also reported in an extensive Iranian study covering the period 1996 - 2021: ceftriaxone, 49.3%; cefepime, 43.6%; ciprofloxacin, 35.3%; gentamicin, 42.1%; amikacin, 30.3% and meropenem, 16.2% *(Khademi F, 2022).*

The results of this study indicate that carbapenems are still the drugs of choice for the treatment of bloodstream infections caused by ESBL-producing and MDR *Enterobacter* spp. Only one carbapenem-resistant *E. asburiae* was identified in the ten-year period, isolated in 2017, a producer of the NDM-1 metallo-carbapenemase. According to literature data, this species is relatively rarely identified as a species of clinical importance, being mostly associated with blood cultures, although after 2017 Florio et al. found its gradual increase *(De Florio, 2018).* The isolate from the present study demonstrated multiple resistance. The emergence of such MDR isolates is of particular concern because of the potential risk of nosocomial dissemination. Between July 2017 and the end of 2021, four carbapenem-resistant *E. cloacae* complex isolates (3 from urine and 1 from fecal sample), identified as VIM metallo-carbapenemase producers, were detected in our hospital *(Niyazi D, 2022; Savova D, 2023).* Although *Escherichia* and *Klebsiella* species are the two most problematic species in terms of carbapenemase and ESBL production in the *Enterobacteriaceae* family, in the USA, for

example, carbapenem-resistant *Enterobacter* spp. are the second most common carbapenem-resistant *Enterobacteriaceae (Chavda KD, 2016).* According to the CDC from 2019, the increased prevalence of carbapenem-resistant *Enterobacteriaceae*, specifically *Enterobacter cloacae* complex, is now a serious public health concern in the United States *(https://www.cdc.gov/drugresistance/pdf/threats-report/2019-ar-threats-report-508.pdf.).*

There are relatively few reports describing MLST types among the *Enterobacter cloacae* complex. The first study on carbapenemase-producing *E. cloacae* clones, was performed by Izdebski on 173 cephalosporin-resistant isolates of *E. cloacae* from Israel and several European countries. The authors identified ST78, ST114, ST108 and ST66 as the most common STs, and the KPC-2 and VIM-1 carbapenemases identified in the study were associated with ST78 and ST114, respectively *(Izdebski R, 2015)*. In contrast, CR *E. asburiae* isolate from blood in the present study, was identified as ST23. The results of a large study on carbapenemase-producing *Enterobacter* spp. from 2008-2014 identified 4 global clones, ST114, ST93, ST90, ST78, and reported VIM carbapenemase as the most common, followed by NDM, KPC, OXA-48, and IMP enzymes *(Peirano G, 2018).* Although the collection of isolates is mainly represented by *E. xiangfangensis* and *E. hormaechei*, the study also reported NDM-producing *E. absuriae* isolate, but of ST435 type *(Peirano G, 2018).* The carbapenem-resistant isolate of *E. asburiae* in the present study also produced AmpC cephalosporinase of the EBS family (ACT-68). The high levels of resistance in this isolate can be explained by the co-production of the three different beta-lactamases, NDM-1 carbapenemase, CTX-M-3 ESBL and ACT-68. In addition, the isolate also demonstrated resistance to colistin, an antimicrobial agent considered one of the few antimicrobial agents of last choice for the treatment of infections caused by such problematic MDR strains. This result is consistent with the study of P. Iglesias published in 2023, covering the period 2014 - 2019, which reported a 12% proportion of carbapenem-resistant *Enterobacter* spp. from blood (*E. xiangfangensis*, *E. hoffmannii*), all producers of the OXA-48 carbapenemase and belonging to the high-risk and globally prevalent STs 66, 171, 78, responsible for the dissemination of genes encoding resistance to both carbapenems and extended-spectrum cephalosporins, and to colistin *(Lumbreras-Iglesias P, 2023).* In this study, Iglesias reported also resistance to colistin in some OXA-48 producing *Enterobacter* spp., mediated by carriage of mcr-9 gene. Several studies reported a higher prevalence of colistin resistance in clinical isolates of *Enterobacter* spp. than in *E. coli* and *Klebsiella* spp., reaching 0.7% in global studies *(Binsker U, 2022).* In this sense, the prevalence of carbapenemase-producing *E. cloacae* complex carrying genes encoding resistance also to colistin, is a serious problem as these are isolates frequently associated with invasive infections. Adequate hospital surveillance and investigation of these problematic bacteria is of utmost importance because of their significant potential for epidemic spread, causing difficult to control nosocomial outbreaks and association with high mortality *(Girlich D, 2021)*

Pseudomonas aeruginosa

P. aeruginosa is one of the most biochemically adaptable bacterial species, widely distributed in the environment and often in hospital environments (respirators, swimming pools, sinks, disinfectant solutions, cleaning objects). It can also be found on human skin, oral mucosa and gastrointestinal tract, especially in patients with prolonged hospitalization and antibiotic treatment. Because of its rich arsenal of virulence factors, *P. aeruginosa*, can cause severe, life-threatening acute and chronic infections, especially in immunocompromised patients; it is a leading etiologic agent of bloodstream infections and sepsis in neutropenic patients and of nosocomial ventilator associated pneumonias *(Wood S, 2023). P. aeruginosa* is among the four most common bacterial pathogens in European hospitals, being associated with various ICU infections (UTIs, pneumonias and bloodstream infections) *(Qin S, 2022).* In

addition to the severe course of the pseudomonal infections, a second challenge is the aetiological treatment of these infections, which is currently often extremely difficult not only because of the innate but also because of the acquired antimicrobial resistance of this pathogen, including the acquisition of resistance even in the course of ongoing antimicrobial chemotherapy (*Qin S, 2022; Wood S, 2023).*

Carbapenems are among the antimicrobials with a pronounced antipseudomonal effect, accepted as strategic drugs for treatment, but unfortunately already with a compromised effect against *P. aeruginosa*. The resistance of *P. aeruginosa* to carbapenems is most often associated with an enzymatic mechanism related to the acquisition of *bla* genes encoding carbapenemases of different classes, as well as mechanisms leading to reduced penetration or accumulation of the antibiotic in the bacterial cell or resulting from a combination of mechanisms. Recent studies on carbapenem-resistant isolates of *Pseudomonas* spp. (including *P. aeruginosa*) obtained from patients after hematopoietic stem cell transplantation at St. Marina University Hospital, Varna, identified $bla_{\text{VIM-2}}$ gene as a major mechanism mediating the resistance to carbapenems *(Niyazi D, 2022; Niyazi D, 2023)*. A study from 2022 on carbapenem-resistant *P. aeruginosa* isolates obtained from various clinical materials of patients hospitalized in the ICUs and COVID-19 clinics of the hospital between 2019 and 2021, did not identified the most common *bla* genes associated with carbapenem resistance in this microbial species, suggesting the presence of mechanisms other than the enzymatic (*Savova D, 2023).*

In the 10-year study period, the susceptibility to the following antipseudomonal agents: piperacillin/tazobactam, ceftazidime, carbapenems, aminoglycosides, and fluoroquinolones was studied among a total of 120 blood isolates of *P. aeruginosa* (Table 19, Figure 17).

In the present study, the proportion of CR isolates over the 10-year period (2011-2020) was 24.2%, and when tracked across years, it was highly variable, ranging from 0% in 2012 and 2013 to 66.6% in 2020, with no statistically significant trend. For the same period, national data show that the proportion of CR isolates of *P. aeruginosa* increases from 29% in 2011 to 42.9% in 2020, significantly exceeding the European average, with only Romania (43.9%) and Slovakia (48.9%) reporting higher levels than Bulgaria in 2020 *(ECDC, 2012; ECDC, 2017; ECDC, 2022).*

Table 19. Antimicrobial resistance of *P. aeruginosa* isolated from blood cultures of patients with laboratory-confirmed bloodstream infections between 2011 and 2020.

AB Group	2011-2020		2011		2012		2013		2014		2015		2016		2017		2018		2019		2020		*p*
	n	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	
piperacillin/tazobactam	120	30.8	7	57.1	13	23.0	16	12,5	8	25.0	15	26.7	14	35.7	19	31.6	11	36.3	8	12.5	9	66.6	0.645
ceftazidime	120	36.0	7	71.4	13	30.7	16	0.0	8	37.5	15	26.7	14	35.7	19	36.3	11	36.3	8	0.0	9	66.6	0.828
carbapenems (meropenem)	120	24.2	7	57.1	13	0.0	16	0.0	8	37.5	15	20.0	14	37.5	19	21.0	11	36.3	8	0.0	9	66.6	0.616
gentamicin*	111	35.1	7	42.8	13	30.7	16	43.8	8	37.5	15	26.7	14	50.0	19	31.6	11	36.3	8	12.5	-	-	0.854
amikacin	120	23.3	7	28.6	13	23.0	16	6.3	8	12.5	15	20.0	14	35.7	19	36.3	11	36.3	8	0.0	9	44.4	0.491
fluoroquinolones	120	40.0	7	42.8	13	30.7	16	43.8	8	37.5	15	40.0	14	50.0	19	31.6	11	36.3	8	12.5	9	77.7	0.666

*susceptibility to gentamicin has been determined until 2019.

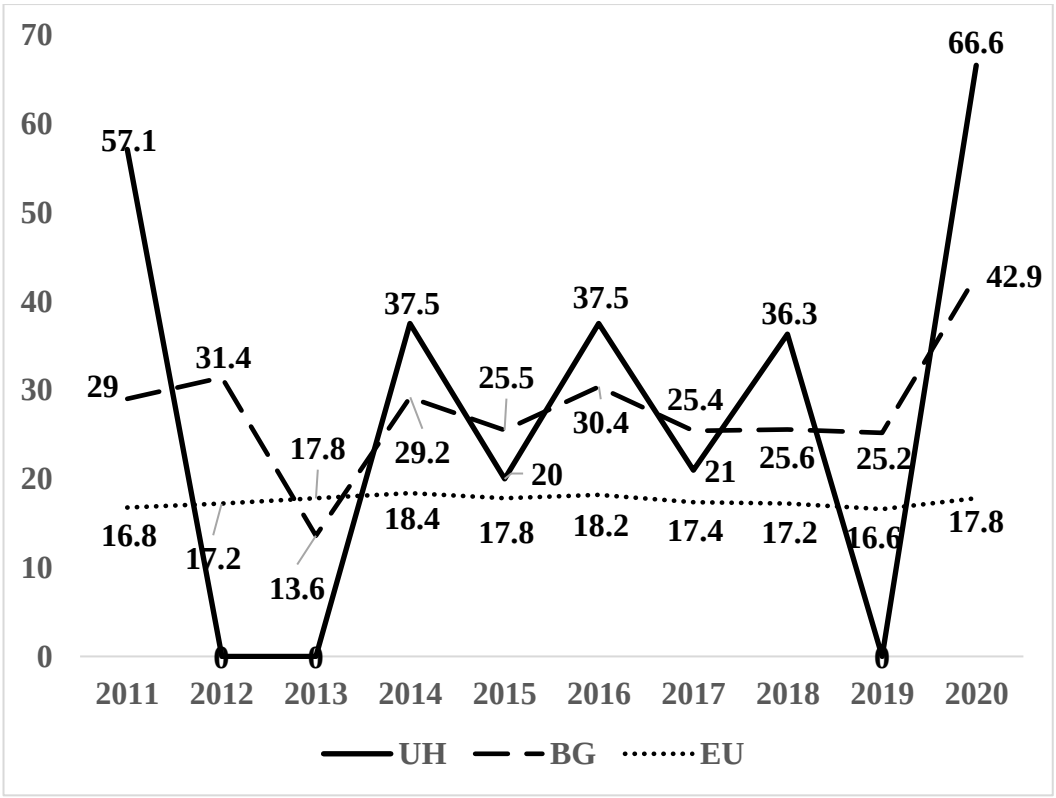

Figure 17. Comparative representation of the relative proportion of carbapenem-resistant *P. aeruginosa* isolated from blood cultures between 2011 and 2020 (%). (source for EU and BG: https://atlas.ecdc.europa.eu/; https://ecdc.europa.eu).

In the same period, average levels of carbapenem resistance among invasive *P. aeruginosa* isolates in Europe fluctuated slightly, between 16.8 and 17.8%, with even a statistically significant decrease in the proportion of carbapenem-resistant isolates demonstrated between 2016 and 2020, a trend that continues in 2021 *(ECDC, 2022a)*. In 2020, the largest number of countries (n=12) are found to have carbapenem resistance levels in the range of 20-30%, with 8 countries having levels below 10% *(ECDC, 2022)*. Overall in Europe for the period 2016-2021, EARS Net reports a trend towards stabilisation of antimicrobial resistance levels in *P. aeruginosa* to the major antipseudomonal agents, including carbapenems *(ECDC, 2022; ECDC, 2022a).* Similar results of a decreasing proportion of carbapenem-resistant *P. aeruginosa* isolates associated with bloodstream infections are reported from other studies on bloodstream infections in the period 2007-2019 *(De Angelis G, 2018; Liu C, 2022; Rothe K, 2019).*

In the present study, more than 25% resistance was found to all other antipseudomonal agents except amikacin, with the most reduced activity of fluoroquinolones, followed by gentamicin, ceftazidime and piperacillin/tazobactam. Between 2011 and 2020 in the EU, the average levels of resistance to fluoroquinolones and aminoglycosides were lower than those we found, ranging between 22.1% and 19.6% for fluoroquinolones and between 16.7% and 9.4% for aminoglycosides, with a significant downward trend over the years. In the same period for Bulgaria, the proportion of fluoroquinolone-, aminoglycoside-, piperacillin/tazobactam- and ceftazidime-resistant invasive isolates of *P. aeruginosa* is similar to that found in this study, being in the high resistance ranges if comparing with the majority of the EU countries monitored, with a statistically significant increasing trend found between 2016 and 2020 for piperacillin/tazobactam (from 40% to 64.3%) *(ECDC, 2022).* In the EU, average ceftazidime resistance rates are also lower than our findings, ranging from 12.8% in 2011 to 15.5% in 2020, with no significant increasing or decreasing trend across years *(ECDC, 2012; ECDC, 2017;*

ECDC, 2022). The results reported by EARS Net for 2020 assign Bulgaria the first position in the proportion of ceftazidime- (42.1%) and fluoroquinolone-resistant (52.9%) invasive *P. aeruginosa* isolates, the second and third position in the proportion of piperacillin/tazobactam (42.1%) and aminoglycoside-resistant isolates (32%) *(ECDC, 2022).*

***Acinetobacter baumannii - calcoaceticus* complex**

Members of the genus *Acinetobacter* are Gram-negative, glucose non-fermenting opportunistic organisms widely distributed in nature. At present, 59 species belong to the genus, with the most clinically significant being *Acinetobacter calcoaceticus - Acinetobacter baumannii* complex, which includes the closely related and difficult to distinguish by phenotypic methods species *A. calcoaceticus, A. baumannii, A. pittii, A. nosocomialis, A. seifertii* and *A. dijkshoorniae.* Of the members of this genus, *A. baumannii* is the species responsible for about 90% of all *Acinetobacter*-associated infections in the human population. This species can be found in the environment, but its natural habitat remains unknown. It is more commonly isolated from clinical materials and hospital environment than from natural sources *(Karah M, 2023; Nguyen M, 2021). A. baumannii* is an MDR opportunistic pathogen with a propensity for clonal spread, causing a variety of acute nosocomial infections and outbreaks, among which VAP, bloodstream infections, but also urinary tract infections, soft tissue and surgical wound infections are the most common. Among the most important risk factors for the development of *A. baumannii*-associated infections are ICU stays, prolonged hospital stays, surgical interventions or other trauma, very elderly patients, prior antibiotic therapy (especially third-generation cephalosporins and carbapenems), and procedures requiring the use of "foreign bodies." Although the proportion of *A. baumannii* infections is lower than that of other ESKAPEEc species, globally over 45% of *A. baumannii* are MDR, with rates exceeding 60% in the USA, Latin America and the Middle East and over 90% in Greece and Turkey. These MDR levels are more than 4-fold higher than the levels in *K. pneumoniae* and *P. aeruginosa (De Oliveira D, 2020).* An aspect in the physiology of *A. baumannii* is its ability to develop resistance very rapidly. From 2011 to 2016, the proportion of carbapenem-resistant *A. baumannii* increased by more than 30% globally *(Xie R, 2018).* Thus, currently, *A. baumannii* infections are commonly caused by MDR-, XDR- or PDR-strains, a phenomenon mediated by different enzymatic and non-enzymatic mechanisms. In 2019, the CDC determines carbapenem-resistant *A. baumannii* as an immidiate public health threat *(https://www.cdc.gov/drugresistance/pdf/threats-report/2019-ar-threats-report-508.pdf).* Carbapenem resistance in *A. baumannii* is primarily associated with the production of carbapenemases, with those of class D being the most common and those of classes A and B much less common.

