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PROSPERITAS VESTRA FINIS NOSTRA!

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Investigation of bacteremia and invasive fungal infections in patients following autologous and allogeneic hematopoietic stem cell transplantation

THESIS SUMMARY

Of a dissertation on the award of educational and scientific degree «Doctor»

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The dissertation contains 187 pages and is illustrated with 19 figures and 20 tables. 430 reference sources are cited, of which 8 in Cyrillic and 422 in Latin.

The dissertation was discussed at a meeting of the Department of Microbiology and Virology of MU - Varna and is referred for public defense in front of the Scientific Jury consisting of:

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The defense of the dissertation will take place on 13.09.2022 at 13:00 in ……..

The materials on the defense are available to those interested in the library of Medical University - Varna.

Contents

Abbreviations

HSC – hematopoietic stem cell
HSCT – hematopoietic stem cell transplantation
HM – hematologic malignancy
GVHD – graft versus host disease
CVC – central venous catheter
CoNS – coagulase-negative staphylococci
GNGNF – Gram-negative glucose non-fermenters
MIC – minimal inhibitory concentration
MRSA – methicillin-resistant *Staphylococcus aureus*
MRS – methicillin-resistant staphylococci
MRCoNS – methicillin-resistant coagulase-negative staphylococci
ESBL – extended-spectrum beta-lactamase
CPR – carbapenem-resistant
MDR – multidrug resistant
CRA – Congo red agar
TT – Christense's tube test
IPA – invasive pulmonary aspergillosis
GM – galactomannan antigen
VRE – vancomycin-resistant enterococci

1. INTRODUCTION

The transplantation of hematopoietic stem-cells (HSC) is one of the revolutionary discoveries in the field of medicine, which led to the treatment of many incurable in the past diseases (*Thomas ED, 1970; Hatzmichael E, 2010*).

Medical conditions using hematopoietic stem-cell transplantation (HSCT) are divided into two groups. The first group includes non-malignant conditions that lead to bone marrow failure such as aplastic anemia, immunodeficiency syndromes, hemoglobinopathies, etc. The second group includes malignant, mainly hematological diseases such as acute and chronic leukemias, myelodysplastic syndrome, lymphomas, and multiple myeloma *(Wingard JR, 2019).*

The HSCs to be transplanted can be extracted from the bone marrow, peripheral blood or umbilical cord of the donor and transferred by infusion into the recipient's body *(Wingard JR, 2010).* Transplanted HSCs serve to replace damaged hematopoietic tissue or to destroy recipient tumor cells (*Negrin RS, 2019).*

There are two main types of HSCT - autologous and allogeneic. In autologous transplantation, the patient's own cells are used, which are returned to the recipient's body by infusion after the tumor cells have been destroyed. Allogeneic transplantation uses foreign HSCs harvested from related or unrelated individuals *(Antin JH, 2013).*

Due to a number of its specific features, HSCT is accompanied by the development of non-infectious and infectious complications. Infectious complications, which are among the leading causes of death in these patients, are associated with different bacteria, fungi, viruses and parasites while the non-infectious complications are due to the drugs used during the conditioning period and are most commonly demonstrated as serositis, mucositis, venoocclusive liver and lung disease, and hemorrhagic cystitis (*Al-Anazi KA, 2010; Weisdorf D, 2019*). Styczynski reported an incidence of bacterial, fungal and viral complications of 33.9%, 22.8% and 38.3%, respectively, with the proportion of them depending on the type of transplant, the conditions at the transplant center, the geographical region, use or no of antimicrobials for prophylaxis and etc. (*Styczynski J, 2016*).

2. PURPOSE AND GOALS

Purpose:

To perform a clinical-microbiological study on bacteremias and invasive fungal infections in patients after autologous and allogeneic hematopoietic stem-cell transplantation performed in the period 01.01.2019 - 31.12.2021 at the Transplantation ward of the Clinical Hematology clinic of University Hospital "St. Marina", Varna.

In this regard, we set the following goals:

1. To study the incidence and risk factors for bacteremia and fungemia in patients after autologous and allogeneic hematopoietic stem cell transplantation, as well as 4-month survival in the entire study group of patients, and analyze the factors that affect it.
2. To study the etiological spectrum of bacteremia and fungemia and to determine the susceptibility of microbial isolates, associated with bloodstream infections to a range of antimicrobial drugs.
3. To study the mechanisms of methicillin resistance in *Staphylococcus* spp. and to 3rd generation cefalosporins and carbepenems in Gram-negative blood isolates by molecular-genetic methods.
4. To study the ability of slime production in clinically relevant *Staphylococcus* spp. blood isolates by phenotypic and molecular genetic-methods, as their major factor of virulence.
5. To study the etiological spectrum of invasive fungal infections.
6. To study the level of fecal colonization with isolates resistant to 3rd generation cefalosporins and carbepenems from order *Enterobacterales*, carbapenem-resistant *Pseudomonas* spp., vancomycin-resistant enterococci, *Stenotrophomonas maltophilia*, and fungi in the studied group of transplanted patients, as well as methods to study the resistance mechanisms to these antimicrobial agents.
7. To study the epidemiological relationship between fecal and blood isolates, demonstrating resistance to 3rd generation cephalosporins, carbapenems and glycopeptides.

3. MATERIALS AND METHODS

3.1. STUDY DESIGN

The present PhD thesis is a prospective clinical, microbiological and epidemiological study on a total of 74 patients admitted to the Transplantation ward of the Clinical Hematology clinic of University

Hospital "St. Marina",Varna in the period January 2019 - December 2021 and underwent HSCT. The study was approved by the Comity for Ethics of Scientific Research with protocol No. 92 / 02.04.2020.

Studied patients

In the period January 2019 - December 2021, 74 patients were admitted to the Transplantation ward of the Clinical Hematology clinic of University Hospital "St. Marina" in Varna and underwent HSCT. Forty-two (56.8%) were men and 32 (43.2%) were women, with the age ranging from 14 to 68 years. In 71 patients (96%) HSCT was preceded by the development of oncological or hematological malignancy (HM), and in three - multiple sclerosis.

3.2. MICROBIAL ISOLATES

A total of 107 non – duplicate microbial isolates were studied (89 bacterial and 18 fungal), obtained from various clinical materials of 74 patients who underwent HSCT (42 from blood and 65 from feces) (Table 1).

Table 1. Distribution of microbial isolates, according to the type of clinical specimen and the microbial type.

Clinical specimen	Microbial type	Number of isolates (n)
Blood	*Escherichia coli*	11
	Klebsiella pneumoniae	2
	Enterobacter cloacae	2
	Pseudomonas aeruginosa	2
	Acinetobacter baumannii	1
	Burkholderia cepacia	1
	Staphylococcus epidermidis	12
	Staphylococcus haemolyticus	4
	Staphylococcus aureus	3
	Staphylococcus hominis	2
	Streptococcus bovis	1
	Candida krusei	1
	Total blood isolates	**42**

Feces	*Escherichia coli*	10
	Enterobacter cloacae	7
	Klebsiella pneumoniae	4
	Pseudomonas putida	4
	Stenotrophomonas maltophilia	3
	Pseudomonas aeruginosa	2
	Pseudomonas composti	1
	Pseudomonas mendocina	1
	Serratia marcescens	1
	Enterococcus faecium	15
	Candida glabrata	7
	Candida albicans	3
	Candida krusei	2
	Candida tropicalis	2
	Candida dubliniensis	1
	Candida kefyr	1
	Candida parapsilosis	1
	Total fecal isolates	**65**
Total clinical specimens (n=95)		**107**

3.3. METHODS FOR MICROBIAL IDENTIFICATION

3.3.1. IDENTIFICATION BY:

- Phoenix 100 (BD, USA)
- MALDI Biotyper® Sirius (Bruker, Germany)
- Polymerase chain reaction (PCR)

3.3.2. IDENTIFICATION OF FUNGAL ISOLATES BY ASSIMILATION TESTS

- AUXACOLOR™ 2 (Bio- Rad, France)

3.4. METHODS FOR DETERMINATION OF SUSCEPTIBILITY TO ANTIMICROBIAL AGENTS

3.4.1. DETERMINATION OF SUSCEPTIBILITY TO ANTIMICROBIAL DRUGS BY AN AUTOMATED SYSTEM

3.4.2. MICRODILUTION METHOD FOR DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATION OF ANTIMICROBIAL AGENTS:

- Sensititre ™ YeastOne ™ YO3IVD AST Plate (Thermo Fisher, USA)
- MIKROLATEST (Erba Lachema, Czech Republic)

3.4.3. DETERMINATION OF SUSCEPTIBILITY TO ANTIMICROBIAL AGENTS BY E-TEST

- E-test (Liofilchem, Italy)

3.5. MOLECULAR-GENETIC DETECTION OF BETA-LACTAM, METHICILLIN AND GLYCOPEPTIDE RESISTANCE

3.5.1. POLYMERASE CHAIN REACTION (PCR)

3.5.2. DNA SEQUENCING

3.6. METHODS FOR DETECTING SLIME PRODUCTION IN *STAPHYLOCOCCUS* SPP.

3.6.1. PHENOTYPIC METHODS

- Cultivation on Congo red agar (Freeman DJ, 1989)
- Christensen's tube test (Christensen GD, 1982)

3.6.2. MOLECULAR-GENETIC METHODS

- icaA, icaD (Petrelli D, 2006)

3.7. DETECTION OF *ASPERGILLUS* GALACTOMANNAN ANTIGEN IN SERUM AND BRONCHO-ALVEOLAR LAVAGE (BAL)

- Platelia™ *Aspergillus* Ag (Bio- Rad, France)

3.8. EPIDEMIOLOGICAL TYPING

- ERIC PCR
- RAPD PCR

3.9. STATISTICAL METHODS

- Descriptive
- Hypothesis testing

4. RESULTS AND DISCUSSION

4.1. EPIDEMIOLOGY OF BLOODSTREAM INFECTIONS (BACTEREMIA AND FUNGEMIA) IN PATIENTS FOLLOWING HSCT.

Characteristics of the studied patients

Demographic and other characteristics of patients, associated with HSCT are shown in Table 2.

Table 2. Characteristics of patients with HSCT.

Characteristics	Patients, n (%)	
	Autologous HSCT (n=49)	**Allogeneic HSCT (n=25)**
Age (95% IQR)	51 (18 – 68)	39 (14 – 65)
Patients < 18 y., n (%)	-	3 (12.0)
Male	27 (55.1)	15 (60.0)
Underlying disease		
Multiple myeloma	26 (52.0)	-
Acute myeloblastic leukemia	-	11 (44.0)
Acute lymphoblastic leukemia	-	5 (20.0)
Non-Hodgkin's lymphoma	13 (26.5)	2 (8.0)
Hodgkin's disease	6 (12.2)	4 (16.0)
Aplastic anemia	-	2 (8.0)
Multiple sclerosis	3 (6.1)	-
Myelodisplastic syndrome	-	1 (4.0)
Proliferative disease	1 (2.0)	-
Previous HSCT		
Autologous	5 (10.2)	5 (20.0)
Allogeneic	-	-
Donor type		
HLA MUD	NA	14 (56.0)
HLA MRD	NA	5 (20.0)
Haplo	NA	6 (24.0)
Stem-cell source		
Bone marrow	-	2 (8.0)
Peripheral blood	49 (100.0)	23 (92.0)

NA - not applicable; **MUD -** matched unrelated donor; **MRD -** matched related donor; **Haplo -** haplo-identical donor**; IQR -** interquartile range;

Bloodstream infections (bacteremia and fungemia)

During the studied period, bloodstream infections were documented in 26 of 74 patients who underwent HSCT (35.1%). Fifteen of them (58%) had one episode of bloodstream infection, in 7 two episodes, in 2 - three episodes were documented and one patient had 4 different episodes. In 25 (96.2%) patients the bloodstream infection occurred before day 30 after HSCT, and in one (3.8%) after 30 days. In 24 (92.3%) patients, episodes of bloodstream infection developed before neutrophil engraftment, including 29 episodes. An episode of fungemia on the 6th day after HSCT was documented in a 61-year-old male patient with underlying myelodysplastic syndrome and subsequent allogeneic HSCT with a source of HSC - matched unrelated donor. The mean period of onset of bloodstream infections was 46.7 (SD ± 15.42) days (Fig. 1).

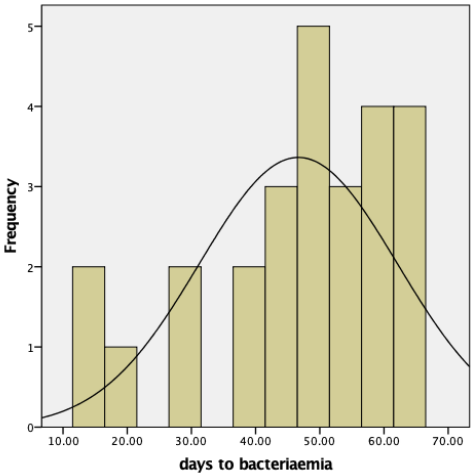

Figure 1. Mean period before bloodstream infection onset following HSCT.