In the 10-year period of the present study, susceptibility to carbapenems, aminoglycosides, fluoroquinolones and trimethoprim/sulphomethoxazole was studied among a total of 171 blood isolates *of A. baumannii - calcoaceticus complex* (Table 20, Figure 19). The resistance to major antimicrobials in the carbapenem-resistant (CR) group of *A. baumannii - calcoaceticus* complex isolated between 2016 and 2020 (n=60), representing 80% of all invasive *A. baumannii* isolates in this period was as follows: sulbactam, 50%; amikacin, 78.3%; gentamicin, 96.6%; tobramycin, 61.7%; ciprofloxacin, 100%; levofloxacin, 97.2%; trimethoprim/sulphomethoxazole, 85%; colistin, 0%. Five percent of these isolates were simultaneously resistant to 3 of the 6 antibiotic groups (carbapenems, aminoglycosides, quinolones, colistin, sulbactam, trimethoprim/sulphomethoxazole), 31.7% (n=19) to 4, and 60% (n=36) to five groups.

Table 20. Antimicrobial resistance of *A. baumannii - calcoaceticus* complex isolated from blood cultures of patients with laboratory-confirmed bloodstream infections in the period 2011 - 2020.

	2011 - 2020		**2011**		**2012**		**2013**		**2014**		**2015**		**2016**		**2017**		**2018**		**2019**		**2020**		
AB Group	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	**n**	**%R**	***p***
carbapenems (meropenem)	171	68.4	12	75.0	17	64.7	22	63.6	30	43.3	15	66.7	19	84.2	19	73.7	13	84.6	8	87.5	16	75.0	0.121
gentamicin	171	82.5	12	83.3	17	88.2	22	59.0	30	76.7	15	73.3	19	84.2	19	100	13	100	8	75.0	16	93.8	0.241
amikacin	171	76.6	12	91.7	17	88.2	22	54.5	30	76.7	15	53.3	19	78.9	19	84.2	13	92.3	8	75.0	16	81.3	0.853
Fluoroquinolones	171	88.3	12	91.7	17	100	22	68.2	30	90.0	15	73.3	19	84.2	19	100	13	100	8	87.5	16	93.8	0.545
TSM	171	67.3	12	50.0	17	94.1	22	59.0	30	66.7	15	60.0	19	68.4	19	78.9	13	92.3	8	75.0	16	31.3	0.809
colistin	75*	0.0	-		-		-		-		-		19	0.0	19	0.0	13	0.0	8	0.0	16	0.0	NA

* colistin susceptibility testing has been performed for all isolates after 2015; **NA**, not applicable; **TSM**, trimethoprim/sulphomethoxazole.

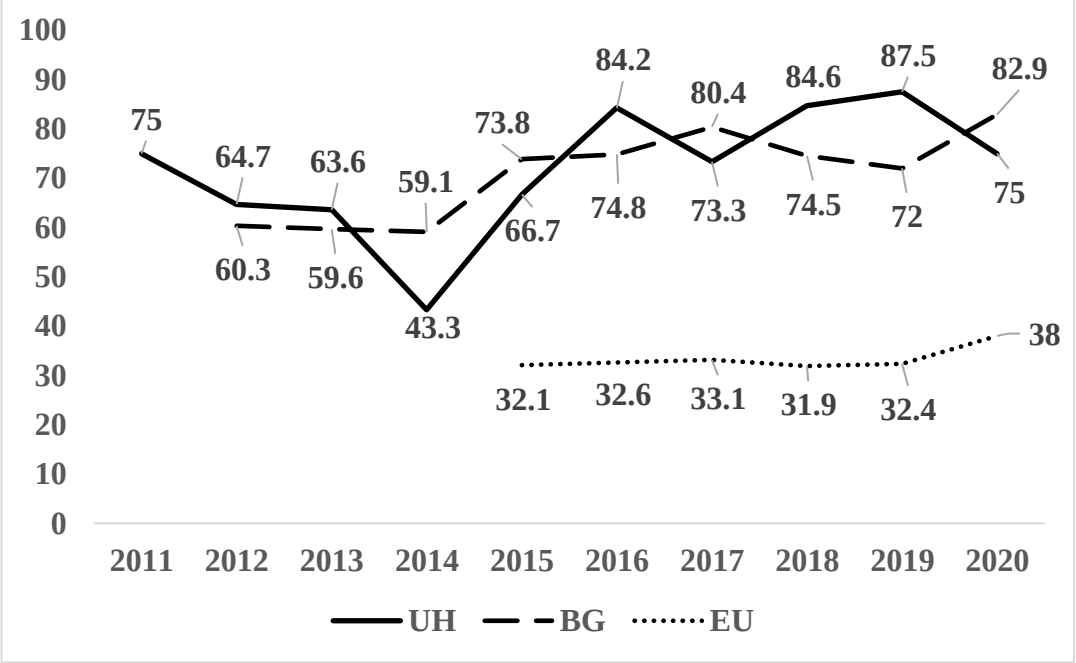

Figure 19. Comparative representation of the relative proportion of carbapenem-resistant *A. baumannii - calcoaceticus* complex isolated from blood cultures between 2011 and 2020 (in %). (source for EU and BG: https://atlas.ecdc.europa.eu/; https://ecdc.europa.eu).
(EU and BG data shown are for *Acinetobacter* spp. isolates).

Genetic mechanisms of resistance to carbapenems in carbapenem-resistant A. baumannii

The genetic mechanisms of carbapenem resistance were studied in a total of 71 *A. baumannii* isolated between 2010 and 2016, of which, twelve isolates were from blood cultures and associated with bloodstream infections (n_{2012}=3, n_{2015}=3, n_{2016}=6). Species identification of the isolates included in this analysis was confirmed by *gyrB* PCR.

To detect the most common OXA carbapenemases in *A. baumannii* multiplex PCR was used. All CR isolates tested were positive for the *bla*$_{\text{OXA-51-}like}$ gene, 35 (49.3%) were positive for the *bla*$_{\text{OXA-23-like}}$ gene (of these, 74.3% (n=26) were positive for *bla*$_{\text{OXA-23-like}}$ only and 25.7% (n=9) were positive simultaneously for *bla*$_{\text{OXA-40/24-like}}$) and 42 isolates (59.2%) were positive for *bla*$_{\text{OXA-40/24-like}}$ (of these, 78.6% (n=33) were positive for *bla*$_{\text{OXA-40/24-like}}$ only and 21.4% (n=9) were positive simultaneously for *bla*$_{\text{OXA-23-like}}$). Genes encoding other OXA-carbapenemases as well as metallo-carbapenemases were not detected. In three CR isolates, no genes encoding carbapenemases other than the intrinsic *bla*$_{\text{OXA-51-like}}$ were demonstrated. In the group of 12 CR blood isolates of *A. baumannii*, the proportion of OXA-carbapenemases was as follows: OXA-51-like, 100% (n=12); OXA-40/23-like, 75% (n=9); OXA-23-like, 41.7% (n=5), with co-production of OXA-23 and OXA-40/23 demonstrated in 16.7% (n=2). *ISAba1* was found upstream of the *bla*$_{\text{OXA-23}}$ gene in all OXA-23 producers.

Epidemiological typing of carbapenem-resistant isolates of A. baumannii

Epidemiological typing of 67 non-duplicate CR *A. baumannii* isolated between 2010 and 2016, eight of which from blood cultures, were performed by repPCR for the isolates from 2010 and 2012 (n_{2010}=1; n_{2012}=21) and by RAPD PCR for the isolates from 2014-2016 (n_{2014}=8; n_{2015}=18; n_{2016}=19). The species identification of all isolates in this analysis, was confirmed by *gyrB* PCR. For comparison purposes, strains, representative of the major *A. baumannii* international clones (IC1-8) were included in the epidemiological analysis of the isolates

obtained from patients in 2010 and 2012. The isolate from 2010 and all 21 isolates from 2012 collected from ICU patients (including three from blood cultures) formed a cluster, demonstrating identical or similar repPCR profiles and IC2 affiliation (Table 21).

Among the remaining 45 CR isolates from the period 2014-2016 (five from blood), four clusters were identified by RAPD PCR as follows: cluster I (25 isolates, 80% genetic relatedness); cluster II (2 isolates, 80% relatedness); cluster III (5 isolates, 81% relatedness), cluster IV (12 isolates, 98% relatedness). One isolate was interpreted as sporadic, demonstrating a unique RAPD profile. All five CR isolates obtained from blood cultures of patients hospitalized in three clinics of the hospital (INU, ICU, Cardio - Surgery) between 2015 and 2016 were assigned to cluster I (Figure 24, Table 21).

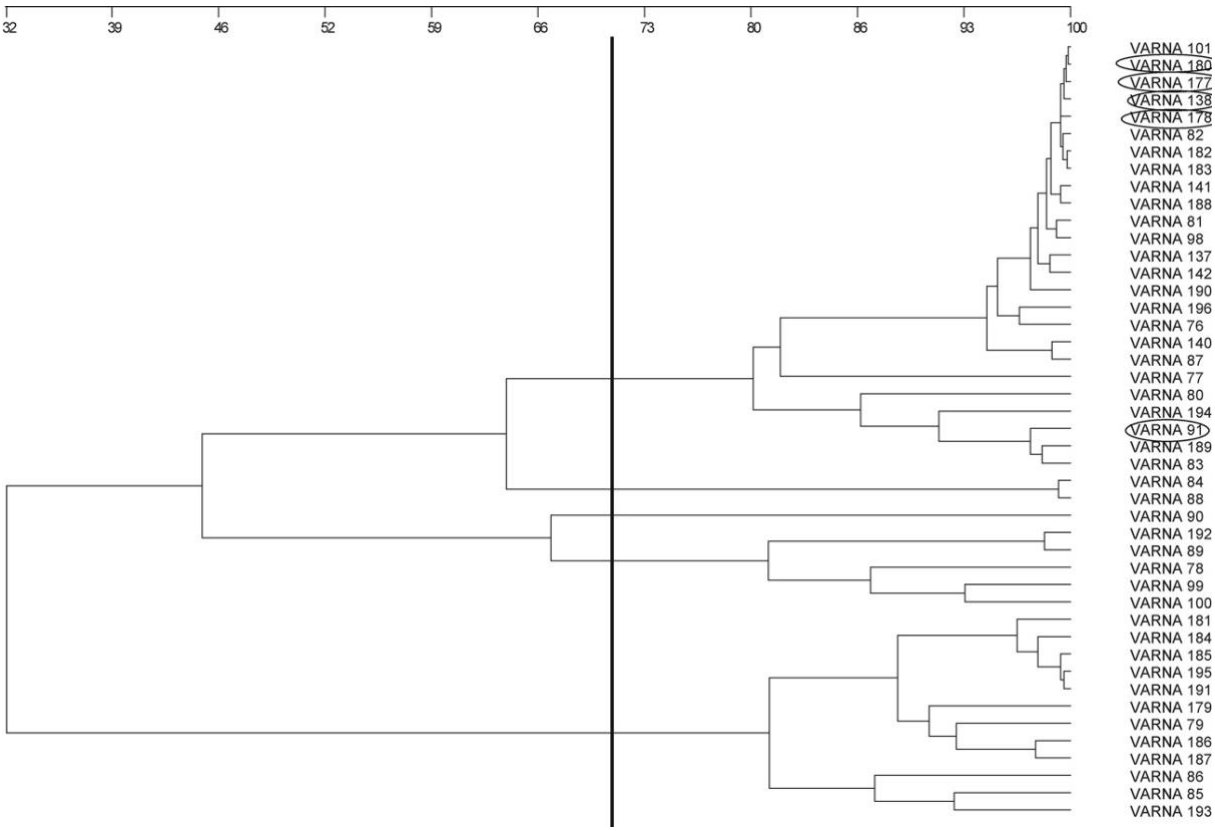

Figure 24. Dendrogram based on RAPD profiles, demonstrating the degree of similarity between carbapenem-resistant *A. baumannii* isolated from clinical samples of patients, hospitalized in St. Marina Hospital, Varna during the period 2014 - 2016*.

*№91, 138, 177, 178 and 180 are *A. baumannii* isolates from blood cultures.

Table 21. Distribution of carbapenem-resistant *A. baumannii* isolates from blood according to their RAPD/repPCR profile, year of isolation, clinic, and carriage of genes encoding carbapenemases.

RAPD/repPCR Profile	Affiliation to IC	Carbapenemase (n)	Year of isolation (n)	Clinic (n)
A (repPCR) (n=3)	IC2	*bla*$_{\text{OXA-23-like}}$ (n=3)	2012$_{n=3}$	ICU$_{n=3}$
Cluster I (RAPD PCR) (n=5)	ND	*bla*$_{\text{OXA-40/23-like}}$ + *bla*$_{\text{OXA-23-like}}$ (n=2)	2015$_{n=1}$, 2016$_{n=1}$	Cardiac Surgery$_{n=1}$ INU$_{n=1}$
	ND	*bla*$_{\text{OXA-40/24-like}}$ (n=3)	2015$_{n=1}$, 2016$_{n=2}$	ICU $_{n=3}$

Abbreviations: ND, not determined; **IC**, International Clone; **ICU**, Intensive Care Unit; **INU,** Intensive Neurology Unit

In the last decade, *A. baumannii - calcoaceticus* complex is among the most frequently isolated MDR organisms from clinical specimens of patients hospitalized at St. Marina Hospital, with the first CR isolates in the hospital being identified in 2009 from wound samples, but presented only as single isolates (data not shown). In the present study on 171 isolates of *A. baumannii - calcoaceticus* complex from blood, very high levels of resistance (> 60%) to virtually all antimicrobials were found over the ten-year follow-up period, with fluoroquinolones (88.3%) and aminoglycosides (76.6 - 82.5%) particularly affected, followed by carbapenems (68.4%). Moreover, 100% of CR isolates from the period 2016-2020 were defined as MDR, and 60% of them were XDR (susceptible only to colistin), confirming the fact that carbapenem resistance in *A. baumannii* alone is associated with extremely problematic treatment of the respective infection due to the lack of therapeutic alternatives.

According to EARS Net data, although no statistically significant trends related to resistance to the monitored antibiotic groups in *Acinetobacter* spp. were found in Europe for the period 2016-2020, in 2020, there is an increase in the European average carbapenem resistance to 38% compared to the previous four years (2016 - 2019), and for 2021 these levels reach 39.9%, resulting in a statistically significant increasing trend in the period 2017 - 2021 *(ECDC, 2022a).* Continuing trends from previous years, in 2021, the highest levels of carbapenem resistance were found in countries in Southern and Eastern Europe (Croatia, 99.5%; Greece, 96.9%; Latvia, 96.1%; Romania, 93.5%; Cyprus, 92.1%; Italy, 86.9%; Hungary, 83%; Poland, 82.7%; Bulgaria, 77.9%), while those in countries in Western and Northern Europe remain below 10% *(ECDC, 2022a).* In addition to the very large increase in the number of reported *Acinetobacter* spp. isolates in both 2020 and 2021, EARS Net reports a doubling (+121%) of invasive *Acinetobacter* spp. isolates for 2021, resistant to each of the three antibiotic groups (carbapenems, aminoglycosides, fluoroquinolones) compared to the 2018-2019 average, with the largest increase in CR isolates (48%). In addition, the largest increase in resistance is found for countries that have traditionally reported high levels of resistance before 2020, confirming the steadily worsening situation with *Acinetobacter* spp. in the last two years, also a result of the COVID-19 pandemic *(ECDC, 2022a).* In line with the findings of the present local study, increasing levels of resistance in the very high ranges to fluoroquinolones (from 69.2% to 82.9%), aminoglycosides (from 58.5% to 76%) and carbapenems (from 60.3% to 82.9%) are reported for Bulgaria between 2012 and 2020 (*ECDC,*

2015; ECDC, 2018; ECDC, 2022). Consistent with the local trends identified in this study, in Europe, a very commonly documented resistance phenotype in *Acinetobacter* spp. is also combined resistance to fluoroquinolones, aminoglycosides and carbapenems, with an average of 36.8% in 2021 and a significant increasing trend between 2017 and 2021 (*ECDC, 2022a).* In Bulgaria in 2020, the relative proportion of MDR *Acinetobacter* spp. with resistance to all three antibiotic groups significantly exceeds the European average (34%), reaching 72.9% *(ECDC, 2022).*

In 2020 and 2021, Bulgaria is the country with one of the highest total antibiotic consumption (hospital and community) in Europe (22.7 - 24.4 DDD/1000 population per day vs. European average of 16.4 DDD/1000), and in the hospital sector it is the country with the highest consumption of strategic antimicrobials (62.6 - 70 DDD/1000 population per day vs. European average of 38.6 - 40.3 DDD/1000) *(ECDC, 2022a).* These facts well correlate with the very high levels of resistance among the microbial species monitored by EARS Net in Bulgaria, incl. *Acinetobacter* spp., the highest for some combinations "bacterial species-antibiotic group" among all European countries. The association between hospital carbapenem consumption and carbapenem resistance levels in *Acinetobacter* spp. has been demonstrated in multiple studies. However, beyond antibiotic use, other potential factors such as ineffective organisation of the infection control activities and health care delivery, as well as climatic and socioeconomic conditions also should be taken into consideration *(Ayobami O, 2020).*

Similar to what was found in this study, and confirming the EARS Net data, GLASS also reported a high proportion of bloodstream infections caused by CR *Acinetobacter* spp. for 2019 based on isolates from 70 countries worldwide - 65.5% *(GLASS, 2021)* The SENTRY Antimicrobial Surveillance Program database provides information about similar proportion of CR *A. baumannii* isolates from bloodstream infections, between 57.5% and 59.6%, and a high proportion of resistant to quinolones (62%), aminoglycosides (43.8 - 55.3%) and colistin (7.9%) isolates *(Di Franco, S, 2021).* A recent global study including invasive and non-invasive carbapenem non-susceptible *Acinetobacter baumannii* complex isolates demonstrated a significantly higher proportion of these isolates in the Asia-Pacific region (~79%), Latin America (~85%) and North America (~45%) compared to European averages *(Gales A, 2019).*

The extremely unfavourable trend of increasing proportion of carbapenem-, MDR and XDR invasive isolates *A. baumannii* in the last decade is also reported by numerous single-center, multicenter, retro- and prospective studies in different countries (Italy, Greece, Turkey, China, South Korea, South and Southeast Asia) *(De Angelis G, 2018; De Socio G, 2019; Hsu L, 2017; Jarlier V, 2019; Liu C, 2019; Liu C, 2022; Yardimci A, 2022).*

Currently, six major groups of class D carbapenemases are associated with the emergence and spread of carbapenem resistance in *A. baumannii* - OXA-51-like, OXA-23-like, OXA-24/40-like, OXA-58-like, OXA-235-like and OXA-143-like *(Abouelfetouh A, 2019).* In the present study, OXA-24/40-like and OXA-23-like carbapenemases (100% associated with *ISAba1*) were shown to be the major mechanism of carbapenem resistance. Logically, the intrinsic $bla_{\text{OXA-51-like}}$ was identified in all tested isolates. Consistent with our results, $bla_{\text{OXA-23-like}}$ gene has been reported to be worldwide dessiminated and most frequent (North America, India, Asia-Pacific countries, South Korea, Europe) *(Al-Hassan L, 2021; Evans B, 2014; Hamidian M, 2019; Hammoudi D, 2015; Nguyen M, 2021; Moubareck C, 2020; Mugnier P, 2010; Nordmann P, 2019; Ramirez M, 2020; Santimaleeworagun W, 2016; Valcek A, 2022; Wibberg D, 2018).* OXA-23 production is the most common mechanism mediating carbapenem resistance in *A. baumannii* isolates also in several studies from the Balkan region (Croatia, Greece, Romania, Turkey, Bulgaria) as well as in the EURECA study (67.7%) investigating isolates from 10 countries (Albania, Croatia, Kosovo, Greece, Italy, Montenegro, Romania, Serbia, Spain and Turkey) in the period 2016-2018 *(Bonnin R, 2011; D'Onofrio V, 2020; Karampatakis T, 2017; Kostyanev T, 2021; Palmieri M, 2020; Petrova A, 2017; Pfeifer*

Y, 2017; Strateva T, 2012; Zeka A, 2014). OXA-23 carbapenemase has been identified as the leading resistance mechanism (96.4%) also among a large collection of carbapenem-resistant isolates from the Mediterranean region (Israel, Greece and Italy) *(Frenk S, 2022)*.

It should be noted that in a number of geographical regions OXA-23 carbapenemase has been identified co-produced with other carbapenemases in clinical isolates *A. baumannii*: OXA-23 and GES-11 (Lebanon, Kuwait, Sudan); OXA-23 and NDM-1 (India, Sudan); OXA-23 and OXA-58 (Tunisia, Sudan); OXA-23, OXA-58 and NDM-1 (Sudan); OXA-23, VIM-2 and NDM-1 (Thailand) *(Al-Hassan L, 2021; Hammoudi D, 2015; Kumar S, 2019; Mathlouthi N, 2018; Santimaleeworagun W, 2016; Zhu L, 2019)*. A study by Niyazi from 2022 on bacterial infectious complications in patients after hematopoietic stem cell transplantation identified carbapenem-resistant isolate of *A. baumannii* from blood possessing *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OX24/40,}}$ *bla*$_{\text{OXA48-like}}$ and *bla*$_{\text{VIM-like}}$ genes, a result that confirms the genetic plasticity of this bacterial species, enabling it to exploit a diversity of resistance mechanisms under conditions of high selective antibiotic pressure *(Niyazi D, 2022; Fournier P, 2006).* Consistent with these reports, the present study also identified co-production of OXA-23 and OXA-24/40 carbapenemases in 12.7%. In addition, *bla*$_{\text{OXA-24/40-like}}$ was identified in 59.2% of carbapenem-resistant isolates. Currently, *bla*$_{\text{OXA-24/40-like}}$ CR *A. baumannii* isolates have been identified worldwide, including isolates associated with nosocomial outbreaks *(Afzal-Shah M, 2001; Chen Y, 2018; Huang L, 2013; Kostyanev T, 2021; Kuo S, 2018; Nasiri M, 2020; Pailhories H, 2016; Pfeifer Y, 2016; Pfeifer Y, 2017; Poirel L, 2010; Sari A, 2013; Todorova B, 2014; Valcek A, 2022).* Similar results to those obtained in the present local study are reported by Lukovic et al. in the first multicentre study on 237 carbapenem-resistant *A. baumannii* in Serbia (43 isolates associated with bloodstream infections) *(Lukovic B, 2020).* In a study from Poland, OXA-24-like (OXA-72) was also shown to be the major mechanism of carbapenem resistance (64%), followed by OXA-23 (23%) and OXA-58 (10.2%) *(Słoczyńska A, 2021).*

Besides the plasmid-encoded carbapenemases OXA-23-like and OXA24/40-like, carbapenemases from OXA-58-like, OXA-143-like and OXA-235-like groups have also been identified in *A. baumannii (Higgins P, 2013; Mathlouthi N, 2018; Sarikhani Z, 2017).* In the present study, no *bla*$_{\text{OXA-58-like}}$ positive isolates were detected, which is in line with other studies demonstrating a shift from OXA-58-like to OXA-23-like and OXA-24/40-like production in *A. baumannii (Adams-Haduch J, 2011; Djahmi N, 2014; Liakopoulus A, 2012; Pournas S, 2017; Rosales-Reyes R, 2017; Schleicher X, 2013; Wang T, 2018)*. OXA-143 and OXA-23 carbapenemases have also been reported to be associated with nosocomial outbreaks in several countries, but they are not currently considered to be leading causes of carbapenem resistance in *A. baumannii (Hamidian M, 2019; Rodriguez C, 2018),* which is confirmed by our results.

Although rare, carbapenem-resistance in *A. baumannii* is also mediated by the production of class A carbapenemases (GES-11, KPC-2, KPC-3) and metallo-carbapenemases (NDM-1, NDM-6, VIM, GIM, SIM, IMP). No CR isolates producing carbapenemases of these classes were detected in the present local study.

The spread of MDR *A. baumannii* is widely associated with its specific propensity for clonal spread, with nine known international clones (IC 1-9) circulating globally, of which IC2 is widely distributed across continents *(Bansal G, 2020; Brito B, 2022; Graña-Miraglia L, 2020; Higgins P, 2010; Jones C, 2015; Mateo-Estrada V, 2021; Pournas S, 2017; Seifert H, 2020 Tomaschek F, 2016).* In addition, MDR and carbapenem-resistant *A. baumannii* are also known for their propensity to cause difficult-to-control nosocomial outbreaks, and these have been documented in the USA, Canada, South America, Europe, Africa, the Middle East, Southeast Asia and Australia *(Dandachi I, 2019; Hamidian M, 2019; Valencia-Martín, R, 2019; Wareth G, 2020).*

The very high relative proportion of carbapenem- and MDR- *A. baumannii - calcoaceticus* complex at St. Marina University Hospital in the period 2011-2020 raises the

question of whether the isolates are genetically related. To clarify the molecular epidemiology of representative isolates of *A. baumannii* (including those associated with bloodstream infections), repPCR and RAPD PCR were used. The results clearly demonstrated intra-hospital dissemination between 2014 and 2016 of clonal OXA-carbapenemase-producing MDR isolates grouped into four clusters, two of which were dominant and persistent over a three-year period (2014-2016), with cluster I comprising 55.6% of isolates, including isolates from blood cultures of patients hospitalized in three different clinics of the hospital. The results demonstrate the ability of A. *baumannii*, once introduced in the hospital environment, to persist there for a long time, causing nosocomial infections and outbreaks. The exceptional resistance to desiccation and disinfectants, together with a complex of virulence factors (biofilm production, AbOmpA protein, etc.) allow this microorganism to persist viable for extended periods on dry surfaces (up to 4 months) and in the hospital environment *(Nguyen M, 2021).* This specific feature contributes greatly to the clonal spread of isolates and predisposes to person-to-person transmission and environmental contamination *(Higgins P, 2010).* These results support previous and recent studies demonstrating successful dissemination and endemicity of certain CR clones of *A. baumannii* producing OXA-23, OXA-40/24 and OXA-58 carabenemases (including IC2) associated with nosocomial infections and outbreaks (bloodstream infections, VAP, etc.) in other Bulgarian hospitals *(Stoeva T, 2008; Stoeva T, 2009; Pfeifer Y, 2017; Stratev A, 2020*). It can be assumed that the introduction and spread of several clones leads to parallel outbreaks and polyclonal endemicity in the hospital, similar to what has been reported by other authors *(Marchaim, D, 2007; Mateo-Estrada V, 2021).*

The results of the epidemiological typing of 21 isolates from 2012 and one from 2010, all from ICU, obtained from patients with infections of different antatomic localization, including bloodstream infections, demonstrated a prolonged nosocomial outbreak associated with OXA-23-producing IC2 and its persistence with endemicity characteristics in this hospital setting. IC2 is currently the most widespread international clone globally documented among all sequenced *A. baumannii* genomes *(Levy-Blitchtein S, 2018; Seifert H, 2020; Valcek A, 2022).* A number of studies have documented the dissemination of OXA-23-producing CR *A. baumannii* belonging to IC2 in different countries *(Al Atrouni A, 2016; Castanheira M, 2014; Cherubini S, 2022; Dandachi I, 2019; Eigenbrod T, 2019; Hamidian M, 2019; Higgins P, 2010a; Kostyanev T, 2021; Müller C, 2019; Tomaschek F, 2019; Zarrilli R, 2013).* Similar to our results, authors from the Balkan and Mediterranean regions (Greece, Italy, Israel, Lebanon and Turkey), Poland and Peru reported IC2 associated with OXA-23 or OXA-24 production as endemic and associated with multiple nosocomial infections and outbreaks (incl. bloodstream infections), and with widespread interhospital dissemination *(Di Popolo A, 2011; Frenk S, 2022; Gogou V, 2011; Levy-Blitchtein S, 2018; Lukovic B, 2020; Palmieri M, 2020; Pournas S, 2017; Słoczyńska A, 2021; Stratev A, 2020).*

3. Etiological spectrum of bacterial bloodstream infections in hospitalized patients with oncohematological diseases in the period 2010-2020.

During the study period in the Laboratory of Microbiology of the University Hospital "St. Marina" - Varna, a total of 3954 blood cultures of patients with oncohematological diseases and suspected infectious complications, hospitalized in the Hematology Clinic and in the Clinic of Pediatric Oncohematology of the hospital, were examined. A total of 457 non-duplicate, clinically significant isolates were isolated and identified from the blood cultures of 442 patients (incl. 17 patients after hematopoietic stem-cell transplantation). The etiological spectrum of bacteremias associated with bloodstream infections is presented in Table 22. The ratio of Gram-negative to Gram-positive bacteria from the total number of bacterial isolates for the period 2010-2020 was 58.8% : 41.2%, with 57.7% : 42.3% in the first five-year period (2010-2014) and 59.2% : 40.8% in the second (2015-2020), respectively.

Table 22. Etiological spectrum of bloodstream infections in hospitalized patients with oncohematological diseases in the period 2010-2020 (comparative presentation).

Etiological spectrum	**Total for the period**	**First period**	**Second period**	***p***
	2010 - 2020г. n (%)	2010 - 2014г. n (%)	2015 - 2020г. n (%)	
Gram negative bacteria	248 (54.3)	75 (53.2)	173 (54.7)	0.764
Gram positive bacteria	174 (38.0)	55 (39.0)	119 (37.7)	0.794
Fungi	35 (7.7)	11 (7.8)	24 (7.6)	0.944
Distribution of isolates by microbial species				
Staphylococcus aureus	79 (17.3)	38 (27.0)	41 (13.0)	**0.0002**
E. coli	73 (16.0)	27 (19.1)	46 (14.5)	0.215
Enterobacter spp.	50 (10.9)	12 (8.5)	38 (12.0)	0.267
Klebsiella spp.	47 (10.3)	15 (10.6)	32 (10.1)	0.865
Enterococcus spp. (*E. faecalis, E. faecium*)	40 (8.8)	10 (7.0)	30 (9.5)	0.4
Candida spp.*; Cryptococcus* spp.	35 (7.6)	11 (7.8)	24 (7.5)	0.936
CoNS	32 (7.0)	3 (2.1)	29 (9.2)	**0.006**
Pseudomonas aeruginosa	27 (5.9)	10 (7.0)	17 (5.4)	0.471
Acinetobacter baumannii	18 (3.9)	4 (2.8)	14 (4.4)	0.418
Serratia marcescens	8 (1.8)	3 (2.1)	5 (1.6)	0.682
Stenotrophomonas maltophilia	6 (1.3)	1 (0.7)	5 (1.6)	0.447
Streptococcus viridans	6 (1.3)	-	6 (1.9)	-
Salmonella spp.	5 (1.0)	2 (1.4)	3 (0.9)	0.659
Streptococcus agalactiae	5 (1.1)	2 (1.4)	3 (0.9)	0.659
Streptococcus pneumoniae	4 (0.9)	-	4 (1.3)	-
Bacteroides spp.	3 (0.7)	1 (0.7)	2 (0.6)	0.928
Corynebacterium spp.	3 (0.7)	-	3 (0.9)	-
Listeria monocytogenes	3 (0.7)	2 (1.4)	1 (0.3)	0.18
Proteus mirabilis	2 (0.4)	-	2 (0.6)	-
Acinetobacter lwoffii	2 (0.4)	-	2 (0.6)	-
Citrobacter koseri	1 (0.2)	-	1 (0.3)	-
Morganella morganii	1 (0.2)	-	1 (0.3)	-
Aeromonas veronii	1 (0.2)	-	1 (0.3)	-
Aeromonas hydrophila	1 (0.2)	-	1 (0.3)	-

Pseudomonas fluorescens	1 (0.2)	-	1 (0.3)	-
Pasteurella multocida	1 (0.2)	-	1 (0.3)	-
Burkholderia cepacia	1 (0.2)	-	1 (0.3)	-
Streptococcus group D	1 (0.2)	-	1 (0.3)	-
Lactobacillus rhamnosus	1 (0.2)	-	1 (0.3)	-
Total	**457 (100.0)**	**141 (100.0)**	**316 (100.0)**	**-**

Patients with oncohematologic diseases (OHD) are at increased risk of developing infectious complications, with neutropenia being the most significant risk factor. Bloodstream infections are among the most severe, life-threatening complications with mortality rates between 18 and 42% (*Wisplinghoff H, 2003*). According to a study by Pagano et al, the incidence of microbiologically proven infections among patients with OHD was 9.4%, with 85% of these being bacterial bloodstream infections *(Pagano L, 2012).* Historically, during the last five decades, significant changes have been identified in the etiological spectrum of microorganisms isolated from the blood cultures of patients with OHD. In the 1960s and 1970s, Gram-negative bacteria were among the most common causative agents of bloodstream infections in this group of patients. In the following 30 years, the proportion of Gram - positive microorganisms (coagulase - negative staphylococci, viridans streptococci, enterococci, *S. aureus*) increased significantly. Factors associated with this change include the increased use of certain chemotherapeutics and the associated development of oral mucositis, severe and prolonged neutropenia, increased use of venous catheters, fluoroquinolone prophylaxis, etc.