The etiological spectrum of documented infections of the blood was dominated by Gram - positive bacteria ($p>0.05$), associated with bacteremia in the study group of transplanted patients. Figure 2 summarizes the rate and the causative agents of these infections.

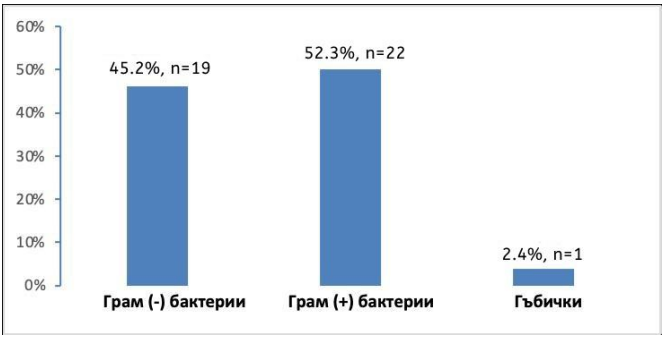

Figure 2. Spectrum of causative agents of bloodstream infections, documented in 26 transplant patients in the studied period (2019 - 2021).

Risk factors for bloodstream infections (bacteremia and fungemia) in patients after HSCT

The cumulative incidence of bloodstream infections after autologous and allogeneic HSCT was 32.0% (95% IQR 26.3% -37.7%) and 38.5%, respectively (95% IQR 14.6% -62.3%).

Risk factors with a potential effect on the occurrence of bloodstream infections in the study group of 74 patients, related to demographic characteristics, underlying disease and features, related to transplantation are presented in Table 3.

Table 3. Risk factors for bloodstream infections (bacteremia and fungemia) in patients after HSCT.

	Patients in total		Patients with bloodstream infection		Patients without bloodstream infections		*p*
	n	%	n	%	n	%	
Total	74	100.0	26	35.1	48	64.9	
Sex							
male	42	56.8	17	69.2	25	52.1	0.15
female	32	43.2	9	30.8	23	47.9	
Type HSCT							
autologous	49	66.2	15	57.7	34	70.1	0.25
allogeneic	25	33.8	11	42.3	14	29.2	
Stem-cell source							
bone marrow	2	2.7	2	3.8	0	0.0	0.17

peripheral blood	72	97.3	24	96.2	48	100.0	
Time to NE							
0-10 days	16	21.6	6	23.1	10	20.8	
11-20 days	51	68.9	17	65.4	34	70.8	0.86
21-30 days	7	9.5	3	11.5	4	8.3	
Time to PE							
0-10 days	11	14.9	2	7.7	9	18.8	
11-20 days	56	75.7	21	80.8	35	72.9	0.43
21-30 days	7	9.5	3	11.5	4	8.3	
Previous HSCT							
No	64	86.3	23	88.5	41	85.4	0.88
Yes	10	13.7	3	11.5	7	14.6	
Fecal colonization							
Yes	4	5.4	4	15.4	0	0.0	**0.005**
No	70	94.6	2	84.6	48	100.0	
Bloodstream infection before HSCT							
Yes	2	2.7	2	7.7	0	0	**0.05**
No	72	97.3	24	92.3	48	100	

HSCT - hematopoietic stem-cell transplantation; **NE** - neutrophil engraftment; **PE** - platelet engraftment;

Previous colonization of the digestive system with members of the family *Enterobacteriaceae*, *Pseudomonas* spp. and *Enterococcus faecium*, showing reduced sensitivity to beta- lactams and glycopeptides, as well as an episode of bloodstream infection before the current HSCT proved to be statistically significant risk factors, associated with the occurrence of bloodstream infections in the studied patient group ($p \leq 0.05$). Pretransplant bloodstream infections caused by *E. coli* (n = 1) and methicillin – resistant *Staphylococcus hominis* (n = 1) were detected in 2 patients (7.8%).

Bloodstream infection with the same bacterial species (*Enterobacter cloacae*) detected by fecal screening and with an identical susceptibility profile was detected in 1 patient (1.4%) after transplantation.

Survivival

The overall 4-month survival among the study group of transplanted patients was 86.5%. There was a statistically significant difference in terms of this survival between the group of patients with autologous and allogeneic transplantation ($p < 0.001$) (Table 4), as well as a statistically significant difference between these two groups in terms of

average survival after transplantation, as autologous HSCT patients have much longer survival than allogeneic HSCT recipients (p = 0.002).

A statistically significant relationship was also found between the type of diagnosis and 4-month survival (p = 0.043) (Table 4), with 100% survival rate of the cases of multiple myeloma, aplastic anemia, multiple sclerosis and idiopathic proliferative disease 4 months after transplantation.

A statistically significant link was also established between 4 months of survival across the entire group of transplanted patients (auto- and allogeneic HSCT) and the variable "previous transplantation", with those patients who had no previous transplant experience (p=0.001) (Table 4) were more likely to live up to 4 months after HSCT.

For the following variables: sex, age of the transplanted patient, type of donor, source of stem-cells, prolonged neutropenia, mucositis, graft versus host disease (GVHD), no effect on 4 months of survival (p>0.05) (Table 4) was proven.

Table 4. Risk factors influencing survival in patients after HSCT.

	Survival at 4th month			
	Survived	**Died**	**Total**	***p***
Allogeneic HSCT				
Median age (SD)	37.9 (17.86)	37.7 (18.23)	37.8 (17.62)	0.971
Count (%)	16 (64.0)	9 (36.0)	25 (100.0)	
Autologous HSCT				
Median age (SD)	53.8 (10.47)	43.0 (0.00)	53.6 (10.47)	0.312
Count (%)	48 (98)	1 (2.0)	49 (100.0)	
Stem-cell source				
Bone marrow	1 (100.0)	0 (0.0)	1 (100.0)	0.691
Peripheral blood	63 (86.3)	10 (13.7)	73 (100.0)	
Prolonged neutropenia				
Yes	4 (57.1)	3 (42.9)	7 (100.0)	0.146
No	16 (84.2)	3 (15.8)	19 (100.0)	
Mucositis				
Proven	4 (100.0)	0 (0.0)	4 (100.0)	0.234
Not proven	16 (72.7)	6 (27.3)	22 (100.0)	
Graft versus host disease (GVHD)				

Yes	1 (100.0)	0 (0.0)	1 (100.0)	0.664
No	21 (84.0)	4 (16.0)	25 (100.0)	
Underlying disease				
MM	26 (100)	0 (0.0)	26 (100.0)	**0.043**
AML	8 (72.7)	3 (27.3)	11 (100.0)	
ALL	4 (80.0)	1 (20.0)	5 (100.0)	
HD	7 (70.0)	3 (30.0)	10 (100.0)	
NHL	13 (86.7)	2 (13.3)	15 (100.0)	
AA	2 (100.0)	0 (0.0)	2 (100.0)	
MS	3 (100.0)	0 (0.0)	3 (100.0)	
MDS	0 (0.0)	1 (100.0)	1 (100.0)	
IPD	1 (100.0)	0 (0.0)	1 (100.0)	
Donor type				
Haplo	3 (50.0)	3 (50.0)	6 (100.0)	0.587
MRD	4 (80.0)	1 (20.0)	5 (100.0)	
MUD	9 (64.3)	5 (35.7)	14 (100.0)	
Previous HSCT				
Yes	4 (50.0)	4 (50.0)	8 (100.0)	**0.001**
No	60 (90.9)	6 (9.1)	66 (100.0)	
Sex				
Male	38 (88.4)	5 (11.6)	43 (100.0)	0.576
Female	26 (83.9)	5 (16.1)	31 (100.0)	
Transplantation type				
Autologous	48 (98.0)	1 (2.0)	49 (100.0)	**< 0.001**
Allogeneic	16 (64.0)	9 (36.0)	25 (100.0)	

SD - standard deviation; **MM** - multiple myeloma; **AML** - acute myeloblastic leukemia; **ALL** - acute lymphoblastic leukemia; **HD** - Hodgkin's disease; **MRD** - matched related donor; **MUD** - matched unrelated donor; **IPD** - idiopathic proliferative disease; **Haplo** - haplo-identical donor; **NHL** - Non-Hodgkin's lymphoma; **AA** - aplastic anemia; **MS** - multiple sclerosis; **MDS** - myelodysplastic syndrome;

Thirty-day mortality after diagnosis of bloodstream infection (bacteremia/fungemia) was 23% (6 out of 26 patients), and in 50% of the patients (3 out of 6) the infection was accepted as the main cause of death.

Discussion

Transplantation of HSCs is an effective therapeutic method for dealing with many malignancies, syndromes with bone marrow failure, as well as immunodeficiency conditions in children, adolescents and adult individuals. More than 50,000 HSCT per year are carried out globally. The development of transplant strategies and the advancement of supportive care lead to an improvement in the outcome of transplantation *(Dandoy CE, 2020)*. However, infectious complications constantly accompany HSCT, with bloodstream infections being one of the most common causes of morbidity and mortality in patients who have undergone HSCT *(Ge J, 2018).*

Bloodstream infections are a severe form of systemic infection caused by the entry of pathogenic microorganisms into the blood circulation. They occur as the most common complication mainly during the early stages of the transplantation and range between 13.6% and 38.9%. According to a large number of literary sources, the development of this type of complication is an essential risk factor for early death after HSCT *(Cao W, 2021).* Main factors, identified as risky, for the development of bloodstream infection after HSCT are absolute neutropenia, mucositis and the presence of a central venous catheter (CVC). Additional risk factors, contributing to the occurrence of this complication are considered the type of underlying disease, severe form of GVHD and the use of corticosteroids *(Youssef A, 2019).*

In this study, by prospective follow-up of patients who underwent HSCT in the period 2019-2021, collecting information and its statistical processing, we identified the risk factors contributing to the development of bloodstream infections in the study group of patients and evaluated the factors influencing the outcome of HSCT.

Over the followed three-year period, 35.1% of the patients developed at least one episode of bloodstream infection, with the average bloodstream infection period being approximately 47 days after HSCT. These data differ significantly from those obtained by other researchers. Ferreira et al. reported an average period of 8 days after HSCT of blood infection among 232 patients (*Ferreira AM, 2018*). Similar data reported by Akinboyo, Youssef and Ge – 15.5 and 4.5 days, respectively (*Ge J, 2018; Akinboyo IC, 2020; Youssef A, 2020*). Results closer to ours were reported in a large-scale cohort retrospective study covering a total of 16,875

allogeneic transplants from different centers conducted between 2009 and 2016 – 29 days (*Dandoy CE, 2020*).

In the current study, the cumulative incidence of bloodstream infections after auto- and allo-HSCT was 32% and 38.5%, respectively. Close to our values are reported in a study of Ferreira et al. – 25.4% (*Ferreira AM, 2018*). Significant differences were observed in the reports of Girmenia et al. and Dandoy et al. In a large-scale Italian study involving data from 54 transplant centers for more than 2500 HSCTs, a cumulative bloodstream infection rate of 9% for auto and 17.3% for allo-HSCT was documented *(Girmenia C, 2017*). The incidence in the study of Dandoy et al. was 21% (*Dandoy CE, 2020).*

To determine the role of potential risk factors for the development of bloodstream infection after HSCT, we used single-factor and multiple regression analysis. After analyzing the data obtained, two factors were identified where statistical significance was detected ($p<0.05$). Fecal colonization of patients with microorganisms demonstrating multidrug resistance and *Candida* spp., as well as an episode of bloodstream infection before HSCT proved to be independent risk factors for the occurrence of invasive bloodstream infection after HSCT. Similar results have been reported in other studies. Youssef et al. in a study conducted in Egypt among pediatric patients, who underwent HSCT between 2013 and 2017, found a statistically significant link between the occurrence of bloodstream infection after HSCT and an episode before the transplantation (*Youssef A, 2020).* Girmenia et al. found statistical significance between colonization with multidrug resistant bacteria and the occurrence of bloodstream infection in both autologous and allogeneic transplant patients (*Girmenia C, 2017).* These data are also supported by a study conducted in Brazil, documenting intestinal colonization with bacteria demonstrating multiple resistance as a risk factor for the occurrence of bloodstream infection (*Ferreira AM, 2018).*

In addition to determining the potential risk factors, influencing the occurrence of bloodstream infections, we also examined the factors that may affect the survival after HSCT. Out of the patients studied, more than 80% survived the first 4 months after HSCT. Patients, who underwent auto-HSCT and those with underlying disease: multiple myeloma, aplastic anemia, multiple sclerosis and idiopathic proliferative disease turned out to have a higher chance of surviving compared to allogeneic HSCT and patients with previous transplantation. Close to ours results are shared by Girmenia et al. The authors reported a 4-month survival rate in approximately 90% of patients, higher survival rates were seen in patients with auto-HSCT. Factors negatively affecting the survival are acute

leukemia, previous transplantation, prolonged neutropenia, etc. *(Girmenia C, 2017).*

In our study, the 30-day mortality after HSCT and bloodstream infection was 23%, with the immediate cause of death being a bloodstream infection in half of the cases. In a study by Girmenia et al. the 30-day mortality after allo-HSCT was 17.9%, in 96% of them the infection was proven to be the main cause. Among auto transplantees, this incidence is much lower (4.1%), as all patients died as a result of bloodstream infection (*Girmenia C, 2017).*

Conclusion

In the current study, fecal colonization and previous bloodstream infection have proven to be independent risk factors for the occurrence of bloodstream infection in patients after HSCT. The cumulative incidence of these infections is 32% for auto- and 38.5% for allogeneic transplants, with an average period of occurrence of the infectious complication of 47 days after the procedure. Thirty-day mortality after diagnosis of bloodstream infection is 23%. A high 4-month survival rate was found among the entire group of studied patients (over 86%), with statistical significance found between 4-month survival and the indicators type of transplantation, underlying disease and lack or presence of previous transplantation, with patients with autologous HSCT and a diagnosis other than leukemia and lymphoma having a better chance of survival rather than patients with allogeneic HSCT and previous transplantation.