In recent years, multiple studies have reported a shift in the etiological spectrum of these infections: from Gram-positive to Gram-negative bacteria, alongside with the increase and successful dissemination of MDR and even pan-drug resistant Gram-negative bacteria *(Kokkayil, 2018; Paul M, 2020).* The change in the ratio of Gram positive to Gram negative bacteraemia is from 60 : 40% at the beginning to 55 : 45% in favour of Gram negative bacteria currently *(Secreto C, 2020).* Among the reasons for this trend of prevalence of Gram-negative microorganisms, several main factors have been identified, the most important among them being the use and duration of antibiotic prophylaxis. Consistent with this trend, for the time period studied (2010-2020), Gram-negative bacteria were more frequent causative agents of bloodstream infections in our center, with the leading bacterial species *E. coli* (16.0%), *Enterobacter* spp. (10.2%), *Klebsiella* spp. *(*10.3%), *P. aeruginosa* (5.9%), and *A. baumannii* (3.9%), with a ratio of Gram negative to Gram positive bacteria of 58.2% : 41.2%. Similar results for Gram-negative to Gram-positive bacteraemias ratio have been reported in studies of patients with OHD after 2000 from Italy, Spain and South Korea: Cattaneo et al, 57.3% versus 33.6%; Trecarichi et al. 52.8% for Gram negative bacteremias; Gudiol et al. 49% versus 41%; Kang et al. 55.6% versus 32.7% *(Cattaneo C, 2012; Gudiol C, 2013a; Kang C, 2012; Trecarichi E, 2015).* In a study by Kaleva et al. on the microbiological spectrum of infectious complications in children with cancer in Bulgaria, covering two earlier periods: 1990-1994 and 1995-2003, the prevalence of Gram-positive bacteremias (53.2%) was found to be higher than Gram-negative (40.3%) and fungemias (6.5%), with the most frequently isolated being coagulase-negative staphylococci, followed by *Klebsiella* spp., *Enterococcus* spp., *E. coli* and *P. aeruginosa (Kaleva B, 2006).* In the same study, the ratio between Gram-positive and Gram-negative blood isolates did not change during the two periods studied, but during the later period the relative proportion of *E. coli* increased and the proportion of α-hemolytic streptococci decreased *(Kaleva V, 2006).*

In the present study, Gram-positive bacteria were found in 38% of cases, with staphylococci (*S. aureus*, 17.3%; *CoNS*, 7.0%) and enterococci (8.8%) being the most common

Gram-positive bacteria associated with bloodstream infections in our patients. *S. aureus* (17.3%), followed by *E. coli* (16.0%) and *Enterobacter* spp. (10.9%) were the most frequently isolated bacterial species among all bacterial species, regardless of their Gram affiliation. Comparing the two periods of the present study, we found a statistically significant trend for a decrease in the proportion of *S. aureus* in the second period (p=0.002), against a clear trend for an increase in the proportion of *CoNS* (p=0.006), most commonly associated with catheter-related bloodstream infections (data not shown), and also an increase of enterococci (9.5% vs. 7%) (p=0.4) in the period 2015-2020. A similar leading position of staphylococci but at the expense of *CoNS* is reported by S. Haddad, A. Amanati, D. Mert, M. Paul et al *(Amanati A, 2021; Haddad S, 2021; Mert D, 2019; Paul M, 2020; Secreto C, 2020)*. C. Secreto et al. report *CoNS* responsible for about 25% of Gram positive bacteraemias and *S. aureus* for about 5% of the cases *(Secreto C, 2020*). Other studies have also reported higher isolation rates of *CoNS* as etiological agents of bloodstream infections: 8.3% *(Kang C, 2012a)*, 14.7% *(Kjellander C, 2012)*, 23.1% *(Chong Y, 2011)*, 24.8% *(Trecarichi E, 2009)*, 43% *(Irfan S, 2008)*, 55.2% *(Saghir, S, 2009)*. With regard to *S. aureus*, the proportion varies considerably in different studies, between 1.3%, 6.9%, 9.5% and 12%, and this bacterial species is not among the top three dominant pathogens in the respective studies (*Cattaneo C, 2012; Chong Y, 2011; Gudiol C, 2010; Irfan S, 2008; Kang C, 2012a, Kjellander C, 2012*). Trecarichi et al. reported that *S. aureus* ranked last in isolation rate (1.6%), which is in contrast with our results (*Trecarichi E, 2015*). Closer to our data is the work of Kang et al. who identified *S. aureus* (9.8%) and *Enterococcus* spp. (9.2%) as the most common Gram-positive bacterial species causing bloodstream infections in patients with OHD *(Kang C, 2012a).*

Comparing the results from the two periods of the present study, the trend of the prevalence of Gram-negative bacteria in the overall spectrum of bloodstream infections was found to persist over time. In the second period, an increased proportion of *Enterobacter* spp. (from 8.5% to 12%) was demonstrated, decreasing E. *coli* (from 19.1% to 14.5%) and *P. aeruginosa* (from 7.0% to 5.4%) (p>0.05). Similar results for prevalence of Gram-negative bacteria in the etiologic spectrum of bloodstream infections in patients with OHD, with leading pathogens *E. coli*, followed by *K. pneumoniae*, *Enterobacter* spp., *P. aeruginosa* and *A. baumannii* are also reported by many other authors *(Amanati A, 2021; Govind Babu K, 2016; Haddad S, 2021; Islas-Munoz, 2018; Kokkayli P, 2018; Mert D, 2019; Paul M, 2020; Tang Y, 2021).* In contrast to our results, A. Carvahlo et al. reported the highest percentage for *Streptococcus viridans* (24.1%) and enterococci (13.3%) in the etiological spectrum and a similar relative proportion of *E. coli* (14.9%) *(Carvahlo A, 2020).*

Although much less frequently isolated than the predominant causative agents of bloodstream infections, isolates belonging to *Listeria monocytogenes* and *Salmonella* spp. have been identified in the present study. Listeria infection affects certain populations, including neonates, very elderly patients, pregnant women, and immunocompromised individuals with deficiency in the cell-mediated immunity. One of the groups with highest risk for listeria sepsis are individuals undergoing immunosuppressive therapy (cancer patients, transplant recipients). Generalization of the infectious process in cases of *Salmonella* spp. after overcoming the local barrier of the lymph nodes in the intestinal tract is also characteristic of immunosuppressed patients and those with immunodeficiencies. Isolates of these bacterial species, although detected in a single blood culture in the respective patients, should always be interpreted as clinically significant.

4. Antimicrobial resistance of the most common causative agents of bacteremia associated with bloodstream infections in hospitalized patients with oncohematological diseases in the period 2010-2020.

The resistance of the most common Gram negative (*E. coli, K. pneumoniae, E. cloacae, P. aeruginosa, A. baumannii - calcoaceticus* complex) and Gram-positive bacterial species (*S. aureus*, *Enterococcus* spp.) causing bacteraemias associated with bloodstream infections in patients with OHD between 2010 and 2020 to major groups of antimicrobial drugs is presented in Tables 23, 24, 25, 26, 27, 28 and 29.

Table 23. Antimicrobial resistance of *S. aureus* isolated from blood cultures of oncohematological patients with laboratory-confirmed bloodstream infections in the periods 2010-2014 and 2015-2020.

AB Group	2010 - 2020		2010-2014		2015-2020		p
	n	%R (n)	n	%R (n)	n	%R (n)	
methicillin	**79**	11.4 (9)	**38**	10.5 (4)	**41**	12.2 (5)	0,818
aminoglycosides	**79**	13.9 (11)	**38**	7.9 (3)	**41**	19.5 (8)	0,136
fluoroquinolones	**79**	8.9 (7)	**38**	7.9 (3)	**41**	9.7 (4)	0,771
glycopeptides	**79**	0.0 (0)	**38**	0.0 (0)	**41**	0.0 (0)	NA
oxazolidinones	**79**	0.0 (0)	**38**	0.0 (0)	**41**	0.0 (0)	NA

Table 24. Antimicrobial resistance of *E. faecalis* and *E. faecium* isolated from blood cultures of oncohematological patients with laboratory-confirmed bloodstream infections in the periods 2010-2014 and 2015-2020.

AB Group	2010 - 2020		2010-2014		2015-2020		*p*
	n	%R (n)	n	%R (n)	n	%R (n)	
E. faecalis							
aminopenicillins	**23**	8.7 (2)	**6**	16.6 (1)	**17**	5.8 (1)	0,418
HLAR	**23**	69.6 (16)	**6**	66.6 (4)	**17**	70.5 (12)	0,857
glycopeptides	**23**	0.0 (0)	**6**	0.0 (0)	**17**	0.0 (0)	NA
oxazolidinones	**23**	0.0 (0)	**6**	0.0 (0)	**17**	0.0 (0)	NA
fluoroquinolones	**23**	82.6 (19)	**6**	66.6 (4)	**17**	88.0 (15)	0,230
E. faecium							
aminopenicillins	**17**	94.1 (16)	**4**	100 (4)	**13**	92.3 (12)	0,568
HLAR	**17**	94.1 (16)	**4**	100 (4)	**13**	92.3 (12)	0,568
glycopeptides	**17**	0.0 (0)	**4**	0.0 (0)	**13**	0.0 (0)	NA
oxazolidinones	**17**	0.0 (0)	**4**	0.0 (0)	**13**	0.0 (0)	NA
fluoroquinolones	**17**	100 (0)	**4**	100 (4)	**13**	100 (13)	NA

Table 25. Antimicrobial resistance of *E. coli* isolated from blood cultures of oncohematological patients with laboratory-confirmed bloodstream infections in the periods 2010-2014 and 2015-2020.

	2010-2020		**2010-2014**		**2015-2020**		
AB Group	**n**	**%R (n)**	**n**	**%R (n)**	**n**	**%R (n)**	***p***
3rd generation cephalosporins	**73**	39.7 (29)	**27**	74.0 (20)	**46**	19.6 (9)	**0,0001**
carbapenems (meropenem)	**73**	1.4 (1)	**27**	0.0 (0)	**46**	2.2 (1)	0,443
gentamicin	**73**	30.1 (22)	**27**	40.7 (11)	**46**	23.9 (11)	0,131
amikacin	**73**	2.7 (2)	**27**	3.7 (1)	**46**	2.2 (1)	0,697
fluoroquinolones	**73**	45.2 (33)	**27**	48.1 (13)	**46**	43.5 (20)	0,697
colistin	**46**	2.2 (1)	**-**	-	**46**	2.2 (1)	NA

Table 26. Antimicrobial resistance of *K. pneumoniae* isolated from blood cultures of oncohematological patients with laboratory-confirmed bloodstream infections in the periods 2010-2014 and 2015-2020.

	2010 - 2020		**2010 - 2014**		**2015 - 2020**		
AB Group	**n**	**%R (n)**	**n**	**%R (n)**	**n**	**%R (n)**	***p***
3rd generation cephalosporins	**45**	57.8 (26)	**15**	67.0 (10)	**30**	53.3 (16)	0,395
carbapenems (meropenem)	**45**	6.7 (3)	**15**	0.0 (0)	**30**	10.0 (3)	0,204
gentamicin	**45**	77.8 (35)	**15**	80.0 (12)	**30**	71.8 (23)	0,802
amikacin	**45**	11.1 (5)	**15**	6.6 (1)	**30**	13.3 (4)	0,503
fluoroquinolones	**45**	51.1 (23)	**15**	67.0 (10)	**30**	43.3 (13)	0,134
colistin	**30**	0.0 (0)	**-**	-	**30**	0.0 (0)	NA

Table 27. Antimicrobial resistance of *E. cloacae* complex isolated from blood cultures of oncohematological patients with laboratory-confirmed bloodstream infections in the periods 2010-2014 and 2015-2020.

	2010 - 2020		**2010 - 2014**		**2015 - 2020**		
AB Group	**n**	**%R (n)**	**n**	**%R (n)**	**n**	**%R (n)**	***p***
3rd generation cephalosporins	**44**	46.8 (25)	**12**	25.0 (3)	**32**	68.8 (22)	**0,009**
carbapenems (meropenem)	**44**	2.3 (1)	**12**	0.0 (0)	**32**	3.1 (1)	0,535
gentamicin	**44**	59.0 (26)	**12**	25.0 (3)	**32**	71.8 (23)	**0,005**
amikacin	**44**	0.0 (0)	**12**	0.0 (0)	**32**	0.0 (0)	NA
fluoroquinolones	**44**	34.0 (15)	**12**	0.0 (0)	**32**	46.8 (15)	**0,004**
colistin	**32**	0.0 (0)	**-**	-	**32**	0.0 (0)	NA

Table 28. Antimicrobial resistance of *P. aeruginosa* isolated from blood cultures of oncohematological patients with laboratory-confirmed bloodstream infections in the periods 2010-2014 and 2015-2020.

AB Group	2010 - 2020		2010 - 2014		2015 - 2020		*p*
	n	%R (n)	n	%R (n)	n	%R (n)	
piperacillin/tazobactam	**27**	11.1 (3)	**10**	20.0 (2)	**17**	5.9 (1)	0,258
ceftazidime	**27**	14.8 (4)	**10**	20.0 (2)	**17**	11.8 (2)	0,562
carbapenems	**27**	7.4 (2)	**10**	0.0 (0)	**17**	11.8 (2)	0,258
gentamicin*	**25**	0.0 (0)	**10**	0.0 (0)	**15**	0.0 (0)	NA
amikacin	**27**	3.7 (1)	**10**	0.0 (0)	**17**	5.9 (1)	0,435
Fluoroquinolones	**27**	7.4 (2)	**10**	0.0 (0)	**17**	11.8 (2)	0,258
colistin	**17**	0.0 (0)	**-**	-	**17**	0.0 (0)	NA

*susceptibility to gentamicin has been determined until 2019.

Table 29. Antimicrobial resistance of *A. baumannii - calcoaceticus* complex from blood cultures of oncohematological patients with laboratory-confirmed bloodstream infections in the periods 2010-2014 and 2015-2020.

AB Group	2010 - 2020		2010 - 2014		2015-2020		*p*
	n	%R (n)	n	%R (n)	n	%R (n)	
carbapenems	**18**	72.2 (13)	**4**	25.0 (1)	**14**	85.7 (12)	**0,017**
gentamicin	**18**	94.4 (17)	**4**	100 (4)	**14**	93.0 (13)	0,582
amikacin	**18**	88.9 (16)	**4**	75.0 (3)	**14**	93.0 (13)	0,317
fluoroquinolones	**18**	94.4 (17)	**4**	100.0 (4)	**14**	93.0 (13)	0,582
colistin	**14**	0.0 (0)	**-**	-	**14**	0.0 (0)	NA

Although some studies have reported a significant reduction in the mortality rate associated with bloodstream infections in patients with OHD from 25% in the 1970s to 6% in recent years due to the use of broad-spectrum antibiotics, one negative consequence of this, however, is the current trend of a steady increase in the proportion of infections caused by MDR bacteria, including bloodstream infections. Because of the limited choice of active antibiotics, infections caused by MDR pathogens are often associated with treatment failure and high mortality *(Secreto C, 2020; Tang Y, 2021).* Currently, the spectrum of MDR pathogens in patients with OHD is limited primarily (but not exclusively) to Gram-negative bacteria *(Secreto C, 2020).* Among the MDR Gram-negative bacteria, members of the *Enterobacteriaceae* family exhibiting an ESBL phenotype, carbapenem- and colistin-resistant isolates of the same family, as well as carbapenem- and colistin-resistant *P. aeruginosa* and *A. baumannii*, are a growing problem in modern infectious pathology and in this specific group of patients. The patients with OHD who are immunosuppressed because of their main underlying disease and repeated, prolonged exposure to broad-spectrum antimicrobials are at particularly high risk for developing bloodstream infections. Multiple studies in recent years have demonstrated that the epidemiology of bloodstream infections in patients with oncohematological diseases is increasingly associated with MDR pathogens, with these microorganisms emerging as the

dominant agents in this type of infection *(Amanati A, 2021; Kokkayil P, 2018).* In this aspect, it should be noted that it is necessary to continuously accumulate and analyze information on locally prevalent pathogens with their resistance profile to support the empirical choice of antimicrobial treatment. Major risk factors for the development of infections caused by MDR bacteria include the presence of previous or current colonization (intestinal tract, nose, throat) with MDR bacteria, previous infection with an MDR pathogen, recent exposure to broad-spectrum antibiotics, prolonged hospital or ICU stay, and the local hospital resistance. In the context of the oncohematologic diseases, it is necessary to assess which patients are at high risk for these factors so that antimicrobial therapy targeting these pathogens to be initiated especially in these patients.

In the 11-year follow-up period, a high relative proportion of 3rd generation cephalosporin-resistant *Enterobacteriaceae* was found [49.4% (80 of 162 isolates)], with the highest proportion in the group of *K. pneumoniae* isolates (57.8%), followed by *E. cloacae* complex (46.8%) and *E. coli* (39.7%). The present study identified 3rd generation cephalosporin resistant enteric bacteria among the most common causative agents of bloodstream infections in patients with OHD, which is confirmed by other similar studies *(Martin M, 2015).* In addition, comparing the periods 2010 - 2014 and 2015 - 2020, a statistically significant trend towards a decrease in the proportion of *E. coli* resistant to 3rd generation cephalosporins (p=0.0001) and an increase in that of *E. cloacae* resistant to 3rd generation cephalosporins (p=0.009) was found in the second period. A number of authors have reported similar and higher proportions for Gram negative bacteria causing bloodstream infections in OHD patients, resistant to 3rd generation cephalosporins (50.5%, 52.4%, 64%, 79.5%) (*Amanati A, 2021; Haddad S, 2021; Mert D, 2019; Tang Y, 2021).*

High levels of resistance to fluoroquinolones (between 34% and 51%) were also found among *Enterobacteriacae* isolates, and a statistically significant trend of increase in the proportion of quinolone-resistant isolates from 25% to 68.8% in the second period (2015-2020) was demonstrated for *E. cloacae* complex. Similar to these results, a number of authors who studied cancer patients also reported quinolone resistance rates among *Enterobacteriaceae* members between 45 and 91% *(Bhusal Y, 2011; Chong Y, 2011; Irfan S, 2008; Mihu C, 2010; Trecarichi E, 2009; Trecarichi E, 2015).* Prophylactic use of quinolones is currently thought to play an important role in the emergence of this type of resistance. Moreover, the wide prophylactic use of fluoroquinolones has contributed significantly not only to the selection of quinolone-resistant bacteria in the gastrointestinal tract, but also to the selection of enteric bacteria producers of ESBLs and MRSA *(Montassier E, 2013).* In addition, the dissemination and circulation of quinolone-resistant strains belonging to family *Enterobacteriaceae* in the community, may explain the isolation of quinolone-resistant enteric bacteria from blood cultures of cancer patients who have not received quinolone prophylaxis.

Amikacin demonstrated very good activity (0% - 11.1% resistance) against all three bacterial species *E. coli*, *K. pneumoniae* and *E. cloacae* complex, in contrast to that of gentamicin (30.1% - 77.8% resistance). Similar to 3rd generation cephalosporin and fluoroquinolone resistance in *E. cloacae* complex, a statistically significant increasing trend was also demonstrated for the proportion of gentamicin-resistant *E. cloacae* (p=0.005) over the 11-year follow-up period.

The resistance to the strategic agent colistin in the whole group of Gram-negative bacteria was very low and was identified in a single isolate of *E. coli.* These results determine amikacin and colistin the drugs of choice in the therapy of bloodstream infections caused by MDR enteric bacteria, however, the potential nephrotoxicity of these antimicrobials must be taken into account, especially in cases of critically ill patients receiving chemotherapy.

The carbapenems (meropenem) are another key group of antimicrobials whose activity was monitored. In the present study, a very high relative proportion of CR *A. baumannii* -

calcoaceticus complex was found (72.2%) and a statistically significant increasing trend (p=0.017) was demonstrated when traced over the years. Significantly lower levels of meropenem resistance were demonstrated in *P. aeruginosa* (7.4%), *K. pneumoniae* (6.7%), *E. cloacae* (2.3%) and *E. coli* (1.4%). Carbapenem resistance in the whole group of Gram-negative bacteria was 9.7% (20/207 isolates), and this type of resistance was demonstrated only among blood isolates obtained from patients after 2014. Other authors have reported similar results for carbapenem resistance among Gram-negative bacteria (12%, 11.5%) (*Haddad S, 2021; Kedzior S, 2021).* In contrast, Amanati et al. reported 39.3% carbapenem resistance among members of family *Enterobacteriaceae* and non-fermenters, 87.5% in *A. baumannii* and 71.6% in *P. aeruginosa (Amanati A, 2021).* An extensive review published in 2017 and including studies from 21 countries concluded that resistance to carbapenems in the *Enterobacteriaceae* (especially *K. pneumoniae*), *P. aeruginosa* and in *A. baumannii* is a serious threat to patients with OHDs worldwide and is associated with high mortality rates *(Righi E, 2017).* There are a number of published reports in the scientific literature on the emergence of carbapenem-resistant *Enterobacteriaceae*, specifically *K. pneumoniae* and *Enterobacter* spp. causing infections in cancer patients, including nosocomial outbreaks and death *(Micozzi A, 2017; Satlin M, 2013; Satlin M, 2014).* In addition, Y. Tang et al. reported that the presence of carbapenem resistance in the causative bacterial agent significantly affects the prognosis of patients with OHDs and bloodstream infections, and this is particularly true for CR *K. pneumoniae*, possibly due to the highly resistant profile of these bacterial pathogens *(Tang Y, 2021).*

In line with global and European trends, during the period 2011-2020 a sustained trend of gradually increasing levels of carbapenem resistance among the most common members of *Enterobacteriaceae* family (*K. pneumoniae, E. coli*) isolated from blood cultures for Bulgaria was reported (<1% to 28.1%) *(ECDC, 2022)*. This is fully consistent with our results for meropenem from 2010 to 2020 for bloodstream infections in patients with OHDs, which reflect the national and local results in the unselected patient population.

P. aeruginosa is an organism that is associated with particularly high mortality in patients with OHDs and remains an important target for initial empiric therapy *(Kedzior S, 2021).* The isolates we studied between 2010 and 2020 showed relatively low levels of resistance to all monitored antipseudomonal antibiotics (< 15%), with fully preserved gentamicin and colistin activity.

In the present study, very high resistance levels of *A. baumannii - calcoaceticus* complex isolates to almost all antibacterials, including carbapenems, were found, which confirms the results reported by EARS-Net, especially for countries such as Bulgaria, Italy, Latvia, Lithuania, Cyprus, Romania, Greece and Croatia *(ECDC, 2022; ECDC, 2022a).* Only colistin demonstrates fully preserved activity against this bacterial species, often being the only active drug against *A. baumannii*, making the choice of adequate antimicrobial therapy in patients with OHDs extremely difficult.

The relatively preserved susceptibility to meropenem of *E. coli, K. pneumoniae, E. cloacae* and *P. aeruginosa* isolates in the present study, and the high levels of resistance to 3rd generation cephalosporins, indicate that the carbapenems remain the drugs of first choice for 3rd generation cephalosporin resistant bacteria associated with bloodstream infections in this particularly at-risk patient group.

Staphylococcus aureus and the bloodstream infections, caused by this bacterial species, are also a serious clinical problem in patients with OHDs. *S. aureus* is associated with increased virulence than coagulase-negative staphylococci if it demonstrates methicillin resistance *(Secreto C, 2020).* The present study found relatively low levels of MRSA (11.4%), similar to those reported by other authors *(Haddad S, 2021; Mert D, 2019)*, although many similar studies report higher proportions of MRSA: 18% for India, 30.2% for Pakistan, 36% for Italy and

48.4% for South Korea *(Irfan S, 2008; Kang C, 2012a; Prabhash K, 2010; Trecarichi E, 2015).* In the present 11-year study, no vancomycin, teicoplanin and linezolid resistant strains were identified, a result that indicate the glycopeptides and oxazolidinones as the agents with the highest activity against *S. aureus*, incl. MRSA.

The enterococci are commensals in the gastrointestinal tract, with the most common causative agents of bloodstream infections being *E. faecalis*, followed by *E. faecium*. The frequent use of vancomycin as empiric therapy in patients with OHDs, also especially in the context of COVID-19, is associated with a strong selective pressure on enterococci as part of the normal intestinal microflora, and respectively with a serious risk for emerging of resistant isolates in this anatomical area, a trend that we have found in recent years in our hospital center (unpublished data). The colonization of the intestinal tract with vancomycin-resistant enterococci (VRE) in this patient group is an important potential source for bloodstream infections. For both two most common enterococcal species (*E. faecalis*, *E. faecium*), evidence of increasing vancomycin resistance, increasing incidence of infections, and higher mortality from VRE-associated bloodstream infections in patients with OHDs have been reported in the scientific literature *(Secreto C, 2020)*. Studies on septic conditions in oncology patients with neutropenia found varying levels of vancomycin resistance in enterococci: 1% in Sweden; 7.5% in Italy; 13% in Pakistan *(Irfan S, 2008; Kjellander C, 2012; Trecarichi E, 2015)*. No enterococci resistant to vancomycin, teicoplanin and linezolid were identified in the present study. A negative result was the persistence over the years of multiple resistance in *E. faecium* isolates: resistance to aminopenicillins, 94.1%; fluoroquinolones, 100% and aminoglycosides (HLAR), 94.1%. A high level of quinolone resistance was also demonstrated in *E. faecalis* (82.6%), ranging between 66.6% and 88% in the two study periods. The pronounced MDR, especially in the isolates of *E. faecium*, makes vancomycin, teicoplanin and linezolid the drugs of choice for treatment.

5. Thirty day lethality in bloodstream infections caused by *S. aureus, S. pneumoniae, E. coli, K. pneumoniae, E. cloacae* complex, *A. baumannii - calcoaceticus* complex and *P. aeruginosa* in hospitalised patients between 2016 and 2020.

Between 2016 and 2020, 798 patients with laboratory-confirmed bloodstream infections caused by *S. aureus, S. pneumoniae, E. coli. K. pneumoniae, E. cloacae* complex, *A. baumannii - calcoaceticus* complex and *P. aeruginosa* that met the inclusion criteria specified in "Materials and Methods", were studied. These patients accounted for 90.2% of all patients with documented episodes of bacteremia caused by the 7 microbial species in 2016-2020 and 54.7% of all patients with bloodstream infections associated with various microbial agents during the same period. The analyzed group was divided into two subgroups: first subgroup: survivors (n=590) within at least 30 days and second subgroup: deceased (n=208) within up to 30 days from the time when the positive blood culture was collected (Table 30).

Table 30. Characteristics of patients with bloodstream infections stratified by 30-day lethality.

Variables	**Total n=798 n (%)**	**Deceased to 30 days n=208 n (%)**	**Favorable outcome n=590 n (%)**	***p***
Men	442 (55.4)	119 (57.2)	323 (54.7)	0,538
Ethnicity				
Bulgarian	663 (83.1)	173 (83.2)	490 (83.1)	
Turkish	88 (11.0)	20 (9.6)	68 (11.5)	
Roma	33 (4.1)	10 (4.8)	10 (3.9)	0,684
another	-	-	-	
Age 65+	389 (48.7)	91 (43.8)	298 (50.5)	0,094
Average age	59.5 (20.9)	62.0 (19.5)	58.6 (21.3)	**0,045**
Average hospital stay	20.3 (25.81)	20.4 (34.72)	20.2 (21.82)	0,934
Type of clinic where the patient was hospitalized				
Clinics with profile "Internal Diseases"	273 (34.2)	55 (26.4)	218 (36.9)	
Hematology clinics	183 (22.9)	50 (24.0)	133 (22.5)	
ICUs	201 (25.2)	72 (34.6)	129 (21.9)	
Other	141 (17.7)	31 (14.9)	110 (18.6)	**0,001**
Place of acquisition of the infection				
community acquired	395 (49.5)	82 (39.4)	313 (53.1)	
nosocomial infections	403 (50.5)	126 (60.6)	277 (46.6)	**0,001**
Type of therapy				
surgical treatment	135 (17.0)	13 (6.3)	122 (20.7)	
treatment in ICU	148 (18.6)	85 (41.1)	63 (10.7)	
surgical and ICU treatment	57 (7.2)	34 (16.4)	23 (3.9)	
none of the above	456 (57.3)	75 (36.2)	381 (64.7)	**<0,001**
Prior hospitalization				
no	492 (61.7)	107 (51.4)	385 (65.3)	
yes (in the same clinic)	112 (14.0)	24 (11.5)	88 (14.9)	
yes (in the same hospital but in another clinic)	132 (16.5)	48 (23.1)	84 (14.2)	
yes (in another hospital)	62 (7.8)	29 (13.9)	33 (5.6)	**<0,001**

The highest relative proportion of bloodstream infections was found in the 61-70 age group (26.2%), followed by 71-80 (24.2%), 51-60 (14.7%) and over 80 (9.9%).

All 21 clinics where the studied patients were hospitalized were divided into four groups according to their profile: Intensive Care Clinics (group I), Clinics with the profile “Internal medicine" (group II), Haematology clinics (group III) and other (group IV). The Hematology Clinic (21.4%) had the highest relative proportion of patients with bloodstream infections caused by the 7 bacterial species, followed by the Nephrology (11.7%), Cardiac Surgery ICU (10%) and the Internal Medicine Clinic (9.6%) (Table 31).

Table 31. Distribution of patients with bloodstream infections by the type of the clinical setting and stratified by 30-day lethality.

Type of clinic	Number of patients n (%)	Patients deceased by the 30th day n (%)
Group I		
Intensive Care Clinics	**201 (25.2)**	**72 (34.6)**
Cardiac Surgery ICU	80 (10.0)	27 (13.0)
ICU	27 (3.4)	17 (8.2)
Intensive Neurology Unit	46 (5.8)	13 (6.3)
Intensive Pediatric Unit	36 (4.5)	10 (4.8)
Intensive Respiratory Unit	10 (1.3)	4 (1.9)
Intensive Cardiology Unit	2 (0.3)	1 (0.5)
Group II		
Clinics with profile "Internal Medicine"	**273 (34.2)**	**55 (26.4)**
Nephrology	93 (11.7)	14 (6.7)
Gastroenterology	26 (3.3)	14 (6.7)
Internal diseases	77 (9.6)	13 (6.3)
Endocrinology	29 (3.6)	1 (0.5)
Cardiology	23 (2.9)	10 (4.8)
Rheumatology	20 (2.5)	3 (1.4)
Pulmonology	5 (0.6)	0 (0.0)
Group III		
Hematology clinics	**183 (22.9)**	**50 (24.0)**
Hematology Clinic	171 (21.4)	50 (24.0)
Paediatric Hematology Clinic	12 (1.5)	0 (0.0)
Group IV		
Other clinics	**132 (16.5)**	**31 (14.9)**
Surgical clinics	54 (6.8)	10 (4.8)
Neurology Clinic	42 (5.3)	18 (8.7)
Infectious disease clinics	21 (2.6)	2 (1.0)
Oncology Clinic	15 (1.9)	1 (0.5)
Paediatric Clinics	6 (0.8)	0 (0.0)
Psychiatry	3 (0.4)	0 (0.0)
Total	**798 (100)**	**208 (100)**

Thirty-day lethality among all 798 patients was 26% (208/798), with 20.8% (82/395) in the group of patients with community-acquired infections and 31.3% (126/403) in those with nosocomial infections (p=0.001).

In the group of deceased patients (n=208), the highest relative proportion was represented by patients from the Hematology Clinic (24%), followed by those in the Cardiac Surgery ICU (13.0%), Neurology clinics (8.7%), ICU (8.2%), Nephrology (6.7%), and Gastroenterology Clinics (6.7%) (Table 31).