4.2. ETIOLOGICAL SPECTRUM OF BLOODSTREAM INFECTIONS

4.2.1. Etiological spectrum

During the studied period, a total of 968 blood cultures of 74 patients following HSCT with suspected infectious complication (chills, headache, fever, fatigue) were examined. A total of 42 clinically significant, non-duplicate isolates from the blood cultures of 26 patients, associated with episodes of bloodstream infection were isolated and identified. The detailed etiological spectrum is presented in Table 5.

Table 5. Etiological structure of bloodstream infections in patients after HSCT in the period 2019-2021.

Etiological agents of the bloodstream infections	n (%)
Gram-positive bacteria	**22 (52.3)**
Staphylococcus epidermidis	12 (54.5)
Staphylococcus haemolyticus	4 (18.1)
Staphylococcus hominis	2 (9.1)
Staphylococcus aureus	3 (13.6)
Streptococcus bovis	1 (4.5)
Gram-negative bacteria	**19 (45.2)**
E. coli	11 (57.9)
Enterobacter cloacae	2 (10.5)
Klebsiella pneumoniae	2 (10.5)
Pseudomonas aeruginosa	2 (10.5)
Acinetobacter baumannii	1 (5.3)
Burkolderia cepacia complex	1 (5.3)
Yeasts	**1 (2.4)**
Candida krusei	1 (100.0)
Total	**42 (100.0)**

Discussion

In the etiological spectrum of bloodstream infections, diagnosed in 26 patients who underwent HSCT in the period 2019 – 2021 a prevalence of Gram-positive bacteria is found (52.3%). Among them leading pathogens are coagulase-negative staphylococci (CoNS), most common being *S. epidermidis* (54.5%). Significantly less often, species with more pronounced pathogenic potential, such as *S. aureus*, are detected (13.6%). Similar results are reported in studies conducted in hematological centers in the United States, Turkey and Egypt involving pediatric and adult patients after HSCT and with HM *(Yemisen M, 2016; Balian C, 2018; Youssef A, 2019; Akinboyo I, 2020).*

Our results show a very low incidence of catalase - negative Gram - positive cocci, as causative agents of bloodstream infections (a single isolate *S. bovis*), which is in line with the data reported by Youssef and Yemisen (*Yemisen M, 2016; Youssef A, 2019*). Our data differ significantly from those obtained by Akinboyo, when examining bloodstream infections

for the period 1997 – 2016, which establishes the dominance of the representatives of the *Enterococcus* and *Streptococcus* genera, as associated with 57.9% of all blood infections caused by Gram - positive bacteria (*Akinboyo I, 2020).*

In this study, the representatives of Gram-negative bacterial species rank second as causative agents of bloodstream infections. Representatives of the *Enterobacteriaceae* family dominate, with *E. coli* being the most common isolate (57.9%). With a much lower relative share are presented glucose non-fermenting Gram-negative bacteria (GNGNF) such as *P. aeruginosa, A. baumannii* and *B. cepacia* (21.1%). The data we received are similar to those reported by Balian and Youssef *(Balian C, 2018; Youssef A, 2019)*. Other authors also identify *E. coli* as the most common causative agent of bacteremia (*Ge J, 2018; Youssef A, 2019; Akinboyo I, 2020*). In contrast to our results, Yemisen reported the dominance of GNGNF (*A. baumannii, S. maltophilia, P. aeruginosa*) over the representatives of the *Enterobacterales* order in an 11-year study (2000-2011) (*Yemisen M, 2016*). The same author for an earlier period (2000 – 2005) established Gram – negative bacteria as the leading causative agents of bloodstream infections, but after 2005 reported a sharp change and increase in the number of Gram – positive bacterial isolates, with the change in the etiological spectrum being explained by the use of various conditioning regimens before transplantation, antibiotic prophylaxis, as well as the change in global antibiotic resistance (*Yemisen M, 2016).*

The etiological spectrum of bloodstream infections in patients after HSCT and HM has been the subject of numerous in-depth studies for years, and over the past 30 years it has shown a tendency to change. In the last century, Gram – positive bacteria were considered the most common infectious agents, leading to bacteremia in neutropenic patients. About 2000 a change in this structure begins, taking into account the dominance of Gram – negative bacteria. For example, for the period 2013 - 2014 Youssef et al. found an increase in the incidence of Gram – negative bacteremias. The change undertaken in the therapeutic and prophylactic regimen, exchange of piperacillin/tazobactam with carbapenem, in the following documented period (2015-2017) reported a decrease in Gram-negative causative agents from 72% to 29%, but this was accompanied by a significant increase in the incidence of Gram – positive bacteremia – from 28% to 70% (*Youssef A, 2019).*

Some authors believe that the type of etiological causative agent of bloodstream infections in patients after HSCT depends on the resources available to different countries and their economic development. For example, China and some developing countries reported a higher incidence

of Gram - negative bacteria compared to Gram – positive ones. On the other hand, the dominance of Gram – positive agents is documented in developed countries (*Ge J, 2018*).

Other authors believe that the higher incidence of Gram-positive bacteremia reported in a number of studies is due to contamination of the samples with skin microflora. It is assumed that prolonged skin trauma, a rare change of CVCs and the difficulty in keeping them sterile, especially in pediatric patients, have an effect on the type of isolated bacteria *(Akinboyo I, 2020*).

In Bulgaria, in a study conducted by Stoeva et al., on the etiological structure of bloodstream infections in patients with HM hospitalized at St. Marina University Hospital – Varna in the period 2010 – 2014 the prevalence of Gram-negative microorganisms is established (54.7%), with the highest proportion of enteric bacteria (*E. coli, K. pneumoniae* and *Enterobacter* spp.). Gram – positive bacteria were isolated in 38%, dominated by *S. aureus* and *Enterococcus* spp. Representatives of genus *Candida* were isolated in 6.9% (*Stoeva T, 2016*). These data differ from the results reported in the current study and are likely related to the change in therapeutic and prophylactic regimens, chemotherapy options, as well as widespread use of CVC in recent years.

The comparative study of Kaleva et al. on the etiological spectrum of infectious complications in children with HM, covering two periods - 1990 - 1994 and 1995 - 2003, established the dominance of Gram - positive bacteria (53.2%) as the causative agents of bloodstream infections compared to Gram - negative ones (40.3%), and mycotic agents were reported in 6.5% of the cases. Most often isolated microorganisms were CoNS, followed by *Klebsiella* spp., *Enterococcus* spp., *E. coli* and *P. aeruginosa*. During the two periods there was no change in the ratio between Gram – positive and Gram – negative causative agents, but in the second period there was a significant increase in the relative share of *E. coli* - associated bacteremias (*Kaleva V, 2006*).

In this study, only one mycotic agent is isolated, causing infection of the blood belonging to the genus *Candida – C. krusei* (2.4%). Similar results are reported by various authors studying the etiology of bloodstream infections. Yemisen et al. reported 6 episodes of fungemia caused by *Candida* spp. (3.1%), with all fungi falling into the nonalbicans group (*Yemisen M, 2016*). In a study by Youssef et al. 141 infectious agents were isolated, but only 1.4% (*C. albicans*, n=2) were fungi (*Youssef A, 2019*). Another 4-year study conducted by Mikulska et al. reported 5 isolates *Candida* spp. (*C. glabrata*, n=1; *C. krusei*, n=1; *C. parapsilosis*, n=2) with an incidence of 3.4% (*Mikulska M, 2012*). From the studied data it becomes

clear that the causative agents of mycotic bloodstream infections are mainly from the nonalbicans group and their frequency varies between 1.4% - 3.4%, data that fully support our results. A number of scientists believe that this phenomenon of displacement of sensitive *C. albicans* by more resistant nonalbicans species is mainly due to the antimycotic prophylaxis used with fluconazole (*Akinboyo I, 2020).*

In a study conducted at St. Marina University Hospital – Varna by Stoeva et al., including data for a period of 4 years (2007 – 2011) for fungemia in patients from intensive and non-intensive units (including patients with HM and febrile neutropenia) there was an increase in the incidence of fungal bloodstream infections, as well as displacement of the spectrum from *C. albicans* to *C.* nonalbicans species (*Stoeva T, 2013).*

Conclusion

Our study found that Gram-positive bacteria dominate (52.3%) over Gram - negative bacterial species (45.2%) as causative agents of bloodstream infections in patients after HSCT and the most common causative agents being representatives of normal skin microflora (CoNS), a fact that we associate with the influence of the prophylactic regimen in the neutropenic period and the use of CVC. *E. coli* prevails among Gram - negative microorganisms. We found a low relative share of fungemia, which is associated with *Candida* nonalbicans species (*C. krusei*, 2.4%).

4.2.2. Susceptibility of blood isolates to antimicrobial agents

Susceptibility to a set of antimicrobial agents of 19 Gram - negative and 22 Gram - positive bacterial isolates has been studied by the Phoenix 100 automated system and microdiluation method for determining the minimum inhibitory concentration (MIC) of colistin.

Gram-negative bacteria

The resistance documented among Gram - negative isolates, representatives of *Enterobacteriacae* family (n=15) in descending order is as follows: 80% ampicillin (n=12) > 53.3% trimethoprim/sulfamethoxazole (n=8) > 46.7% ciprofloxacin (n=7) > 40% amoxicillin/clavulanate (n=7) n=6) > 33.3% levofloxacin (n=5) > 26.6% gentamicin (n=4), cefuroxime (n=4) and cefotaxime/ceftazidime (n=4) > 20% piperacillin/tazobactam (n=3) and cefepime (n=3). Isolates resistant to imipenem, meropenem, ceftazdime/avibactam, amikacin and colistin have not been detected.

The *A. baumannii* isolate demonstrates multidrug resistance to β-lactams (including carbapenems), quinolones and aminoglycosides.

Susceptibility to colistin is preserved. In *P. aeruginosa* and *B. cepacia* complex isolates, the typical for these bacterial species of intrinsic resistance to ampicillin, amoxicillin/clavulanate, cefuroxime and cefotaxime and preserved antibacterial susceptibility to the other tested agents was documented.

Gram-positive bacteria

The resistance profile of 21 Gram - positive bacterial isolates, representatives of the genus *Staphylococcus* (CoNS, n=18; *S. aureus*, n=3) to major groups of antimicrobials in decreasing order is as follows: 90.5% penicillin (n =19) > 85.7% cefoxitin (n=18) > 71.4% erythromycin (n=15) > 62% ciprofloxacin (n=13) > 52.4% gentamicin (n=12) > 47.6% doxycycline (n=10) > 42.9% trimethoprim/sulfamethoxazole (n=9) > 38.1% clindamycin (n=8). Isolates resistant to glycopeptides (vancomycin, teicoplanin) and linezolid have not been detected.

Susceptibility among *S. aureus* isolates to all antibiotics except penicillin is fully preserved. Fully preserved sensitivity to the tested antimicrobial agents (penicillin, cefoxitin, clindamycin, ciprofloxacin, vancomycin, teicoplanin and linezolid) also demonstrates the only blood isolate *Streptococcus bovis*.

Isolates from genus *Candida*

Susceptibility to a set of antimycotic agents (fluconazole, voriconazole, itraconazole, isavuconazole, caspofungin, micafungin, anidulafungin and flucytosine) has been studied by a microdiluation method for determining MIC (Sensititre YO3IVD).

For the studied period of time only one fungal blood isolate is cultivated and identified, which is a representative of the genus *Candida* - *C. krusei* and represents 2.4% of all blood infections.

In addition to the typical intrinsic resistance of this species to fluconazole (MIC > 64 μg/ml), the following MIC values of the other antimycotics as follows were reported: voriconazole, 1 μg/ml; itraconazole, 0.5 μg/ml; isavuconazole, 0.06 μg/ml; anidulafungin, 0.06 μg/ml; caspofungin, 0.5 μg/ml. According to the European and American standards used to account for susceptibility to antimycotics, the isolate demonstrates a preserved sensitivity to voriconazole, itraconazole, micafungin and anidulafungin. Due to the lack of generally accepted standards for caspofungin, isavuconazole and flucytosine, the MIC established for these agents were not interpreted.