Thirty-day lethality calculated according to the type of the clinic where the patient was hospitalized was as follows: Intensive Care clinics, 35.8%; Internal Medicine clinics, 20.1%; Hematology clinics, 27.3%; and other, 23.5% (Table 32).

The highest proportion of deceased male patients was in the age group over 80 years (35.7%), while for females - in the age group 20-39 years (30.4%).

Table 32. Thirty-day lethality in bloodstream infections caused by *S. aureus, S. pneumoniae, E. coli. K. pneumoniae, E. cloacae, A. baumannii - calcoaceticus* complex and *P. aeruginosa* in the period 2016-2020 according to the type of the clinic where the patient was hospitalized.

Type of clinic	Number of patients with blood infection	Number of deceased patients by day 30	30-day lethality % (95% CI)
Group I			
Intensive Care Clinics	**201**	**72**	**35.8 (29,2 - 42,9)**
Cardiac Surgery ICU	80	27	33. 8 (23,5 - 45,2)
ICU	27	17	63.0 (42,4 - 86,7)
Intensive Neurology Unit	46	13	28.3 (16 - 43,4)
Intensive Pediatric Unit	36	10	27.8 (14,2 - 45,2)
Intensive Respiratory Unit	10	4	40.0 (12,2 - 73,2)
Intensive Cardiology Unit	2	1	50 (1,3 - 98,7)
Group II			
Clinics with profile "Internal Diseases"	**273**	**55**	**20.1 (15,6 - 25,4)**
Nephrology	93	14	15.0 (8,5 - 24)
Gastroenterology	26	14	53.8 (33,4 - 73,4)
Internal diseases	77	13	16.9 (9,3 - 27,1)
Endocrinology	29	1	3.4 (0,1 - 17,8)
Cardiology	23	10	43.5 (23,1 - 65,5)
Rheumatology	20	3	15.0 (3,21 - 37,9)
Pulmonology	5	0	0.0 (0,00 - 52,2)
Group III			
Hematology clinics	**183**	**50**	**27.3 (21,01 - 34,4)**
Hematology Clinic	171	50	29.2 (22,5 - 36,7)
Paediatric Haematology Clinic	12	0	0.0 (0,00 - 26,5)
Group IV			
Other clinics	**132**	**31**	**23.5 (16,6 - 31,7)**
Surgical clinics	54	10	18.5 (9,25 - 31,4)
Neurology Clinic	42	18	42.9 (27,7 - 59)
Infectious disease clinics	21	2	9.5 (0,12 - 23,8)
Oncology clinic	15	1	6.7 (0,17 - 32)
Paediatric clinics	6	0	0.0 (0,00 - 45,9)
Psychiatry	3	0	0.0 (0,00 - 70,8)
Total	**798**	**208**	**26.0**

Table 33 shows the lethality by day 30 in patient groups divided by the bacterial species causing the bloodstream infections. The highest 30-day lethality was associated with infections caused by *A. baumannii* (56.3% [43-68]), with a statistically significant difference found when comparing this indicator for infections caused by *A. baumannii* on one hand and those caused by *S. aureus, S. pneumoniae, E. coli. K. pneumoniae, E. cloacae* (p=0.026).

Table 33. Thirty day lethality in patients with bloodstream infections during the period 2016 - 2020, grouped according to the bacterial species.

Bacterial species	Total number of patients with bloodstream infection	Number of deaths by the 30th day	30-day lethality % (95% CI)
S. aureus	235	43	18.3 (14-24)
E. soli	210	49	23.3 (18-30)
K. pneumoniae	139	40	28.8 (21-37)
E. cloacae	83	18	21.7 (13-32)
A. baumannii	64	36	56.3 (43-68)
P. aeruginosa	53	19	35.8 (23-50)
S. pneumoniae	14	3	21.4 (5-51)

Among all studied patients with bloodstream infections (n=798), *S. aureus* was the most common etiologic agent (29.4%), followed by *E. coli* (26.3%) and *K. pneumoniae* (17.4%), and the most frequently isolated antibiotic-resistant organisms were ampicillin-resistant *E. coli (*126/798) and 3rd generation cephalosporin-resistant *K. pneumoniae* (91/798). *E. coli* (23.6%; 49/208), *S. aureus* (20.7%; 43/208) and *K. pneumoniae* (19.2%; 40/208) were associated with the highest number of deaths.

When 30-day lethality was evaluated against the combination "bacterial species/antibiotic", aminoglycoside, quinolone and meropenem-resistant *A. baumannii - calcoaceticus* complex were associated with the highest number of deaths (15.9-16.3%; 34/208; 33/208), followed by ampicillin-resistant *E. coli (*15.9%; 33/208) and 3rd generation cephalosporin-resistant *K. pneumoniae* (14.9%; 31/208) (Table 34). Bloodstream infections caused by 3rd generation cephalosporins resistant *E. coli* were associated with a statistically significant higher risk of lethality (p=0.006), as were the infections caused by *P. aeruginosa* resistant to ceftazidime, piperacillin/tazobactam, meropenem and aminoglycosides ($p<0.05$).

Table 34. *S. aureus, S. pneumoniae, E. coli, K. pneumoniae, E. cloacae* complex, *A. baumannii - calcoaceticus* complex, and *P. aeruginosa* - associated bloodstream infections in 798 hospitalized patients between 2016 and 2020, stratified by 30-day lethality and antimicrobial resistance.

	Total number of patients n (%)	**Died by the 30th day n (%)**	**Favorable outcome n (%)**	***p***
Bacterial species				**<0.001**
Staphylococcus aureus	**235 (29.4)**	**43 (20.7)**	192 (32.5)	0.77
MRSA	31 (13.2)	6 (2.9)	25 (4.2)	0.870
erythromycin resistant	59 (25.1)	13 (6.3)	46 (7.8)	0.437
clindamycin resistant	48 (20.4)	13 (6.3)	35 (5.9)	0.094
aminoglycoside resistant	42 (17.9)	7 (3.4)	35 (5.9)	0.830
fluoroquinolone resistant	17 (7.2)	4 (1.9)	13 (2.2)	0.523
E. coli	**210 (26.3)**	**49 (23.6)**	161 (27.3)	0.983
ampicillin resistant	126 (60.0)	33 (15.9)	93 (15.8)	0.248
3rd generation cephalosporin resistant	57 (27.1)	21 (10.1)	36 (6.1)	**0.006**
piperacillin/tazobactam resistant	20 (9.5)	7 (3.4)	13 (2.2)	0.263
aminoglycoside resistant	42 (20.0)	13 (6.3)	29 (4.9)	0.222
fluoroquinolone resistant	78 (37.1)	23 (11.1)	55 (9.3)	0.129
Klebsiella pneumoniae	**139 (17.4)**	**40 (19.2)**	99 (16.8)	0.562
3rd generation cephalosporin resistant	91 (65.5)	31 (14.9)	60 (10.2)	0.076
piperacillin/tazobactam resistant	78 (56.1)	24 (11.5)	54 (9.2)	0.577
meropenem resistant	9 (6.5)	1 (0.5)	8 (1.4)	0.446
aminoglycoside resistant	63 (45.3)	21 (10.1)	42 (7.1)	0.347
fluoroquinolone resistant	84 (60.4)	27 (13.0)	57 (9.7)	0.340
***Enterobacter cloacae* complex**	**83 (10.4)**	**18 (8.7)**	65 (11.0)	0.983
3rd generation cephalosporin resistant	55 (66.3)	14 (6.7)	41 (6.9)	0.277
piperacillin/tazobactam resistant	48 (57.8)	13 (6.3)	35 (5.9)	0.188
meropenem resistant	1 (1.2)	1 (0.5)	0 (0.0)	0.217
aminoglycoside resistant	47 (56.6)	12 (5.8)	35 (5.9)	0.424
fluoroquinolone resistant	47 (56.6)	13 (6.3)	34 (5.8)	0.181
***Acinetobacter baumannii-calcoaceticus* complex**	**64 (8.0)**	**36 (17.3)**	28 (4.7)	**0.026**
meropenem resistant	54 (84.4)	33 (15.9)	21 (3.6)	0.90
aminoglycoside resistant	61 (95.3)	34 (16.3)	27 (4.6)	1
fluoroquinolone resistant	61 (95.3)	34 (16.3)	27 (4.6)	1
Pseudomonas aeruginosa	**53 (6.6)**	**19 (9.1)**	34 (5.8)	0.313
ceftazidime resistant	18 (34.0)	10 (4.8)	8 (1.4)	**0.04**
piperacillin/tazobactam resistant	20 (37.7)	11 (5.3)	9 (1.5)	**0.038**
meropenem resistant	18 (34.0)	10 (4.8)	8 (1.4)	**0.04**

aminoglycoside resistant	20 (37.7)	11 (5.3)	9 (1.5)	**0.038**
fluoroquinolone resistant	21 (39.6)	11 (5.3)	10 (1.7)	0.077
Streptococcus pneumoniae	**14 (1.8)**	**3 (1.4)**	11 (1.9)	0.670
penicillin resistant	5 (35.7)	1 (0.5)	4 (0.7)	1.000
Total	**798 (100)**	**208 (100)**	**590 (100)**	

The univariate regression analysis applied found that there was a statistically significant association between the probability of survival to day 30 and the following factors: age, type of clinic where the patient was hospitalized, site of acquisition of the infection (hospital or community), prior hospitalization, type of therapy administered, bacterial species, and infectious syndrome. All demographic characteristics excluding age (sex, ethnicity, education, social status) were unrelated to the risk of dying by day 30 and were not significant risk factors for lethality in the patient group studied.

A model was constructed in which all 7 factors independently having a significant effect on the probability of dying/survival were included, namely: age, type of clinic, bacterial species, infection acquired in hospital or in the community, prior hospitalization, type of infectious syndrome, surgical and/or intensive care treatment performed. Of the seven independent variables, only age was a quantitative variable. Reported simultaneously, these variables statistically significantly predicted the probability of survival to and beyond day 30: chi-square test=296.026, *df*=48, p<0.00001. According to Wald's criterion, one of these independent variables, "*hospital- or community-acquired infection*," was reported as statistically insignificant and therefore excluded from the regression model, and a reduced regression model with six independent variables was constructed:

- age, p=0.001
- type of clinic, p=0.007
- bacterial species, p = 0.001
- prior hospitalization, p=0.029
- clinical (infectious) syndrome, p =0.001
- surgical and/or intensive care treatment, p<0.00001

The analysis proved the reduced regression model to be statistically significant: the model explained between 31.1% (Cox & Snell) and 45.5% (Nagelkerke) of the variance of the dependent variable deceased/alive and correctly classified 81.9% of the observations (106 of 208 deceased and 546 of 590 alive were accurately predicted and classified). Wald's criterion indicates that all six independent variables significantly affect the 30-day survival prognosis. i.e. they are factors independently having a significant effect on the probability of dying/surviving (predictors of death). The values of the regression coefficients and odds ratios are presented in Table 35. The probability of a favourable outcome decreased with increasing the age (p=0.001).

Table 35. Multivariate regression analysis predicting the probability of 30-day lethality in patients with bloodstream infections caused by *S. aureus, S. pneumoniae, E. coli. K. pneumoniae, E. cloacae* complex, *A. baumannii - calcoaceticus* complex and *P. aeruginosa*, 2016 - 2020.

Variable	**B**	***p* value**	**Odds ratio Exp B**	**95% CI for EXP(B)**
Age	**-0,027**	0,001	**0,973**	0,958 - 0,989
Clinic		0,005		
Nephrology	**-0,975**	0,366	**0,377**	0,045 - 3,131
Oncology Clinic	**0,002**	0,999	**1,002**	0,056 - 18, 051
Internal Diseases	**-1,418**	0,188	**0,242**	0,029 - 1,997
Pulmonology	**18,098**	0,999	**72401648**	0
Intensive Respiratory Unit	**-0,457**	0,725	**0,633**	0,05 - 8,051
Cardiology	**-2,354**	0,043	**0,095**	0,0 - 0,9241
Intensive Cardiology Unit	**-4,031**	0,027	**0,018**	0,001 - 0,629
Rheumatology	**-1,299**	0,345	**0,273**	0,018 - 4,053
Haematology Clinic	**-1,878**	0,079	**0,153**	0,019 - 1,239
Endocrinology	**1,366**	0,364	**3,921**	0,205 - 75, 061
Gastroenterology	**-2,811**	0,012	**0,06**	0,007 - 0,545
Neurology Clinic	**-1,242**	0,26	**0,289**	0,033 - 2,503
Intensive Neurology Unit	**0,277**	0,803	**1,319**	0,15 - 11,594
Pediatric Clinics	**16,679**	0,999	**17526341**	0
Intensive Pediatric Unit	**-1,924**	0,119	**0,146**	0,013 - 1,644
Pediatric Hematology Clinic	**16,848**	0,999	**20739139**	0
Surgical Clinics	**-1,202**	0,311	**0,301**	0,029 - 3,079
ICU	**-1,383**	0,233	**0,251**	0,026 - 2,433
Cardio-Surgery ICU	**-2,077**	0,102	**0,125**	0,01 - 1,511
Psychiatry	**18,331**	0,999	**91448497**	0
Bacterial species		0		
K. pneumoniae	**-1,052**	0,196	**0,349**	0,071 - 1,719
A. baumannii-calcoaceticus complex	**-2,39**	0,005	**0,092**	0,017 - 0,495
E. coli	**-1,332**	0,099	**0,264**	0,054 - 1,283
E. cloacae complex	**-0,77**	0,366	**0,463**	0,087 - 2,459
P. aeruginosa	**-1,675**	0,052	**0,187**	0,035 - 1,013
S. aureus	**-0,531**	0,511	**0,588**	0,121 - 2,862
Prior hospitalization		0,03		
None	**0,916**	0,01	**2,5**	1,239 - 5, 043
Previous hospitalization in the same clinic	**0,808**	0,09	**2,244**	0,881 - 5,716
Previous hospitalization in the same hospital but in another clinic	**0,353**	0,405	**1,423**	0,62 - 3,265

Clinical syndrome		0,001		
Respiratory	**-0,938**	0,353	**0,391**	0,054 - 2,829
Gastrointestinal	**-1,792**	0,056	**0,167**	0,027 - 1,045
Urological	**-0,33**	0,723	**0,719**	0,116 - 4,465
Central Nervous System	**-1,583**	0,095	**0,205**	0,032 - 1,316
Toxic-infectious	**-1,772**	0,047	**0,17**	0,029 - 0,98
Cutaneous	**0,207**	0,869	**1,23**	0,105 - 14, 41
Cardiological	**-0,69**	0,494	**0,501**	0,07 - 3,617
ICU and/or surgical treatment		0		
No ICU and/or surgical treatment	**2,399**	0	**11,009**	3,983 - 30, 433
Surgical treatment	**2,746**	0	**15,578**	5,892 - 41, 185
ICU treatment	**-0,19**	0,705	**0,827**	0,31 - 2,208
Constant	**3,936**	0,019	**51,218**	

Abbreviations: ICU, Intensive Care Unit

Next, a univariate regression analysis was performed to determine the factors predicting the likelihood of dying by day 30 for each of the 7 groups of patients grouped according to the bacterial species causing the infection. The following variables were identified as statistically significant, independent predictors of lethality: for infections caused by ***E. coli*** **-** "prior hospitalization", "surgical and ICU treatment", "resistance to 3rd generation cephalosporins"; infections caused by ***S. aureus*** – "age" and "ICU/surgical treatment"; infections caused by ***K. pneumoniae****:* "surgical/ICU treatment"; infections caused by ***P. aeruginosa***: "surgical treatment" and "resistance to gentamicin, ceftazidime, ciprofloxacin and meropenem"; infections caused by ***A. baumannii - calcoaceticus*** **complex**: "surgical treatment".

For the cases of *S. pneumoniae* and *E. cloacae* associated infections, there were no independently significant predictors of risk of death by day 30.

In a constructed model simultaneously including all three variables independently associated with the risk of dying in cases of *E. coli* infections, these factors remained significantly associated with the risk of death ($p<0.0001$). Similarly, for *S. aureus* infections, in a model simultaneously including the variables "age" and "ICU/surgical treatment", these factors remained significantly associated with the risk of dying ($p<0.0001$). However, in the case of *P. aeruginosa*, of the variables "surgical treatment" and "resistance to gentamicin, ceftazidime, ciprofloxacin and meropenem" included in the pooled model, only the type of treatment remained a statistically significant risk factor (Table 36).