Discussion

In the current study, the susceptibility to antimicrobials of 42 non-duplicate clinically significant microbial isolates from blood cultures of 26 patients after HSCT and implemented CVC was studied by automated and microdilution MIC methods.

CoNS are the most commonly isolated bacteria from blood cultures in the studied group of patients, as well as the most commonly isolated Gram - positive bacteria (81.8%), significantly exceeding the share of *S. aureus* isolates (13.6%). In the studied collection of staphylococcal isolates (n=21), the level of methicillin resistance was the highest (85.7%) compared to resistance levels to the other groups of tested antibiotics. All CoNS were identified as methicillin-resistant, but methicillin-resistant *S. aureus* (MRSA) were not detected. Methicillin - resistant staphylococci (MRS) are among the problematic pathogens for treatment, as they are resistant to all β-lactams (including carbapenems) and often demonstrate a profile of multiple-drug resistance. Along with β-lactam resistance, these isolates showed significantly reduced sensitivity to other antimicrobial groups, ranging from 44.4% for sulfonamides and lincozamides to over 80% for macrolides. It is a well-known fact that resistance to methicillin has been documented more frequently in CoNS than in *S. aureus*. Our results are consistent with data reported by other authors and support this statement *(Busca A, 2012).*

An alarming result is the established high percentage of staphylococcal isolates with resistance to macrolides, quinolones and aminoglycosides (over 50%), agents usually preferred in infections caused by Gram-positive bacteria and appearing an alternative to β-lactams. A number of guidelines for patients who have undergone HSCT and are at an early post-transplantation stage or in cases of an episode of febrile neutropenia, the recommend prophylaxis or therapy include quinolones *(Verlinden A, 2020).* The observed significantly reduced sensitivity to these antimicrobials (62%) has the potential to compromise preventive and treatment strategies in these patients.

A reasonable choice for empirical therapy of CoNS-related bacteraemia are glycopeptide antimicrobials vancomycin and teicoplanin, which are usually with preserved activity and MRSA/ methicillin-resistant coagulase-negative staphylococci (MRCoNS). In case of colonization of CVC by these microorganisms, its replacement is recommended, due to the ability of bacteria to form a biofilm, through which antibiotics can't diffuse (*Balletto E, 2015).* Our study did not prove resistance to strategic glycopeptides and oxazolidinones (linezolid) in all 21 isolates. However, it should be considered that cases of glycopeptides-resistant staphylococci have already been described *(Ghahremani M, 2018).*

A positive result of this study is that unlike CoNS, *S. aureus isolates* demonstrate a preserved sensitivity to all antimicrobials except penicillin.

Among the Gram-negative bacteria, associated with bacteremia in this study, the largest relative share is of the representatives of the *Enterobacteriaceae* family (78.9%). The β-lactam antibiotics are some of the most commonly used antimicrobial agents in the clinical practice, especially in cases of empirical therapy. In our study, the highest incidence of resistance to these antimicrobials among enteric bacteria was reported for aminopenicillins (ampicillin, 80%; amoxicillin/clavulanic acid, 40%). Resistance to the broader spectrum cephalosporins of 2nd (cefuroxime) and 3rd generations (cefotaxime/ceftazidime) (26.6%), followed by piperacillin/tazobactam and cefepime (20%) is lower. The results we got differ significantly from other similar studies. Barman et al. conducted a two-year study studying the etiological spectrum of bloodstream infection in patients who underwent HSCT and their resistance profile (*Barman P, 2020*). They reported a complete lack of sensitivity to ampicillin among enteric bacteria, as well as reduced sensitivity to other β-lactams: amoxicillin/clavulanic acid (93.6%), piperacillin/tazobactam (85.5%), third and fourth generation cephalosporins – 97.6% and 89.4%, respectively (*Barman P, 2020*). Like Barman, Lubwama et al. reported increased resistance among *E. coli* isolates, *K. pneumoniae* and *Enterobacter* spp., causative agents of bloodstream infections in patients with febrile neutropenia and oncological diseases, to widely used β-lactam antibiotics:

over 90% for ampicillin and amoxicillin/clavulanic acid, 85% for second and third generation cephalosporins and 65% for piperacillin/tazobactam (*Lubwama M, 2019*). These data are also supported by Ge et al., who for five years (2012–2017) examined 336 patients with HM who underwent HSCT. The authors report a complete lack of sensitivity to ampicillin among *E. coli* isolates and significantly reduced to the remaining β-lactams (76.9% - 100%) *(Ge J, 2018).*

Beta - lactam antibiotics are agents with good tolerability, a wide spectrum of action and a bactericidal effect. These qualities make them one of the most commonly preferred antibiotics, both in hospitalized patients and in outpatients. Their frequent use, however, determines the occurrence of bacterial resistance to them. The most common and well-studied mechanism, which determines the occurrence of β - lactam resistance is associated with the production of enzymes, hydrolyzing these antimicrobial agents. Among the most problematic isolates are those exhibiting resistance to broad-spectrum cephalosporins of the third and fourth generations, which is most often mediated by the production of broad-spectrum beta-lactamases (ESBLs).

In this study, the proportion of ESBL producers among the collection of blood isolates from the *Enterobactreiaceae* family was 20%, which was detected by the molecular-genetic methods used (see section 4.3). In one *E. coli* isolate, resistance to third generation cephalosporins, but preserved sensitivity to fourth generation (cefepime) is documented. It is likely that this phenomenon is due to hyperproduction of acquired Class C AmpC beta-lactamase. A similar result was reported by Yemisen et al. (20.6%) (*Yemisen M, 2016).* Unlike our data, other transplant centers report a much higher incidence of ESBL producers, associated with bacteremia. Lubawa et al. reported that 41% of the enterobacteria tested in their study were ESBL producers (*Lubawa M, 2019*). According to Gustinetii et al. the most common ESBL producers among enterobacteria are *E. coli* and *K. pneumoniae*. In their systematic study, an increase in the incidence of these isolates was reported, with this percentage ranging from 11% to 69% in some countries. In a study conducted in South Korea involving neutropenic patients, ESBL producing *E. coli* and *K. pneumoniae* were responsible for

26% of infections associated with resistant bacteria, and in an Italian cohort study the incidence of these isolates was significantly higher – about 40% (*Gustinetti G, 2016).*

Carbapenem antibiotics (meropenem, imipenem) are among the recommended first-choice agents when an ESBL producer is documented as the causative agent of bloodstream infections. Following these recommendations, in the past there has been a decrease in the number of ESBL – associated invasive infections, but only a few years after the introduction of this practice, reports of enteric bacteria showing resistance to these strategic antibiotics began to increase (*Logan LK, 2012; Labaste F, 2019*). Along with ESBL producers, bacteria carrying genes for resistance to carbapenems are some of the most problematic ones for treatment. Internationally, data on an ever-increasing incidence of carbapenem-resistant (CPR) bacteria have been reported in recent years, which is why in 2017 WHO declared bacterial species resistant to carbapenems (*A. baumanii, P. aeruginosa*, representatives of the *Enterobacteriaceae* family) a global threat and included them in the first group of agents with high priority for the creation of new antimicrobials with activity against them (WHO, 2017). Among the group of representatives of the *Enterobacteriaceae* family in this study, CPR isolates, causative agents of bacteremia were not documented. Many authors report different data from our results. For example, Cao et al. analyzed for 4 years episodes of bloodstream infections in the pre-engraftment period in patients after HSCT and reported an incidence of 17.9% of bacteremias caused by CPR enterobacteria (*Cao W, 2021).* In other hematological centers, the incidence of this type of infection is much higher. According to Lubwama et al. 45% of the causative agents of bloodstream infections in their study were CPR *Enterobacteriaceae*, similar results are reported by Barman et al. (22.9% - 44.8%) (*Lubwama M, 2019; Barman P, 2020).*

Due to its wide antimicrobial spectrum, fluoroquinolones are among the first choice agents for prophylaxis in neutropenic patients with oncological diseases. Moreover, in a large number of studies it has been reported that fluoroquinolones are more effective than trimethoprim/sulfamethoxazole in reducing infectious complications. In

2007 The European Conference on Infections approved antibacterial prophylaxis with fluoroquinolones and included them in recommendations for the prevention and treatment of leukemia patients, with it is being recommended for high-risk patients with neutropenia and an expected duration of neutropenia for more than seven days (*Servidio AG, 2021).* Our results show high levels of quinolone resistance – over 40% for ciprofloxacin and over 30% for levofloxacin. Other authors report much higher levels, ranging between 70% - 95.2% (*Ge J, 2018; Lubwama M, 2019; Barman P, 2020*). Similar data are reported by Mukulska et al. – 73% resistance to quinolones among Gram – negative bacteria causing bloodstream infections in patients with HSCT (*Mikulska M, 2012*). In contrast to these results, a study of Yemisen et al. reported only 10% Gram – negative bacteria with a lack of sensitivity to ciprofloxacin/levofloxacin (*Yemisen M, 2016*).

Along with quinolones, aminoglycosides are an alternative to β-lactam antibiotics, and in many cases are also used in combination with them in order to achieve a synergistic effect and improve the outcome of the infection in septic patients. In our study, resistance to gentamicin was over 20%. However, a positive result is the established fully preserved activity of amikacin among enteric blood isolates. Other authors reported significantly higher levels of resistance to gentamicin (67.3% - 75%) and amikacin (28.6% - 50%) (*Ge J, 2018; Lubwama M, 2019; Barman P, 2020*).

In this study, among the antimicrobials with the highest activity against isolates, representatives of the *Enterobacteriaceae* family are the cephalosporin antibiotic of the 3rd generation - ceftazidime, potentiated with the new beta-lactam inhibitor avibactam, as well as colistin, considered strategic antimicrobials and those of last choice in cases of infections caused by multidrug-resistant (MDR) bacteria and CPR enterobacteria. However, there have already been reports of resistant to these antibiotics bacteria *(Gogry FA, 2021; Xu T, 2021).*

The GNGNF group in this study, presented by *A. baumanii, P. aeruginosa* and *B. cepacia* as a cause of bacteremia in patients who underwent HSCT are the third group after staphylococci and the

representatives of the *Enterobacteriaceae* family for the studied period. In three of these isolates, *P. aeruginosa* and *B. cepacia*, only their typical natural resistance (aminopenicilins, first and second generation cephalosporins) was identified, with no evidence of acquired resistance to other antibiotics. It should not be forgotten that infections caused by these microorganisms are among the most problematic for treatment precisely because of their intrinsic and often acquired antibiotic resistance, and are also associated with high mortality (*Ku NS, 2011; Montero MM, 2020*). The innate resistance of these bacterial species should not be ignored and, if empirical use of antimicrobials is necessary, anti-pseudomonal activity should be selected.

The only blood isolate *A. baumannii* in this study demonstrated a phenotype of MDR: complete lack of sensitivity to all tested antimicrobials (β-lactams, quinolones, aminoglycosides and sulfonamides), except colistin. Like CPR enterobacteria, CPR *A. baumannii* and *P. aeruginosa* are considered a serious threat to public health precisely because of the lack of active agents against them and therefore adequate therapeutic behavior, which for patients after HSCT is of critical importance. For MDR *A. baumannii*, causative agents of bloodstream infections in patients after HSCT also reported Barman, and establishes an 84% share of these isolates (*Barman P, 2020).* Other authors such as Mikulska, Wang and Youssef report significantly lower rates - 35%, 16% and 21%, respectively (*Mikulska M, 2009; Wang L, 2015; Youssef A, 2019).*

Our study documents one episode of fungemia caused by *C. krusei*. Recently published recommendations for primary prophylaxis in patients after HSCT are for the use of antimycotics depending on the risk factors (type of transplantation, underlying disease, expected duration of neutropenia, etc.) (*Teh BW, 2021).* In patients at high risk, the drug of choice is posaconazole or its alternative voriconazole, while in patients at low risk, fluconazole is the first choice (Teh BW, 2021). When proving or suspecting an invasive infection caused by *Candida* spp. echinocandins are the agents of choice, with an alternative liposomal amphotericin B (*Rahi MS, 2021*). A number of authors report that a large proportion of the fungi of the genus *Candida* are sensitive to voriconazole and echinocandins, but

resistant to fluconazole (*Mikulska M, 2012; Barman P, 2020*). Our isolate demonstrates the typical for this species natural resistance to fluconazole, but retains sensitivity to the recommended for therapy and prevention antimycotics (triazols and echinocandins). Unfortunately, in the scientific literature there are already reports of resistance to voriconazole and echinocandins in invasive *Candida* isolates (*Phu TT, 2019; Posteraro B, 2020*).