Table 36. Risk factors for 30-day lethality in patients grouped according to the bacterial species causing the bloodstream infection.

Variable	B	*p* value	Odd Ratio Exp(B)	95% CI for EXP(B)	
				Lower	Upper
K. pneumoniae					
no surgical / ICU treatment	1.546	.002	4.692	1.727	12.747
surgical treatment	21.203	.998	1615474842.851	.000	.
ICU treatment	-.405	.483	.667	.215	2.067
Constant	.000	1.000	1.000		
E. coli					
prior hospitalization		.036			
no prior hospitalization	1.282	.127	3.604	.695	18.688
ICU/surgical treatment		.000			
no surgical/ICU treatment	2.632	.026	13.897	1.366	141.422
resistance to 3rd generation cephalosporins	-1.088	.023	.337	.132	.862
Constant	-1.658	.243	.191		
S. aureus					
no surgical/ICU treatment	2.666	.000	14.386	3.309	62.539
surgical treatment	21.873	.997	3158788063.095	.000	.
ICU treatment	.447	.565	1.564	.341	7.175
Age	-.038	.000	.962	.943	.982
Constant	1.727	.059	5.624		
P. aeruginosa					
no surgical/ICU treatment	2.238	.087	9.377	.720	122.035
surgical treatment	3.616	.019	37.180	1.827	756.508
ICU treatment	.786	.585	2.194	.131	36.700
resistance to aminoglycosides	-21.523	1.000	.000	.000	.
resistance to ceftazidime	-1.773	.246	.170	.008	3.406
resistance to fluoroquinolones	22.338	1.000	5028474008.979	.000	.
piperacillin/tazobactam resistance	-.798	.567	.450	.029	6.900
Constant	-.803	.500	.448		
A. baumannii - calcoaceticus **complex**					
no surgical/ICU treatment	1.030	.325	2.800	.361	21.727
surgical treatment	1.946	.033	7.000	1.173	41.759
ICU treatment	.272	.771	1.312	.210	8.184
Constant	-1.253	.118	.286		

Abbreviations: ICU, intensive care unit.

The present study demonstrates a high 30-day lethality (26%) in an unselected population of hospitalized patients with bloodstream infections caused by *S. aureus, S. pneumoniae, E. coli. K. pneumoniae, E. cloacae* complex*, A. baumannii - calcoaceticus* complex and *P. aeruginosa*. When comparing the results with those in the scientific literature, highly variable data were found, probably influenced by study design, site of acquisition of infection, and different distribution of pathogens in the etiological spectrum, differences in the prevalence of antimicrobial resistance, as well as differences in health care delivery in the respective country, with mortality rates ranging between 10% and 19% for community-acquired bloodstream infections, between 17% and 28% for nosocomial infections, and reaching 35-60% in the ICUs *(Alonso-Menchén D, 2022; Bassetti M, 2016; Pérez-Crespo P, 2021; Pérez-Crespo P, 2021a; Verway M, 2022).* The present study also found a higher 30-day lethality rate in nosocomial infections (31.3%) compared to that of community-acquired infections (20.75%) (p=0.001), with the relative proportion of patients with nosocomial infections who died reaching 60.6% in the whole group of deceased. The 30-day lethality rate in the intensive care clinics averaging 35.8%, reaching 63% in the ICU. Of the clinics with a "non-intensive" profile, the highest 30-day lethality was found in the Hematology clinics (27.3%). The results of studies on mortality in patients with oncohaematological diseases with bloodstream infections are very variable, ranging between 12 and 42%, and this rate is often higher in cases of Gram negative infections compared to Gram positive ones *(Garcia-Vidal C, 2018; Nørgaard M, 2006; Trecarichi E, 2014, Trecarichi E, 2015; Tumbarello M, 2009; Wisplinghoff H, 2003).*

In terms of bacterial species studied, 30-day lethality was associated with the greatest number of infections caused by *E. coli,* followed by *S. aureus* and *K. pneumoniae*. Similarly, a population-based study in Canada on mortality associated with bloodstream infections found that of the ESKAPEEc group of pathogens, *S. aureus*, *E. coli* and *K. pneumoniae* are associated with the greatest number of deaths on day 30 *(Verway M, 2022).* The results are very similar also to data reported in 2022 from a large study on global, regional and national mortality associated with 33 pathogens (including ESKAPEEc) and 11 infectious syndromes (including bloodstream infections) *(Ikuta K, 2022).* The study found that regardless of geographic region, *S. aureus* was the leading cause of fatal bloodstream infections (mortality rate 3.9/100,000), being associated with 23% of deaths from bloodstream infections in the HICs-super region in 2019. In addition to *S. aureus,* 4 other bacterial species are globally associated with the highest mortality rates as causes of bloodstream infections - *K. pneumoniae* (3.42/100 000), *A. baumannii* (3.20/100 000), *E. coli* (3.13/100 000) and *P. aeruginosa* (2.10/100 000). Consistent with the results of this study, the data for Bulgaria indicate that the highest mortality rates for bloodstream infections are associated with *E. coli* (20.37/100 000), *S. aureus* (9.15/100 000) and *K. pneumoniae* (7.63/100 000) and they are among the highest globally *(*https://vizhub.healthdata.org/microbe/). Of note is the high proportion of deaths associated with *K. pneumoniae* (19.2%) in the present work, which is consistent with other studies demonstrating this pathogen as the fastest growing threat in Europe in terms of morbidity and mortality, a bacterial species primarily associated with intra- and inter-hospital transmission *(David S, 2019).*

When the 30-day lethality indicator was evaluated against the combination "organism/antimicrobial agent", it was found that the patients with bloodstream infections caused by aminoglycoside, quinolone, and meropenem-resistant *A. baumannii - calcoaceticus* complex had the highest proportion in the deceased group (15.9 - 16.3%)*,* followed by ampicillin-resistant *E. coli (*15.9%) and *K. pneumoniae* resistant to 3rd generation cephalosporins (14.9%). In contrast to these results, the study cited above reported that MRSA (1.56/100 000) and 3rd generation cephalosporin-resistant *E. coli (*0.77/100 000) are associated with the highest mortality globally, and CR *A. baumannii* (0.75/100 000) and CR *K. pneumoniae* (0.72/100 000) occupied the next positions *(Ikuta K, 2022).*

Mortality rates in Bulgaria for bloodstream infections caused by 3rd generation cephalosporin-resistant *E. coli* (2.37/100,000), 3rd generation cephalosporin-resistant *K. pneumoniae* (1.66/100 000), CR *K. pneumoniae* (1.13 / 100 000) and CR *A. baumannii* (0.71/100,000) are among the highest *(https://vizhub.healthdata.org/microbe/).* ECDC study in the period 2016-2020 found that *E. coli* continues to be the most frequently reported pathogen in EARS-Net and consequently associated with the highest burden over this period, with the largest contribution of the infections caused by *E. coli* resistant to 3rd generation cephalosporins, followed by MRSA and *K. pneumoniae* resistant to 3rd generation cephalosporins, together responsible for 58.2% of the total DALYs, although in this period in many countries CR *Acinetobacter* spp., CR *P. aeruginosa* and CR *K. pneumoniae* are among the antibiotic-resistant bacteria with relatively high DALYs, especially in 2020 *(ECDC, 2022c).* The present study demonstrates a low proportion of MRSA (2.9%) and CR *K. pneumoniae* bloodstream infections (0.5%) in the group of deceased patients in general, and a low proportion of deceased patients with MRSA and CR *K. pneumoniae* in the groups of patients with *S. aureus* (6 of 235) and *K. pneumoniae* associated infections (1 of 139) respectively. Particularly high mortality rates associated with MRSA bloodstream infections were reported for HICs (2.52/100 000), the USA (2.66/100 000), Greece (3.58/100 000) and Italy (2.18/100 000), with Greece also showing a high mortality rate for CR *K. pneumoniae* associated bloodstream infections (1.56/100 000) *(Ikuta K, 2022; https://vizhub.healthdata.org/microbe/).*

In this study, demographic, clinical and microbiological variables were evaluated to identify predictors of lethality. Multivariate regression analysis was applied and six variables: age, type of clinic, prior hospitalization, type of therapy administered (ICU and/or surgery), bacterial species, and infectious syndrome, were found to be independent predictors of death. Various studies have reported multiple clinical factors (underlying diseases, prior antibiotic therapy, severity of bacteremia) and patient-related factors (age, sex, prior hospitalization) as indicators independently associated with fatal outcome in cases of bloodstream infections, although more often these are studies focusing on specific patient subpopulations rather than the general population *(Babich T, 2020; Diallo K, 2018; Rac H, 2020).* Similar to our findings, Jin et al. demonstrate age, ICU stay and clinical symptoms (also cancer disease and length of hospital stay) as independent predictors of death in the general population *(Jin L, 2021)*.

The present study demonstrates that infections caused by *A. baumannii - calcoaceticus* complex (53.6%) and *P. aeruginosa* (35.8%) were accompanied by the highest rates of lethality, while those caused by *E. coli* resistant to 3rd generation cephalosporins and *P. aeruginosa* resistant to ceftazidime, piperacillin/tazobactam, meropenem and aminoglycosides were associated with a statistically significant higher risk of lethality. These results are consistent with those reported by other authors in single-center and multicenter hospital-based studies *(Hattori H, 2021; Jin L, 2021).* Wisplinghoff *et al*. reported a 40% mortality rate in nosocomial bloodstream infections caused by *A. baumannii (Wisplinghoff H, 2012*), and Wong *et al.* - about 60% in bloodstream infections and nosocomial pneumonias caused by CR *A. baumannii (Wong D, 2017)*. Among ESKAPEEc pathogens, in a large population-based study, M. Verway reported the highest 30-day mortality for bloodstream infections caused by *Pseudomonas* spp. (24.7%), *S. aureus* (22.8%) and *Klebsiella* spp. (17.6%), and significantly lower than our finding for *Acinetobacter* spp. (15.5%) *(Verway M, 2022).* A lower 30-day lethality (30%) for *Acinetobacter* - bloodstream infections is also demonstrated in a study by R. Patel *(Patel R, 2019).* In the present study, *K. pneumoniae* is the bacterial species for which after *A. baumannii* and *P. aeruginosa*, the highest 30-day lethality was reported (28.8%). Rates close to these are reported by T. Xiao (25.6%), but this author demonstrates a much higher 28-day lethality in cases of bloodstream infections caused by CR *K. pneumoniae* (55.8%) compared to those associated with carbapenem-susceptible isolates (13.9%) *(Xiao T, 2020)*. A

Chinese study on *K. pneumoniae*-associated bloodstream infections reported increasing mortality rates associated with these infections over the years, from 14% in 2014 to 44% in 2019, also in terms of CR *K. pneumoniae* bloodstream infections - from 38% in 2014 to 74% in 2019 *(Li Y, 2020).* In the present study, only one case caused by CR *K. pneumoniae* that resulted in lethality by day 30 was found, while in the entire group of deceased patients, after ampicillin-resistant *E. coli* and CR *A. baumannii - calcoaceticus* complex, 3rd generation cephalosporin-resistant *K. pneumoniae* (14.9%) were the most frequently documented. In contrast to *E. coli*, in the cases of *K. pneumoniae* bloodstream infections, resistance to none of the antibiotic groups was associated with a higher risk of lethality. There are also similar studies in the scientific literature reporting that resistance in *K. pneumoniae* (specifically to carbapenems) has not been identified as a risk factor for mortality in infections associated with this bacterial species, with some authors reporting increased mortality as a result of hypervirulent strains of *K. pneumoniae* that are otherwise sensitive to all antibiotics except ampicillin *(Li Y, 2020; Wang X, 2018).* Similarly, a study of X. Peng on risk factors and outcomes of bloodstream infections caused by ESKAPEEc pathogens in hospitalized children found a 14.4% mortality rate, with no evidence of a statistically significant difference in mortality between MDR ESKAPEEc and non-MDR ESKAPEEc infections, leading the authors to conclude that in this study group, MDR ESKAPEEc were not a risk factor for unfavourable outcomes. Surgical treatment, ICU stay, mechanical ventilation and thrombocytopenia were identified as such factors *(Peng X, 2021),* some of which were also confirmed in the present study.

In assessing the 30-day lethality relative to a specific etiologic agent, multivariate regression analysis identified in the cases of *E. coli* infections, the variables "prior hospitalization", "surgical/ICU treatment" and "resistance to 3rd generation cephalosporins" as independent predictors of death; for *S. aureus* infections, such variables were "age" and "ICU/surgical treatment", and in the cases of *P. aeruginosa* and *A. baumannii - calcoaceticus* complex - independent predictive factors are only the variable "surgical treatment". In general, statistical analysis showed that the absence of surgical and/or ICU treatment and the absence of prior hospitalization were associated with a higher chance of a favorable outcome in all cases, regardless of bacterial species, i.e. significantly reduced the risk of dying. Similar to what we found for *E. coli*, an earlier study conducted in 10 European hospitals demonstrated that in patients with bloodstream infections caused by members of *Enterobacterales*, resistance to 3rd generation cephalosporins significantly increased the chance of death (*Stewardson A, 2016).*

The analysis in the present study did not demonstrate a statistically significant association between methicillin resistance in *S. aureus* and mortality in MRSA bloodstream infections, which is consistent with that reported by other authors, although there are studies reporting methicillin resistance in *S. aureus* infections as a risk factor for mortality *(Stewardson A, 2016).*

In the present study, 60.3% of bloodstream infections were diagnosed in patients over 60 years of age, with the highest relative proportion in the 61-70 age group. Similar to these data, other authors also found more than 50% of bloodstream infections in patients 65 years and older, which is associated with existing serious underlying diseases and reduced potential of the natural resistance and immunity mechanisms *(Leibovici L, 1993; Skogberg K, 2012; Uslan D, 2007).* In the present study, more than 50% of the deaths occurred in the over 60 age group and the mean age among those who died by day 30 was higher than that of the survivors. Univariate and multivariate regression analyses demonstrated a statistically significant association between the probability of survival to day 30 and the variable "age", defining it as an independent predictor of mortality outcome, which has also been demonstrated by other authors *(Kontula K, 2021)*. In most studies, the mortality associated with bloodstream infections in the elderly population (over 65 years) is higher compared to the younger group,

and this applies to both the 30-day (between 16-50%) and 90-day mortality (20-85%), as well as 1 year after the infection (30-31%) (*Laupland K, 2021; Yahav D, 2016).* A recent population-based study by Cassini demonstrates that the burden associated with bloodstream infections is highest in children under 1 year of age, and in people over 65 years of age, with an increasing trend between 2007 and 2015 in both the incidence (from 239 238 to 602 609) and mortality associated with these infections (from 11 144 to 27 249) *(Cassini A, 2019).* This trend continues also in the period 2016-2020, and in the 65+ age group, infections caused by *E. coli* resistant to 3rd generation cephalosporins are associated with the highest burden *(ECDC, 2022c).* In the present study, 30-day lethality increased with age, and this was true for males, whereas females had the highest lethality at ages 20-39. At ages after 60y lethal cases were more frequent among males, but the difference was statistically insignificant.

IV. FINDINGS

On the basis of the results obtained from the complex microbiological and epidemiological studies on antimicrobial resistance among the leading causative agents of bacteremias associated with bloodstream infections at St. Marina University Hospital over a 10-year period and the lethality associated with them and after the analysis, the following major conclusions can be made:

1. The proportion of positive blood cultures over the ten-year study period was 16%, with 9.9% of true positive (clinically significant) blood cultures.

2. In the period 2011-2020, the etiological spectrum of laboratory-confirmed bacterial bloodstream infections in the unselected patient population was dominated by Gram-negative bacteria (58.9%), but there was a statistically significant trend of increasing proportion of Gram-positive bacteria and decreasing proportion of Gram-negative bacteria and fungi over the years. The most frequently isolated bacterial species from blood cultures over the entire period was *S. aureus (*17.2%), followed by *E. coli* (14.6%), *K. pneumoniae* (12.0%), *E. cloacae* complex (8.0%), *A. baumannii* - *calcoaceticus* complex (6.3%) and *E. faecalis (*6.3%). The proportion of ESKAPEEc pathogens in the etiological spectrum was very high, reaching 66.8%, and that of EARS Net monitored bacterial species was 64.7%. In the course of the 10-year follow-up, a statistically significant trend of increasing relative proportion of *Staphylococcus aureus* and *Streptococcus viridans* isolates and decreasing proportion of *Klebsiella pneumoniae* was demonstrated.

3. The decade-long study demonstrates the emergence, dissemination and persistence over time of problematic MDR Gram negative bacteria associated with bloodstream infections and to a much lesser extent Gram positive microorganisms, confirming the European and global trends of the recent years.

4. The relative proportion of 3rd generation cephalosporin and fluoroquinolone resistant *E. coli* from blood is high (more than 25%), but without a significant increasing or decreasing trend over the years. The activity of aminopenicillins against *E. coli* was significantly reduced, with resistance to this antibiotic group being highest (63.2%). Carbapenems and amikacin had preserved activity against *E. coli* (resistance <1% and <5%, respectively), making them a suitable choice for empiric therapy in cases of bloodstream infections associated with this bacterial species.

5. A very high level of resistance to 3rd generation cephalosporins was demonstrated among *Klebsiella pneumoniae* isolates (74.9%), significantly exceeding the proportion of those resistant to the same antibiotic group *E. coli* and higher than among *Enterobacter* spp. Third generation cephalosporins were the group with the most reduced activity against *K. pneumoniae*. Very high levels of resistance (over 50%) are also reported for fluoroquinolones and gentamicin, although the latter shows a statistically significant trend of decreasing

resistance from 73% in 2011 to 40.9% in 2020. Carbapenems and amikacin are the antimicrobials with the highest activity against *K. pneumoniae* (11.8% and 13.9% resistance, respectively). Over the 10-year study period, the most dynamic and dramatic changes were evidenced in carbapenem resistance, which increased from 0% in 2011 to 32.7% in 2014 and 18.2% and 13.6% in 2019 and 2020. In the group of carbapenem-resistant *K. pneumoniae*, colistin and amikacin demonstrate the best in vitro activity (6% and 17.9% resistance), making them the drugs of choice for treatment in the cases of infection caused by carbapenem-resistant isolates.

6. *bla*$_{\text{CTX-M-15}}$ is identified as a major mechanism of resistance to 3rd generation cephalosporins in carbapenem-resistant *K. pneumoniae* blood isolates, whereas *bla*$_{\text{KPC-2}}$ and, to a much lesser extent, *bla*$_{\text{NDM-1}}$ mediate the enzymatic mechanism of carbapenem resistance in these isolates. Carbapenem-resistant *K. pneumoniae* are associated with 5 different ST types: ST15, ST76, ST151 and ST1350 for KPC-2 producers and ST11 for NDM-1 producing isolates. A widespread intra-hospital dissemination of KPC-2 and/or CTX-M-15 producing ST15 *K. pneumoniae* was found. This clone persisted for several years, demonstrating high cross-transmission, epidemic and invasive potential. In addition to ST15, resistance to 3rd generation cephalosporins and carbapenems has been associated with intra-hospital dissemination of other ST types coexisting with the dominant ST15. In this aspect, although the clonal spread of ST15 contributes significantly to the dissemination of carbapenem-resistant *K. pneumoniae*, non-ST15 strains also emerge and contribute.

7. This study demonstrated a very high relative proportion of *Enterobacter* spp. blood isolates resistant to 3rd generation cephalosporins (more than 65%), as well as high levels of resistance to fluoroquinolones and gentamicin (more than 45%). The proportion of amikacin- and carbapenem-resistant *Enterobacter* spp. remains very low, making these antimicrobials an appropriate choice for the treatment of invasive infections caused by *Enterobacter* spp. NDM-1 metallo-carbapenemase is the mechanism mediating carbapenem resistance in the only carbapenem-resistant *Enterobacter* spp. isolate for this period (*E. asburiae*).

8. In the group of blood isolates of *Pseudomonas aeruginosa*, resistance of more than 20% to all antipseudomonal drugs was demonstrated, with the strongest reduction in the activity of fluoroquinolones (40%). There were no statistically significant trends of decreasing or increasing resistance across years for any of the antimicrobial groups studied. Carbapenems and amikacin were the most active agents among the antipseudomonal agents studied, although the resistance rates were in the range of 20-30%, suggesting careful use of these antimicrobials in cases of suspected *P. aeruginosa* infection.

9. Throughout the 10-year study period, blood isolates of *A. baumannii - calcoaceticus* complex consistently demonstrated very high levels of resistance to all antimicrobials tested (over 60%), with the exception of colistin. Compared to all other Gram-negative species, the proportion of carbapenem-resistant *A. baumannii - calcoaceticus* complex was the highest (68.4%), with 60% of the isolates demonstrating an XDR phenotype. Carbapenem resistance was associated with the presence of *bla*$_{\text{OXA-24/40-like}}$ and/or *bla*$_{\text{OXA-23-like}}$ genes in association with *ISAba1*. The high relative proportion of carbapenem-resistant isolates of *A. baumannii* is associated with intrahospital dissemination and the persistent presence of several OXA-producing MDR *A. baumannii* clones with endemic characteristics, including IC2.

10. A relatively low level of MRSA invasive isolates was found in the period 2011-2020, with a proportion lower than the European average and very close to the Bulgarian average in 2020. With the exception of macrolides, all other antimicrobial groups retain good activity, with a proportion of non-susceptible isolates below 20%.

11. There is a significant trend of increasing proportion of vancomycin-resistant *Enterococcus faecium* from blood - from 0% in the period 2011-2018 to 11.1% in 2019 and 18.2% in 2020. *E. faecium* demonstrates very high levels of resistance to aminopenicillins (95.3%),

fluoroquinolones (84.7%) and HLAR (90.3%). In contrast to *E. faecium*, the proportion of ampicillin-resistant *E. faecalis* remains low (9.4%), making the aminopenicillin group an appropriate choice for therapy of *E.* faecalis-associated infections. The activity of fluoroquinolones over the same period among *E. faecalis* is significantly reduced (over 35%), with a high proportion of HLAR (over 45%). Despite the significant trend of increasing resistance to vancomycin, glycopeptides and linezolid are still the antibiotics with the best activity against *Enterococcus* spp. isolates.

12. In the period 2011-2020, the etiological spectrum of bacteremias associated with bloodstream infections in patients with oncohematological diseases was dominated by Gram-negative over Gram-positive bacteria (54.3% vs. 38.0%), with the most common bacterial pathogens being *S. aureus* (17.3%), *E. coli* (16.0%), *Enterobacter* spp. (10.9%), *Klebsiella* spp. (10.3%), and *Enterococcus* spp. (8.8%). There was a statistically significant trend for a decrease in the relative proportion of *S. aureus* and an increase in that of coagulase-negative staphylococci in the etiologic spectrum of bloodstream infections in this group of patients.

13. The study, performed in the group of patients with oncohematological diseases, demonstrated the emergence and persistence over the years of problematic MDR microorganisms, predominantly Gram-negative bacteria, associated with bloodstream infections in this patient population, a phenomenon that is a reflection of the increasing antimicrobial resistance in the community and follows the trend in the unselected population of hospital patients with bloodstream infections. There is a persistent trend over time of high levels of resistance to 3rd generation cephalosporins among members of *Enterobacteriaceae* family (49.4%) and the emergence since 2014 of invasive carbapenem-resistant isolates from the same family, with the most affected species in terms of both types of resistance being *K. pneumoniae* (57.8% and 6.7%, respectively). A statistically significant trend of increasing resistance to 3rd generation cephalosporins, aminoglycosides and fluoroquinolones was demonstrated in the group of *Enterobacter cloacae* complex isolates; persistence of a high relative proportion of carbapenem-resistant *A. baumannii - calcoaceticus* complex and a statistically significant trend towards an increase in this resistance, as well as an increase in the proportion of MDR *Enterococcus faecium.* Similar to the general patient population, a positive finding was the low relative proportion of MRSA blood isolates. No *Staphylococcus* spp. and *Enterococcus* spp. isolates resistant to glycopeptide antibiotics and oxazolidinones were identified during the study period, making these antibiotics the drugs of choice for initial treatment in cases of suspected staphylococcal or enterococcal bloodstream infection or febrile neutropenia until an etiologic diagnosis is made.

14. A high 30-day lethality (26%) was found in the study group of 798 patients with bloodstream infections caused by *S. aureus, S. pneumoniae, E. coli. K. pneumoniae, E. cloacae, A. baumannii - calcoaceticus* complex, and *P. aeruginosa*, and this rate was higher in cases of nosocomial infections compared with those acquired in the community (31.3% vs. 20.8%). With the exception of age, all other demographics did not prove to be significant risk factors for lethality in the patient group studied. The highest 30-day lethality was found in the Intensive care units of the hospital (35.8%), followed by the Hematology clinics (27.3%).

15. *E. coli* (23.6%), *S. aureus* (20.7%) and *K. pneumoniae* (19.2%) were associated with the highest number of deaths. In a comparison between bacterial species, the highest 30-day lethality was demonstrated for infections caused by *A. baumannii - calcoaceticus* complex (53.6%). When 30-day lethality was evaluated against the combination "bacterial species/antimicrobial agent", aminoglycoside, quinolone and meropenem-resistant *A. baumannii - calcoaceticus* complex (15.9-16.3%) were associated with the highest number of deaths, followed by ampicillin-resistant *E. coli (*15.9%) and 3rd generation cephalosporin-resistant *K. pneumoniae* (14.9%).

16. The variables "age", "type of clinic", "prior hospitalization", "type of therapy administered

(ICU and/or surgical treatment)", "bacterial species" and "infectious syndrome" were identified as independent factors that significantly influenced the prognosis of 30 day survival (predictors of death). Grouped according to bacterial species, in cases of *E. coli* infections, the factors "resistance to 3rd generation cephalosporins", "prior hospitalization", and "surgical/ICU treatment" were significantly associated with the risk of death, whereas for *S. aureus* infections, these factors were "age" and "surgical/ICU treatment". For *P. aeruginosa* and *A. baumannii - calcoaceticus* complex infections, a statistically significant predictor of death was the variable "surgical treatment", and in bloodstream infections caused by *K. pneumoniae* – "surgical/ICU treatment".

V. CONTRIBUTIONS OF THE DISSERTATION

Original contributions

1. A detailed analysis of the etiological spectrum of bacteraemias and antimicrobial resistance of ESKAPEEc pathogens in an unselected group of hospitalised patients with laboratory-confirmed bloodstream infections over a decade was performed, and trends over time were assessed and compared with European and global trends.
2. A detailed analysis of the etiologic spectrum of bacteremias and antimicrobial resistance of ESKAPEEc pathogens in a group of oncohematological patients with laboratory-confirmed bloodstream infections over an 11-year period was performed, and trends over time were assessed. The results provide a basis to define the recommendations for empiric antimicrobial treatment in cases of febrile neutropenia or suspected infectious complications in this group of severely immunocompromised patients.
3. The 30-day lethality (general and specific) and the risk factors for fatal outcome in bloodstream infections caused by 7 bacterial species (*S. aureus, S. pneumoniae, E. coli, K. pneumoniae, E. cloacae* complex, *A. baumannii - calcoaceticus* complex, *P. aeruginosa)* among 798 hospitalized patients over a 5-year period were studied, as well as the heavy burden with which these diseases are associated has been demonstrated.
4. The study expands the available data and scientific information on antimicrobial resistance among the leading bacterial causes of bloodstream infections and the burden of these infections in Bulgaria and can serve as a basis for policy making aimed at limiting and controlling the problem at local and national level.
5. The study has led to the generation of high quality and comparable data and their integration into a very large international database aimed at assessing the burden of antimicrobial resistance in various infections, including bloodstream infections.

Contributions of a confirmatory nature

1. The molecular genetic mechanisms of carbapenem resistance among the most important and problematic pathogens (*K. pneumoniae, A. baumannii*) associated with clinically relevant bacteremias were investigated, demonstrating their widespread intrahospital dissemination and association with certain sequence types and international clones.

Contributions of scientific - applied nature

1. The demonstrated depth of the problem of "Antimicrobial resistance" and "Lethality" accompanying bloodstream infections in hospitalized patients, including severely immunocompromised patients, clearly demonstrates the need to introduce modern methods of rapid microbial identification and antimicrobial susceptibility testing into the laboratory diagnosis of these life-threatening infections. The direct results of this will be a reduction in the mortality rate of infections caused by multidrug-resistant

bacteria and an increase in the number of microbiological tests performed, in particular those for antimicrobial suscetibility testing.

VI. SCIENTIFIC PUBLICATIONS RELATED TO THE THESIS

1. **Antimicrobial Resistance Collaborators.** Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. **Lancet 2022**; 399: 629–55 Published Online January 20, 2022 https://doi.org/10.1016/ S0140-6736(21)02724-0. **IF 202.731**
2. **European Antimicrobial Resistance Collaborators.** The burden of antimicrobial resistance in the WHO European region in 2019: a cross-country systematic analysis. **Lancet Public Health 2022**; 7: e897–913 Published Online October 13, 2022 https://doi.org/10.1016/ S2468-2667(22)00225-0. **IF 72.427**
3. **Temenuga Stoeva**, Rumyana Markovska, Ivan Mitov. Molecular epidemiology of *Klebsiella pneumoniae* blood isolates resistant to 3rd generation cephalosporins and/or carbapenems collected from patients in a Bulgaria University Hospital. **Доклади на Българската академия на науките, 2021**, vol 74, No 10, 1469-1478. **IF 0.326**
4. R. Markovska, **T. Stoeva**, P. Stankova, L. Boyanova, D. Dimitrova, R. Gergova, I. Mitov. First report of *Enterobacter asburiae* isolate, producing NDM-1 and a novel ACT-68 enzyme in Bulgaria. **Infectious Diseases, 2019**, 51(8). **IF 2.191**
5. R. Markovska, **T. Stoeva**, L. Boyanova, P. Stankova, E. Keuleyan, M. Murdjeva, M. Lesseva, G. Nedelcheva, A. Petrova, D. Ivanova, K. Mihova, R. Kaneva, I. Mitov. Multicentre investigation of carbapenemase-producing *Klebsiella pneumoniae* and *Escherichia coli* in Bulgarian hospitals–Interregional spread of ST11 NDM-1-producing *K. pneumoniae*. **Infection, Genetics and Evolution, 2019**, 69: 61-67. **IF 2.26**
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8. R. Markovska. **T. Stoeva**. I. Schneider. L. Boyanova, V. Popova, D. Dacheva, R. Kaneva, A. Bauernfeind, V. Mitev, I. Mitov. Clonal dissemination of multilocus sequence type ST15 KPC-2-producing *Klebsiella pneumoniae* in Bulgaria. **APMIS 2015**, 123, 10:887-94. **IF 1.933.**
9. R. Markovska, I. Schneider, **T. Stoeva**, K. Bojkova, L. Boyanova, A. Bauernfeind, I. Mitov. First identification of KPC-2 and VIM-1 producing *Klebsiella pneumoniae* in Bulgaria. **Diagnostic Microbiology & Infectious Disease 2013**, 77, № 3, 252-253. **IP 2.568**
10. **Т. Стоева**, Д. Ниязи, И. Мичева. Инфекции на кръвта при пациенти с онкохематологични заболявания: етиологичен спектър и профил на антибиотична резистентност (2015 - 2020). **Хематология, 2022**, VOL. LVIII 2022 № 1-2; 38-44. **SJR2022 0.101, Q4.**

11. Д. Ниязи, И. Мичева, **Т. Стоева.** Бактериални и микотични усложнения при пациенти след хематопоетична стволово-клетъчна трансплантация. **Хематология 2020,** vol. LVI, № 1-2; 26-31. **SJR2020 0.101, Q4.**
12. Г. Неделчева, **Т. Стоева**, Р. Марковска, Д. Димитрова, П. Станкова, И. Митов. Антибиотична чувствителност на клинично значими изолати *Klebsiella pneumoniae*, изолирани от пациенти, хоспитализирани в УМБАЛ „Света Марина“ - Варна за периода 2014-2017г. **Обща медицина, 2019**, брой 3; 9-16. **SJR2019 0.113, Q4.**
13. D. Niyazi, I. Micheva, D. Savova, M. Georgieva, E. Ismail, **T. Stoeva.** Species diversity and antimicrobial resistance of *Staphylococcus* spp. isolates associated with catheter – related bloodstream infections in patients after hematopoietic stem cell transplantation. **Scripta Scientifica Medica, 2022**;54(2):13-17.
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