Conclusion

The tests for sensitivity to antimicrobial drugs found a high relative share of methicillin – resistant staphylococci among the causative agents of bloodstream infections (44%), as well as high levels of resistance to ampicillin, ciprofloxacin and trimethoprim/sulfamethoxazole among enteric bacteria. The share of third-generation cephalosporins-resistant representatives of the *Enterobacteriaceae* family is 26.7%, with the share of ESBL producers being 20%. With the best activity are imipenem/meropenem, piperacillin/tazobactam, amikacin and vancomycin, making them suitable agents for initial empirical treatment in cases of febrile neutropenia and septic state. The agents ceftazidime/avibactam and colistin retain their effectiveness and can be used when infections occur due to microorganisms with multiple resistance. In cases of implemented CVC and suspected catheter – associated infection, it is advisable to start glycopeptide therapy because of the high relative share of MRCoNS in the etiological spectrum of bloodstream infections in patients after HSCT.

4.3. STUDY ON THE MECHANISMS OF RESISTANCE TO THIRD-GENERATION CEPHALOSPORINS AND CARBAPENEMS IN GRAM-NEGATIVE BACTERIA AND THE MECHANISMS OF METHICILLIN RESISTANCE IN *STAPHYLOCOCCUS* SPP. FROM BLOOD BY MOLECULAR-GENETIC METHODS

In the studied collection of 42 blood isolates, 4 Gram-negative isolates (*E. coli*, n=2; *E. cloacae*, n=1; *A. baumannii*, n=1) demonstrated resistance to third and fourth generation cephalosporins, and one of them to carbapenems (*A. baumannii*). Eighteen CoNS were identified phenotypically as methicillin – resistant. All of the 22 isolates were

subjected to molecular - genetic testing by PCR to detect the carriers of genes that mediate the relevant resistance type (the most common genes in the *Enterobacteriaceae* family, encoding ESBLs – CTX-M, SHV, TEM and carbapenemases (VIM, IMP, KPC, NDM, OXA-48, OXA-23, OXA-58, OXA-24), as well as *mecA* and *mecC* genes, associated with methicillin resistance. In 3 isolates (*E. coli*, n=2 and *E. cloacae*, n=1), DNA sequencing was performed to document the specific type of beta-lactamase.

PCR for detecting genes, encoding CTX-M, SHV and TEM ESBLs

In 3 bacterial isolates from blood cultures (*E. coli*, n=2; *E. cloacae*, n=1) PCR was used to detect $bla_{\text{CTX-M}}$, bla_{SHV} and bla_{TEM} genes. In all three isolates, an amplified product of 585 bp size, corresponding to $bla_{\text{CTX-M-like}}$ was obtained, and in two of them, *E. coli*, n=1 and *E. cloacae*, n=1, the presence of $bla_{\text{TEM-like}}$ (1075 bp) (Figure 3A) was also demonstrated. $bla_{\text{SHV-like}}$ carriers have not been documented.

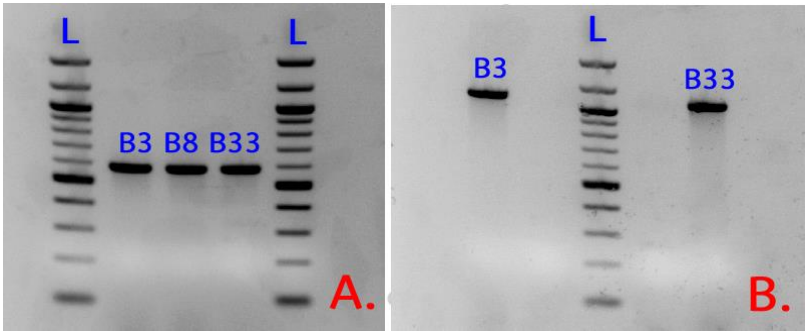

Figure 3A. PCR detection of *bla* genes, associated with beta-lactam resistance. A. Detection of $bla_{\text{CTX-M}}$ (585 bp); **B.** Detection of bla_{TEM} (1075 bp**); L,** 100 bp DNA marker**, B3** and **B8** *(E. coli);* **B33,** *E. cloacae***.**

PCR for detecting genes, encoding VIM, IMP, KPC, NDM, OXA-48, OXA-23, OXA-58, OXA-24 carbapenemases

In a bacterial blood isolate (*A. baumannii*), PCR is applied to demonstrate bla_{VIM}, bla_{IMP}, bla_{KPC}, bla_{NDM}, $bla_{\text{OXA-48}}$, $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24/40}}$, $bla_{\text{OXA-58}}$ genes. Amplification products with a size of 390 bp, 438 bp, 246 bp, 501 bp, corresponding to $bla_{\text{VIM-like}}$, $bla_{\text{OXA-48-like}}$, $bla_{\text{OXA-24/40-like}}$ and $bla_{\text{OXA-23-like}}$ (Figure 3C) were obtained.

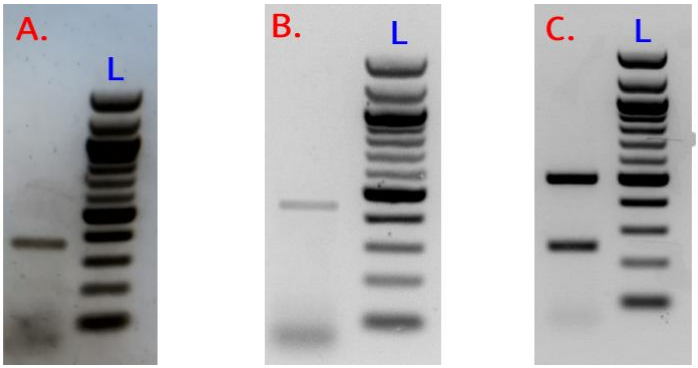

Figure 3B. PCR detection of *bla* genes in blood isolate *A. baumannii*. A. bla_{VIM} (390 bp); **B.** bla_{OXA-48} (438 bp); **C.** bla_{OXA-23} (501 bp), $bla_{OXA-24/40}$ (246 bp). **L,** 100 bp DNA marker

DNA sequencing of genes, encoding ESBLs

All CTX-M and TEM positive isolates were tested with CTX-M-P1/P2 and TEM A/B primers and gave a positive result, which confirmed the presence of CTX-M 1-group and $bla_{TEM-like}$ genes. By nucleotide sequencing, the presence of CTX-M-15 and TEM-1 in the corresponding isolates was identified.

PCR for detecting methicillin resistance

All *Staphylococcus* spp. isolates (n=21) were subjected to PCR to detect the *mecA* and *mecC* genes that mediate methicillin resistance. In 18 isolates (85.7%), all phenotypically defined as methicillin resistant, a PCR product of 162 bp size (Figure 3C) corresponding to the *mecA* gene is displayed. *mecA* positive were all CoNS. Among the *S. aureus* isolates (n=3), all phenotypically defined as methicillin sensitive, *mec* genes were not detected. All of the tested isolates were negative for the *mecC* gene.

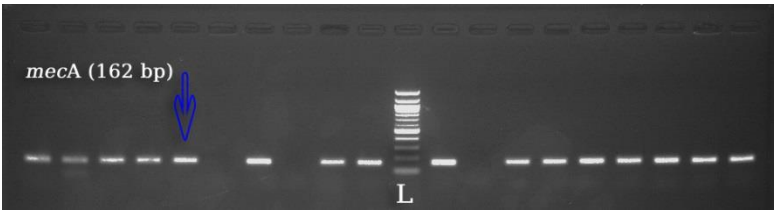

Figure 3C. PCR detection of *mecA* and *mecC* genes, associated with methicillin resistance. L, 100 bp DNA marker**.**

Discussion

In recent years, we have seen a continuous increase in the number of bacteria worldwide, exhibiting multiple resistance, particularly this phenomenon is noticeable among Gram-negative microorganisms – representatives of the *Enterobacteriaceae* family, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, which are also among the most medically important bacterial species. Due to the occurrence of resistance to antimicrobials, often part of the empirical regimens, infections caused by MDR bacteria are often associated with a delay in the initiation of adequate therapy, which in turn leads to an unfavorable outcome of the infectious complication, especially in the case of immunocompromised patients, such as these after HSCT.

The main mechanism of resistance among representatives of the order *Enterobacterales* to β-lactam antibiotics is associated with the production of β-lactamases of various molecular classes (A, B, C, D), some of which have the potential to hydrolyze third-generation cephalosporins known as ESBLs. These enzymes are an ever-growing and evolving group, and so far there are over 200 variants related to three main groups – TEM, SHV and CTX-M (*Rawat D, 2010*). After TEM and SHV ESBLs, CTX-M are the third largest group of broad-spectrum enzymes represented by CTX-M-15 and CTX-M-3, most often detected in bacterial species of the *Enterobacteriaceae* family (*E. coli, K. pneumoniae*) (*Markovska R, 2012; Bevan ER, 2017; Markovska R, 2017*). According to some authors, this group of enzymes is the most widespread ESBLs (*Markovska P, 2012; Markovska R, 2014; Mirkalantari S, 2020*). *bla* genes, responsible for the production of ESBLs, are usually localized in large plasmids, which often carry other genes, encoding resistance to different groups of antibacterial drugs (aminoglycosides, sulfonamides, tetracyclines, quinolones) (*Patterson DL, 2000; Wang M, 2004; Mammeri H, 2005*). For this reason, multiple antibiotic resistance is a common feature of ESBL – producing enteric bacteria, making them a serious therapeutic problem for the clinical practice

(*Rawat D, 2010*). In the current study, among 15 blood isolates, representatives of the *Enterobacteriaceae* family, three showed (*E. coli*, n=2; *E. cloacae*, n=1) resistance to third and fourth generation cephalosporins. Molecular-genetic experiments confirmed that in all three isolates resistance to broad-spectrum cephalosporins is mediated by the presence of CTX-M-15 ESBL. The detected TEM-1 beta-lactamase in two of the isolates (*E. coli, E. cloacae*) is an enzyme with narrow-spectrum activity, hydrolyzing penicillins and cephalosporins of the first generation without activity on broad-spectrum cephalosporins (*Salverda M, 2010*). In a study by Dimitrova et al. between 2014 and 2017, among patients with HM, a series of bacteremias with causative agent *E. cloacae* have been documented, with the authors reporting $bla_{\text{CTX-M-15}}$ genes in 93% of isolates, mediating resistance to third and fourth generation cephalosporins (*Dimitrova D, 2019b*).

In the last decade, CPR *A. baumannii* was assessed as one of the most important, problematic to treat and associated with high mortality causative agents of intra-hospital infections - most often pneumonia associated with artificial ventilation and bloodstream infections (*Strateva T, 2019*). The difficulty in treating these infections is the result of the impressive resistance profile of these bacteria (multiple or pan-resistance) and respectively the presence of an extremely limited number of antibacterial agents with an uncertain effect, such as colistin and tigecycline (*Piperaki ET, 2019*). Resistance of *A. baumannii* to β-lactams (incl. carbapenems) is mediated most often by the enzyme mechanism, associated with the production of β-lactamases, some of them with carbapenemase activity, of classes B (metal-beta-lactamases) and D (oxacillinases). Metal-carbapenemases of class B are the most problematic due to their very wide hydrolytic spectrum: hydrolyzing all β-lactam antibiotics except aztreonam. Class D oxacillinases are a large group of enzymes with variable activity against different β-lactams (incl. carbapenems), some of which have been documented almost exclusively in *A. baumannii* – OXA-23, OXA-58, OXA-24/40 (*Diene SM, 2014*). Other Class D enzymes, such as OXA-48, have little hydrolyzing activity against carbapenems and are more common in representatives of the enteric bacteria (especially *K. pneumoniae*) (*Evans BA, 2014*). The therapy of infections, caused by CPR *A. baumannii*, which usually demonstrate a profile of multiple or pan-resistance is severely hampered. Currently, it is recommended to use colistin, tigecycline, sulbactam, minocycline and some new agents, including cefiderocol and eravacycline (*Piperaki ET, 2019*).

In this study, the only isolate *A. baumannii* (with a characteristic of multidrug-resistant), causative agent of bacteremia in a patient after

allogeneic HSCT was shown to be a carrier of four *bla* genes at the same time, encoding carbapenemases of two molecular classes of enzymes - $bla_{\text{VIM-like}}$ (class B) and $bla_{\text{OXA-48-like}}$, $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24/40-like}}$ (Class D), a fact which confirms the genetic plasticity of this bacterial species, enabling it to benefit from the diversity of resistance mechanisms in the face of high selective antibiotic pressure. Strateva's research and collective study on CPR *A. baumannii* in 4 University Hospitals in Bulgaria reports a dissemination of $bla_{\text{OXA-58}}$, $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24/40}}$, including in blood isolates (*Strateva T, 2019*). The first reports of $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-58}}$ in *A. baumannii* in Bulgaria were made by Stoeva et al. (*Stoeva T, 2008; Stoeva T, 2009; Stoeva T, 2014*). Later Petrova et al. for four years tracked CPR *A. baumannii*, causative agents of infections in a university hospital, and reported $bla_{\text{OXA-23}}$ as the most commonly associated enzyme with resistance to carbapenems (*Petrova AP, 2017*). Many authors from different geographic regions report $bla_{\text{OXA-23}}$ as the most common in CPR *A. baumannii*, but also report an increase in the number of isolates carrying $bla_{\text{OXA24/40}}$ and $bla_{\text{OXA-58}}$ genes mediating resistance to carbapenem antibiotics (*Ji S, 2014; Ayibieke A, 2020*). Unlike the mentioned OXA enzymes, the OXA-48 beta-lactamase with a characteristics of a carbapenemase is detected relatively rare in *A. baumannii*. The first such isolate was documented in 2013 in Portugal (*Goncalves D, 2013*). In addition, globally reports of *A. baumannii*, producers of Class B enzymes (mainly VIM and IMP), are also increasing, with the incidence varying from geographic region to region (*Nikibakhsh M, 2021*).

The mechanism of methicillin resistance in the representatives of the genus *Staphylococcus* is most often mediated by the *mecA* gene, located on a mobile genetic element called Staphylococcal Chromosome Cassette *mec* (SCCmec). The isolates carrying this gene produce alternative penicillin – binding proteins (PBP2a), which have a low affinity for β-lactam antibiotics (*Cikman A, 2019*). In 2005 another gene, also associated with methicillin resistance, has been identified - $mecA_{\text{LGA251}}$, a homolog of the classic *mecA* and named in 2012 *mecC* (*Cikman A, 2019*). In this study, all MRCoNS were carriers of the *mecA* gene. In a study by Gergova et al., including *Staphylococcus* spp. isolates from blood and punctates, a high incidence of MRCoNS (93.9%) and MRSA (39.3%) was detected and the resistance to β-lactam antibiotics in all isolates, was mediated by the *mecA* gene (*Gergova R, 2019*).

Conclusion

The main mechanism of resistance to 3rd generation cephalosporins in blood isolates of the *Enterobacteriaceae* family in this study is associated with the production of CTX-M-15 ESBL, a result that confirms the geographical widespread of this type of ESBLs. A carbapenem-resistant *A. baumannii* was documented as a carrier of four *bla* genes, encoding carbapenemases of class B ($bla_{\text{VIM-like}}$) and class D ($bla_{\text{OXA-48-like}}$, $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24/40-like}}$). Methicillin resistance, demonstrated in all CoNS, is associated with the *mecA* gene.

4.4. SLIME PRODUCTION BY *STAPHYLOCOCCUS* SPP.

All twenty-one clinically significant *Staphylococcus* spp. (n=18, CoNS; n=3, *S. aureus*), isolated from blood cultures and associated with bloodstream infections in 17 patients with implemented CVC, were subjected to phenotypic and molecular-genetic tests to detect slime production. The two phenotypic methods used (Congo red agar, CRA and Christensen's test, TT) were positive in 13 isolates (61.9%) (including strong and weak slime producers) (Figure 4 and 5, Table 6).

In 10 (47.6%) of the isolates *(S. epidermidis*, n=9, *S. haemolyticus*, n=1) the presence of *ica* genes (Figure 6) was documented by PCR. In *S. aureus* and *S. hominis*, *icaA* or *icaD* genes were not detected.

The three methods used (CRA, TT, PCR) were positive in 5 (23.8%) of the tested isolates. Five *ica* carriers demonstrated different combinations, obtained with phenotypic methods (Table 6). Three *ica* – negative isolates (*S. aureus*, *S. hominis* and *S. haemolyticus*) are positive only with the TT test. Two other *ica* negative isolates demonstrate slime production only with CRA phenotypic test (Figure 5). Out of all isolates, 15 (71.4%) were documented as slime producers using the phenotypic and/or PCR method.

In the whole collection, 6 isolates (28.6%) were negative for slime production by all three methods.

All *ica* – positive isolates are also *mecA* carriers. A statistically significant relationship was found between the carriers of *ica* and *mecA* genes (p=0.002).

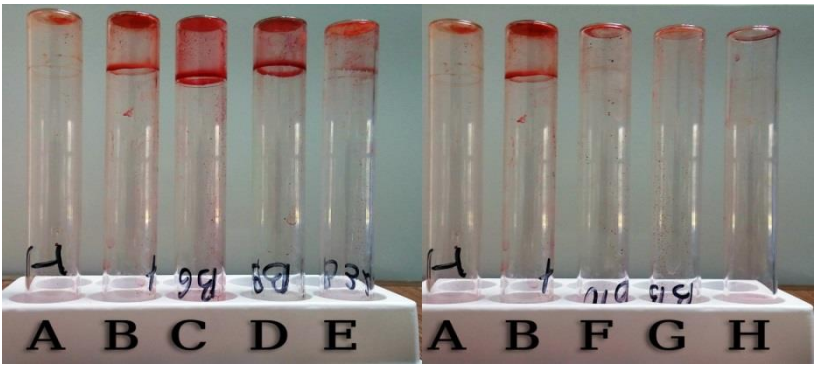

Figure 4. Christensen's test. A, negative control**; B,** positive control**; C** and **D:** strong slime producers**; E,** weak slime producer**; F, G** and **H:** non-producers

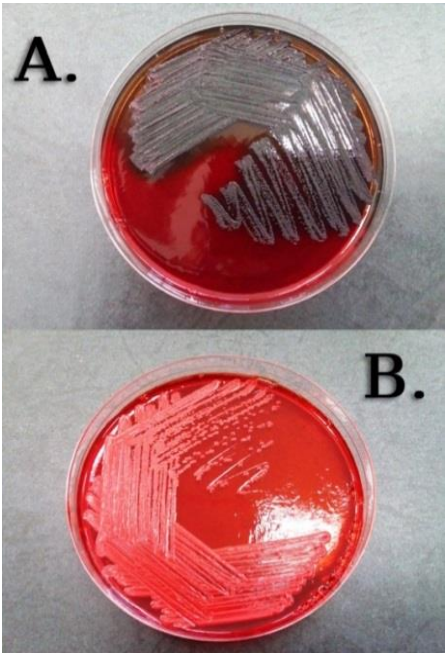

Figure 5. Congo red agar test. A. Positive result**; B.** Negative result**.**

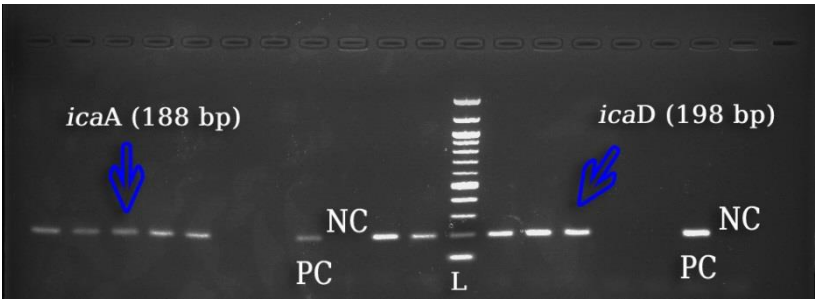

Figure 6. PCR detection of *icaA* and *icaD* genes. PC, positive control (*S. epidermidis* ATCC 35984**); NC,** negative control **(***S. epidermidis* ATCC 12228**); L,** 100 bp DNA marker

Table 6. Results of phenotypic and PCR tests for slime production in 21 clinically significant *Staphylococcus* spp. from blood cultures of patients after HSCT.

Bacterial isolates	*ica*A	*ica*D	CRA	TT	*mec*A	Susceptibility to FOX*
S. epidermidis, n=4	**(+)**	**(+)**	**(+)**	**(+)**	**(+)**	R
S. haemolyticus, n=1	**(+)**	**(+)**	**(+)**	**(+)**	**(+)**	R
S. epidermidis, n=1	**(+)**	**(+)**	(-)	**(+)**	**(+)**	R
S. epidermidis, n=2	**(+)**	**(+)**	**(+)**	(-)	**(+)**	R
S. epidermidis, n=2	**(+)**	**(+)**	(-)	(-)	**(+)**	R
S. haemolyticus, n=1	(-)	(-)	(-)	**(+)**	**(+)**	R
S. hominis, n=1	(-)	(-)	(-)	**(+)**	**(+)**	R
S. aureus, n=1	(-)	(-)	**(+)**	(-)	(-)	S
S. aureus, n=1	(-)	(-)	(-)	**(+)**	(-)	S
S. aureus, n=1	(-)	(-)	**(+)**	(-)	(-)	S
S. epidermidis, n=3	(-)	(-)	(-)	(-)	**(+)**	R
S. haemolyticus, n=2	(-)	(-)	(-)	(-)	**(+)**	R
S. hominis, n=1	(-)	(-)	(-)	(-)	**(+)**	R

CRA - Congo red agar test; **TT –** Christensen's test in a tube**; FOX -** cefoxitin **(***indicator of methicillin resistance**)**

Discussion

Bacterial bloodstream infections in patients after HSCT are often associated with the presence of CVC or damage to mucous membranes/skin. The use of CVC leads to a disruption of the integrity of the skin, with the subsequent colonization of the foreign body most often by resident skin microflora, such as CoNS, bacteria with weak virulent potential (*S. epidermidis, S. hominis, S. haemolyticus*, etc.). Colonization of CVC is an important risk factor for catheter-related infections and bacteremia. CoNS and especially *S. epidermidis* are among the most commonly isolated bacteria, being responsible for approximately 25% of all described episodes of bacteremia in patients with HSCT, while more virulent species (*Staphylococcus aureus*) are isolated at a much lower rate (5%) (*Mikulska M, 2014*). In the current study, bacteremias, associated with different types of staphylococci were documented at 46.7%, dominating slightly over the Gram - negative ones.

It is believed that the increased number of this type of infection is associated with the introduction of biopolymers into the medical practice (polyvinyl chloride, polyethylene, polyurethane, etc.) and their frequent use in high-risk patients (neutropenic, transplanted, etc.) (*Petrelli D, 2006*). CoNS's ability to adhere to the surface of artificial medical devices as a result of slime production is their most significant virulence factor that mediates the development of these infections (*Fey PD, 2011*). In addition, slime increases bacterial resistance to antimicrobial agents and the patient's immune system, causing difficult-to-eliminate infections (*Klingenberg C, 2005; Pourmand MR, 2011*). A number of studies have shown that in the initial phase of microbial attachment to the artificial surface, polysaccharide intercellular adhesin (PIA), mediated by the *ica* operone, which consists of four main genes (*icaA, icaB, icaC, icaD*), plays a direct role (*Fitzpatrick F, 2005; Fey PD, 2011*). In laboratory practice, various phenotypic and molecular-genetic methods have been introduced to detect slime production, such as Congo red agar, Christensen's test, PCR, etc. (*Christensen GD, 1982; Freeman DJ, 1989*).

In addition, a large proportion of slime-producing *S. epidermidis*, associated with catheter-related infections, are identified as carriers of genes mediating methicillin resistance (*Kozitskaya S, 2005*). Many authors report a higher rate of methicillin – resistant CoNS, causing bacteremia than for those caused by MRSA (*Hong J, 2013; Mikulska M, 2014; Weisser M, 2017*).

Due to the high relative share of bacteremias caused by representatives of the genus *Staphylococcus* in the studied group, we set the goal of investigating by various methods the ability to produce slime by

these isolates as their main factor of pathogenicity and virulence. Among the collection of twenty-one clinically significant *Staphylococcus* spp. isolates, obtained from blood cultures of 17 patients, all with CVC and febrile neutropenia, as slime producers by two phenotypic and one molecular-genetic methods, a total of 15 isolates (71.4%) were detected. Thirteen isolates (61.9%, 9 CoNS and 3 *S. aureus*) were documented as producers of slime by phenotypic CRA and/or TT. Close to our results were reported by Prasad and Hussein, who identified between 65% - 69% slim-producing *S. epidermidis* by CRA from a large number of tested isolates (*Prasad S, 2012; Hussein RM, 2018*).

In the current study, ten CoNS (47.6%) carried both *icaA* and *icaD* genes. In line with our results, Pinheiro et al. detected *icaA/icaD* at 43.9% (*Pinheiro L, 2014*). The formation of biofilm is related to the production of PIA, polysaccharide, which mediates the initial stages of slime production. PIA is a product of *icaADBC* operon, consisting of four genes. Among them, *icaA* and *icaD* are the most important. Recent studies have shown a strong link between the presence of these genes and the formation of a large amount of slime (*El-Mahallawy HA, 2009*). Other authors also found the *icaC* gene important because its co-expression with *icaA* and *icaD* leads to the synthesis of a longer oligomer that acts as a starting point for attaching polysaccharide residues. The role of *icaB* is not yet clear, but it is most likely related to the synthesis of secretory protein not directly related to PIA (*Oliveira A, 2010; Gowrishankar S, 2016*).

In our study, three PCR positive *S. epidermidis* isolates demonstrated a lack of slime production by the CRA method. In addition, four *ica* positive *S. epidermidis* had a negative TT. Similar results have been reported in other studies (*Liberto MC, 2009*). It is believed that despite the lack of slime production, the presence of *ica* genes determines microorganisms as potential producers. Ruzicka et al. report that 20% of their *ica* operon carriers do not produce slime phenotypically (Ruzicka F, 2004). Liberto et al. undertake a study to demonstrate the presence of mRNA as a product of *ica* operon expression. They found five *icaA/icaD* positive isolates lacking phenotypic expression. No *icaA* mRNA and/or *icaD* mRNA were found in four of them, which may explain the lack of slime production (*Liberto MC, 2009*). Other authors have suggested that the lack of *icaC* in some *icaA* positive staphylococci may lead to a lack of slime production (*Ziebuhr W, 1999*).

Slime production has been shown to be linked to other factors also. Zmantar et al. demonstrate that the environmental conditions in which bacteria are incubated can affect the slime production in *ica* positive staphylococci (anaerobic conditions, increased amount of NaCl, etc.)

(*Zmantar T, 2008*). In our study, the *icaA* and *icaD* genes were not found in 5 isolates that had positive phenotypic tests (CRA and/or TT). We assume that other mechanisms are related to slime production in these staphylococci. Some authors recommend searching for *aap*, *atlE* and *bhp* genes, associated with an alternative route of slime production *(Petrelli D, 2006; Oliveira A, 2010).*

In the current work, 10 staphylococcal isolates are carriers of both the *mecA* gene and the *icaA/icaD* genes. Similar results have been reported by other authors, and it is believed that the co-expression of *ica* and *mecA* genes is a sign of increased virulence and resistance in *Staphylococcus* spp. (*Petrelli D, 2006; Zhou S, 2013*). *mec*, mediated methicillin resistance among staphylococci is a major health problem, since a large proportion of isolates carrying these genes are found in the hospital environment and colonize patients, and do not respond to the most commonly used in the medical practice β-lactams (*Mathur T, 2006; Rocchetti TT, 2018*). In this sense, when initiating empirical therapy in the case of bacteremia, associated with CoNS, account should be taken of the fact of the wide distribution of methicillin resistance among these staphylococcal species and to start treatment with antibiotics that more easily penetrate through the slime (ciprofloxacin, rifampicin).

The predominant bacterial species in this study is *S. epidermidis*, and most *icaA/icaD* positive isolates belong to the species *S. epidermidis* (n=9, 42.9%). These results confirm *S. epidermidis* as the most commonly associated with catheter-related infections microorganism due to its ability to produce slime and acquire resistance to β-lactams (*Zhou S, 2013*).

In this study, we found a statistically significant correlation between the *icaA/icaD* carrier and the *mecA* gene. Like our data, Zhou et al. observed a statistically significant relationship between the *ica* carriers and the *mecA* gene ($p<0.05$) (*Zhou S, 2013*). In another study, Cafiso et al. found that *ica* positive staphylococci were more likely to demonstrate resistance to various antimicrobials (oxacillin, aminoglycosides, fluoroquinolones, macrolides and sulfonamides) than the *ica* negative ones. They report a strong link between the presence of *mecA* and *ica* operon ($p<0.05$) (*Cafiso V, 2004; Shrestha LB, 2018*). These data are in line with our results that *ica* gene carriers are potentially more resistant to the effect of antimicrobial agents.

Conclusion

Our study documents a high relative share of slime-producing or potential slime-producing CoNS (71.4%), associated with *ica* genes, among which the most common species is *Staphylococcus epidermidis*. In order to

detect alternative routes of production of slime, it is advisable to use phenotypic and genetic methods of examination. All *ica*-positive isolates are also methicillin resistant (*mecA* positive), which is associated with difficult-to-treat and eradicate isolates.

4.5. ETIOLOGICAL SPECTRUM OF INVASIVE MYCOTIC INFECTIONS

For the period 2019 - 2021 among the 74 patients, invasive bacterial and mycotic infections were diagnosed in 26 (35.1%). Invasive mycotic infection was documented in 15.3% (n=4) of these patients, with blood infection reported in one (3.8%) (see section 4.2) and invasive pulmonary aspergillosis (IPA) - in three patients (11.5%). Invasive mycotic infections caused by other mycotic agents (*Mucorales* sp., *P. jirovecii*, *Cryptococcus* sp. etc.) were not documented.

Following the criteria for inclusion (set out in the section "Materials and methods"), in the period 2019 - 2021 aspergillus galactomanan antigen (GM) in serum and/or BAL were investigated in a total of 33 patients after HSCT (44.6%) (Table 7). The age of patients varies between 14 years - 63 years, with twelve (36.4%) of them being women and 21 (63.6%) men. Six (18.2%) of the patients had autologous, and 27 (81.8%) had allogeneic HSCT. Piperacillin/tazobactam therapy was initiated in 4 patients prior to GM testing and 29 received quinolone agents or trimethoprim/sulphamethoxazole.

Table 7. Distribution of the tested patients, depending on their diagnosis.

Underlying disease	n (%)
Acute myeloblastic leukemia	8 (24.2)
Non-Hodgkin lymphoma	7 (21.2)
Hodgkin’s disease	6 (18.2)
Acute lymphoblastic leukemia	5 (15.2)
Multiple sclerosis	3 (9.1)
Aplastic anemia	2 (6.1)
Multiple myeloma	1 (3.0)
Myelodisplastic syndrome	1 (3.0)
Total	**33 (100.0)**

Of the 33 patients studied, 74 clinical materials were obtained: 70 serum and 4 samples of BAL. Depending on the results of the GM test, patients are subdivided into 3 groups (Table 8).

A positive GM score was reported in 3 patients (9.1%) (group I), with optical density (OD) measured in serum samples ranging between 1.6 and 3.37. In one of these patients, BAL was also studied, which was interpreted as positive.

Patients who reported a negative GM score (16 serum and 1 BAL samples) but with data from the imaging study, susceptive of IPA (n=5) (group II), were interpreted as possible cases of IPA. Patients with a negative GM test and lack of clinical data (incl. imaging findings) were categorized as "no IPA" (n=25) (group III). None of the cases investigated were categorized as proven. Molds of the genus *Aspergillus* were not isolated when cultivating BAL samples.

Table 8. Results of the *Aspergillus* galactomanan antigen test.

Patients, count (n)	**Tested serum samples (n)**	**Testes BAL samples (n)**	**Imaging findings**	**Category (probable, possible, proven, no IPA)**
group I n=3 **(positive)** (patients with ID 21, 13, 33 - for more details check table 9)	n=5* positive (of 15 samples) (OD=1.02 – 3.37)	n=1 positive (OD=2.09)	+	Probable
group II n=5 **(negative)**	n=16* (negative)	n=1 (negative)	+	Possible
group III n=25 **(negative)**	n=39* (negative)	n=2 (negative)	-	no IPA

ID - patient identification number**, ***More than one serum sample per patient has been examined**; OD -** optical density**; "+",** presence of a finding**; "-",** lack of finding**.**

No antimycotic therapy was initiated in patients categorized as a possible case of IPA (Group II). On follow-up of these patients, the GM test remained negative.

Ethiotropic therapy with voriconazole with an initial dose of 6 mg/kg and maintenance – 4 mg/kg every 12 hours was initiated in 3 patients with a positive GM score in serum sample and/or BAL (ID 13, 21, 33) identified as probable cases of IPA (Group I) (Table 9).

In a patient with ID 21, two episodes of elevated serum GM (8 serum samples tested) (Tables 8 and 9) were documented. During the first episode (01.07.2020), no IPA-specific imaging data were found. According to the new recommendations of the European Organization for Research and Treatment of Cancer and the Consortium for Training and Research of the Mycoses Research Group, this case is categorized as "no IPA", but treatment with voriconazole has nevertheless been initiated. When monitoring the results, the test became negative and the therapy was discontinued, since it was assumed that serum GM was false-positive, influenced by the mucositis of the digestive system, the β – lactam antibiotic use, the infusions of biological products and electrolytes. The second episode occurred 4 months (09.11.2020) after the first, being confirmed by imaging studies and accompanied by clinical symptoms. It is classified as a probable case and treatment with voriconazole has been initiated immediately in the appropriate dosage.

Table 9. Characteristic of patients with a positive result for *Aspergillus* galactomanan.

Patient					**Data**			
ID	**Sex**	**Age**	**Underlying disease**	**HSCT type**	**Serum**	**BAL**	**WBC count**	**DD**
21	female	14 y.	ALL	AHSCT	01.07.20 (+, 2.31 ODI) 09.11.20 (+, 1.6 ODI)	-	**0.14 x 10^9** **0.64 x 10^9**	19.11.20
13	female	39 y.	HD	AHSCT	31.08.20 (+, 2.73 ODI)	-	**0.87 x 10^9**	01.09.20
33	male	47 y.	AML	AHSCT	11.10.21 (+, 3.37 ODI) 19.10.21 (+, 1.02 ODI)	04.11.21 (+, 2.9 ODI)	**0.01 x 10^9** 16.17 x10^9 6.57 x 10^9	01.12.21

ID – identification number; **HSCT** - hematopoietic stem-cell transplantation; **BAL** - bronchoalveolar lavage; **WBC** - white blood cells; **DD** – death date; **ODI** - optical density index; **ALL** - acute lymphoblastic leukemia; **HD** – Hodgkin's disease; **AML** - acute myeloblastic leukemia; **AHSCT** – allogeneic hematopoietic stem-cell transplantation.

Fatal outcome was documented in all three patients, with IPA identified as the main cause of death in two of them.

Discussion

Aspergillosis is an exogenous mycotic infection caused by widespread molds belonging to the genus *Aspergillus*, with *A. fumigatus*, *A. flavus*, *A. niger* and *A. terreus* are the most important and clinically significant species (*Denning DW, 1998*). Infections, associated with *Aspergillus* spp. can range from local skin forms to severe high-mortality infections such as IPA (*Kousha M, 2011; Bozhkova M, 2018*). Very rarely these molds can cause invasive diseases in immunocompetent individuals (*Denning DW, 1998; Gabrovska N, 2019*).

The main risk factors for IPA are prolonged neutropenia (< 500 cells/μl for more than 10 days), HM, chemotherapy, HSCT, long-term (> 3 weeks) and high-dose corticosteroid therapy and progressive AIDS (*Kousha M, 2011*).

The detection of GM antigen of *Aspergillus* and beta-D-glucan by immunoenzyme and molecular-genetic methods such as PCR are currently used to diagnose IPA (*White P, 2015*). These methods possess high specificity and sensitivity. The GM antigen test, relatively cheap and fast, is specifically adapted for the diagnosis of IPA in patients with neutropenia (*Viscoli C, 2004*).

Invasive infection caused by *Aspergillus* molds is one of the most severe complications in patients with neutropenia, HM and those with HSCT. Early detection and timely launch of etiological therapy are essential for the outcome of the disease and therapeutic success (*Shannon VR, 2015*).

The incidence of IPA in patients with HM and HSCT varies, with average reaching 12% - 15% (*Wald A, 1997; Marr KA, 2002; Fukuda T, 2003; Morgan J, 2005; Van de Peppel R, 2018*). In our study, the incidence of IPA in the studied patients was 11.5% (n=3).

In a large-scale retrospective multicenter study conducted to better characterize invasive mycotic infections in patients with hematological malignancies without HSCT, it was reported that the highest percentage (94% of cases) of IPA was observed in patients with acute leukemia (myeloblastic and lymphoblastic) (*Pagano L, 2006*). The same study reported that patients with acute leukemia had a higher risk of developing IPA and its complications. Like these results, we found that 66.7% (n=2) of our patients with a positive GM test were patients with acute leukemia.

In this study, all patients (n=3) with a positive GM test underwent allogeneic HSCT. According to the literature, the incidence of IPA in

individuals with HSCT is variable. Depending on the transplant center, the incidence of IPA among patients undergoing allogeneic HSCT varies from 4 to 24%, with most studies reporting 8-15%. Unlike allogeneic HSCT, the incidence of IPA after autologous HSCT was significantly lower, ranging from 1 to 2% (*Wirk B, 2009*). IPA-related mortality in HSCT recipients can reach a dramatic 50-80% (*Lin SJ, 2001*).

A false-positive result was found in this study. This patient was followed up, retested and later reported a negative result. One possible explanation for this phenomenon is the fact that at the time of microbiological examination the patient was treated with piperacillin/tazobactam, mucositis of the digestive system has been documented and there were numerous infusions of biological products and electrolytes. Like our observations, other authors report false-positive results, associated with parenteral administration of piperacillin/tazobactam or amoxicillin/clavulanic acid (*Mennink-Kersten MA, 2004; Walsh TJ, 2004; Mattei D, 2004*). Other causes of false-positive tests have also been described. Besides *Aspergillus* spp. other clinically significant fungi (*Fusarium* spp, *Penicillium* spp, *Histoplasma capsulatum*) also possess GM in their cell wall. In cases of infection caused by these organisms due to cross-reactivity, serum GM test can be false-positive (*Huang YT, 2007; Wheat LJ, 2007; Tortorano AM, 2012*). Alban Aubry et al. recommend monitoring twice a week patients with a positive GM test, but without clinical symptoms of IPA to confirm or reject the diagnosis. These authors recommend a 5-day interval between discontinuation of antimycotic therapy (if initiated) and the next test of GM. The same research group created a kinetic model and found that the half-life of GM from contaminated β-lactam antibiotics in serum was 2.4 days, while the mean time to become negative serum GM test was 5.5 days (*Aubry A, 2006*).

Only one of the patients studied tested positive both in serum sample and BAL. Numerous studies have been conducted on the clinical significance of the GM test in BAL (*Bergeron A, 2010; Fisher C, 2014; Boch T, 2016; Marchesi F, 2019).* Since the lung is the site of the primary infection, it is believed that the appearance of GM in BAL precedes its appearance in the bloodstream (*Steinbach W, 2012*). Bergeron et al. conducted a study to detect GM in BAL samples obtained from high-risk patients with HM and reported average sensitivity (57.6%) and high specificity (95.6%) of the ELISA test (*Bergeron A, 2010*). In a comparative study, Boch et al. reported a much higher sensitivity for GM testing in BAL (85%) compared to serum test (23%) and specificity of 88% for both tests (*Boch T, 2016*). According to ESCMID-ECMM-ERS guidelines of 2017, the detection of GM in BAL samples has excellent credibility, reaching

sensitivity and specificity of 100% and 90.4% respectively when using appropriate optical density indices (*Ullman A, 2018*). Other authors demonstrate in experimental conditions that GM values in BAL are increased earlier after the onset of IPA than in serum (*Hope W, 2010*). The advantage of the GM test in BAL is that antimycotic therapy initiated before microbiological examination does not affect the test and does not lead to false-negative results (*Fisher C, 2014*). Disadvantages are mostly associated with false positive results in cases when β-lactam antibiotics are used or in cases of colonization of the respiratory tract, but without the development of a clinical disease (*Husain S, 2008*). Fisher et al. report that the study of both serum and BAL samples for GM and their interpretation with clinical data can significantly improve the diagnosis of IPA (*Fisher C, 2014*).

It should be noted, however, that performing bronchoscopy for the purpose of examining BAL in critically ill patients or patients with HM is associated with a high risk of complications such as bleeding, respiratory failure or pneumothorax (*Svensson T, 2017*).

In addition to the immune-enzyme method of detecting *Aspergillus* GM, the detection of beta-D-glucan by the same method (specificity 89.4% and sensitivity 76.9%), as well as PCR are also used to diagnose invasive mycotic infections (*White P, 2015*). Many studies demonstrate the advantage of the combined use of these methods (*Reinwald M, 2012; White PL, 2013; Hoenigl M, 2014*). Hoenigl et al. reported 100% sensitivity and specificity of 95% – 98% when the GM test was used in combination with PCR (*Hoenigl M, 2014*).

Several guidelines for the prevention and treatment of invasive mycotic complications (incl. IPA) in patients with HSCT have been published and used in clinical practice: the International Guidelines for the Prevention of Infectious Complications in HSCT recipients (*Tomblyn M, 2009*), guidelines for the use of antimicrobials in neutropenic cancer patients of the Infectious Diseases Society of America (IDSA) (*Freifeld AG, 2011*) and IDSA guidelines for the diagnosis and treatment of aspergillosis (*Patterson TF, 2016*). Two main strategies of management of IPA have been adopted: 1) initiation of primary prophylaxis and 2) lack of prevention, but patients are tested at least twice a week with the help of laboratory biomarkers. The centre's experience usually determines which of the two strategies will be used. The choice of antifungal agents for primary prophylaxis depends on whether the individual is neutropenic or has a restored neutrophil count, the presence of GVHD or whether immunosuppressive therapy has been initiated. In individuals with neutropenia and allogeneic HSCT, posaconazole (200 mg three times a day) or voriconazole (200 mg twice a day) is recommended, while in patients

with severe GVHD and immunosuppressive therapy, posaconazole, vorcionazole or itraconazole (400 mg/day) are the agents of choice.

In any high-risk patient, the occurrence of symptoms suspicious of IPA should be an indication for the rapid initiation of adequate antimycotic therapy. In patients with allogeneic HSCT and neutropenia, initial therapy with voriconazole (2x6 mg/kg IV on day 1 and 2-4 mg/kg IV from day two) is recommended. If treatment with voriconazole cannot be initiated, liposomal amphotericin B as an alternative antimycotic agent is recommended. Also liposomal amphotericin B is prescribed as part of prevention schemes (*Ullman A, 2018*). In our study, patients at risk of infectious complications were routinely tested for IPA without initiation of prophylaxis. Antifungal therapy with voriconazole was initiated in 3 patients identified as cases of probable IPA.

Despite the introduction of some new agents with anti-mold activity, IPA-related mortality by literature data remains high, ranging from 50% to 90% (*Kousha M, 2011*). In this study, IPA was accepted as the main cause of death in two of the three patients who tested positive for GM test. In a prospective study from 2004 to 2009, Nicolle et al. found that the mortality rate at the first and third months in patients with IPA and HM was 13% and 43% respectively (*Nicolle MC, 2011).* In addition, another prospective Italian study involving patients with allogeneic HSCT reported a mortality rate of 46.3% after IPA (*Girmenia C, 2014*).

Conclusion

IPA is an infectious disease with severe course and high mortality, which should always be suspected in patients with neutropenia, continuous fever, typical imaging findings and lack of response to antibacterial therapy. Rapid diagnosis and immediate initiation of adequate antimycotic therapy are crucial for the course and outcome of the disease. The current study on invasive mycotic infections in patients after HSCT for the period 2019 – 2021 documents incidence of 11.5% (n=3) and mortality 66.7% (n=2). Our results demonstrate that *Aspergillus* GM ELISA is a reliable method that can be used both to diagnose and monitor a patient's response to ethiotropic therapy. In the absence of contraindications, simultaneous examination of serum and BAL for the detection of *Aspergillus* GM is recommended.

4.6. FECAL COLONIZATION BY FUNGI AND HARD-TO-THREAT BACTERIA: SPECIES DIVERSITY AND SUSCEPTIBILITY TO ANTIMICROBIAL AGENTS

In order to monitor the fecal colonization status of patients with fungi and problematic bacteria as one of the important risk factors for potential and severe infectious complications during the studied period, a total of 242 fecal samples from 74 patients with HSCT were examined. As problematic microorganisms for treatment, we adopted bacterial isolates, demonstrating resistance to the third and fourth generation cephalosporins, carbapenems and glycopeptides (usually with a phenotype of multipledrug-resistant) and *Stenotrophomonas maltophilia*.

The implementation of microbiological fecal screening is part of the protocol introduced in the clinic for monitoring the pretransplantation colonization status of the mucous membranes of patients and, if necessary, in the post-transplant period. A total of 65 microbial isolates were isolated and identified from samples of 36 of the 74 patients studied (49%): 17 mycotic and 48 bacterial isolates meeting the definition of "problematic". In 20 (55.6%) patients were isolated only bacteria, in 8 (22.2%) – only fungi, and in 9 (25%) both fungi and bacteria. The species diversity of colonizing agents is presented in Table 10.

Table 10. Species diversity of problematic microbial agents, colonizing the gastrointestinal tract of 36 patients after HSCT in the period 2019-2021.

Microorganisms, isolated from fecal samples	**n (%)**
Gram – negative bacteria	**33 (50.8)**
E. coli	10 (30.3)
Enterobacter cloacae	7 (21.2)
Klebsiella pneumoniae	4 (12.1)
Pseudomanas putida	4 (12.1)
Stenotrophomonas maltophilia	3 (9.0)
Pseudomonas aeruginosa	2 (6.0)
Pseudomonas mendocina	1 (3.0)
Pseudomonas composti	1 (3.0)
Serratia marcescens	1 (3.0)

Gram – positive bacteria	**15 (23.0)**
Enterococcus faecium	15 (23.0)
Yeasts	**17 (26.1)**
Candida glabrata	7 (41.2)
Candida albicans	3 (17.6)
Candida tropicalis	2 (11.8)
Candida. krusei	2 (11.8)
Candida kefyr	1 (5.8)
Candida dubliniensis	1 (5.8)
Candida parapsilosis	1 (5.8)
Total	**65 (100.0)**

4.6.1 Susceptibility of fecal microbial isolates to antimicrobial agents

Sensitivity to a set of antimicrobial drugs of 33 Gram - negative and 15 Gram - positive bacterial isolates has been studied by the automated Phoenix 100 system, microdiluation MIC method for colistin, vancomycin, teicoplanin, and that of 17 mycotic isolates by microdilution method and E-test.

Gram – negative bacteria

The documented resistance among Gram - negative isolates, from the order *Enterobacterales* (n=22) in descending order is as follows: 100% for cefotaxime/ceftazidime (n=22) and cefepime (n=22) > 68.1% gentamicin (n=15) > 54.5% ciprofloxacin (n=12) > 45.5% piperacillin/tazobactam (n=10) > 41% for levofloxacin (n=9) and trimethoprim/sulfamethoxazole (n=9) > 4.5% for amikacin (n=1), ceftazidime/avibactam (n=1) and meropenem/imipenem (n=1).

Among the isolates *Pseudomonas* spp. (*P. putida*, n=4; *P. aeruginosa*, n=2; *P. mendocina*, n=1; *P. composti*, n=1) a complete lack of sensitivity to all β-lactam antibiotics (incl. carbapenems) was found. Increased resistance is documented to quinolones, aminoglycosides and ceftazidime/avibactam. In all three *S. maltophilia* isolates was found lack of susceptibility to trimethoprim/sulfamethoxazole.

Resistance to colistin has not been demonstrated in the whole Gram - negative isolates group (n=32).

Gram – positive bacteria

All 15 isolates, initially identified as vancomycin-resistant *E. faecium* by Phoenix BD, demonstrated a lack of sensitivity to all β-lactam antibiotics, aminoglycosides and quinolones. The sensitivity to linezolid was fully preserved. In these isolates, the microdilution method for determining the MIC of vancomycin and teicoplanin (Erba Lachema, Czech Republic) was used as confirmatory. All isolates demonstrated a lack of sensitivity to both agents (> 16 μg/ml). In order to confirm the species affiliation, a PCR for detection of *ddl* genes, encoding specific D-alanine-D-alanine ligases in *E. faecium* and *E. faecalis* is applied. For all 15 isolates, a 658 bp product, corresponding to $ddl_{E.\ faecium}$ is amplified (Figure 7).

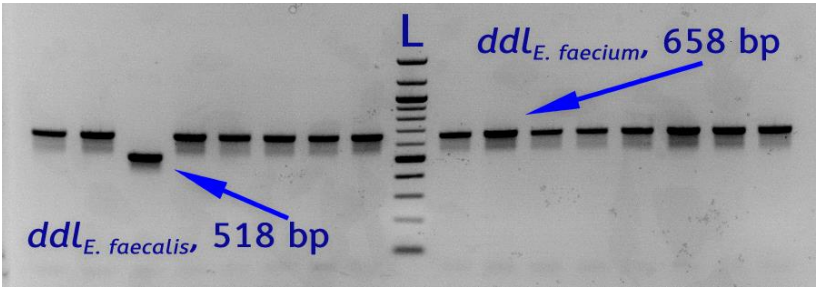

Figure 7. Multiplex PCR for type identification of *Enterococcus* spp. L, marker 100 bp.

Yeasts

Seventeen mycotic agents, isolated from fecal samples of 17 patients (Table 11) are subjected to tests to determine their sensitivity to antimycotic drugs by a microdiluation MIC method and an E-test.

Among isolates for which generally accepted MIC values were reported, the highest resistance was observed to fluconazole (70.6%, n=12), followed by voriconazole (37.5%, n=6), itraconazole (31.3%, n=5) and anidulafungin (26.7%, n=4).

Against two of the antimycotics (caspofungin and micafungin) a lack of sensitivity was not found. For two other antimycotic agents (isavuconazole and flucytosine) for which no standard was adopted, MICs ranging from 0.01 to 32 μg/ml were demonstrated.

Candida albicans isolates (n=3) demonstrated a fully preserved sensitivity to all antimycotics.

In four isolates (*C. glabrata*, n=2; *C. tropicalis*, n=1; *C. krusei*, n=1) (23.5%) resistance was demonstrated simultaneously to antimycotics of the azole group (fluconazole, voriconazole, itraconazole) and echinocandins (anidulafungin). The antimycotic sensitivity of isolates is presented in Tables 11 and 12.

Table 11. Sensitivity to antimycotic drugs of more often isolated representatives of the genus *Candida*.

Antimycotics		***C. glabrata*** (n=7)	***C. albicans*** (n=3)	***C. krusei*** (n=2)	***C. tropicalis*** (n=2)
Fluconazole	**MIC µg/ml**	32 - > 256	0.12 – 0.50	NA	256
	AC MIC µg/ml	0.001 - 16	2 - 4		2 - 4
	S (n, %)	0.0	n=3, 100%		0.0
	R (n, %)	n=7, 100%	0.0		n=2, 100%
Voriconazole	**MIC µg/ml**	0.25 - 2	0.03 – 0.04	0.25 - 2	32
	AC MIC µg/ml	1*	0.06 – 0.25	1*	0.12 – 0.25
	S (n, %)	n=5, 71.4%	n=3, 100%	n=1, 50%	0.0
	R (n, %)	n=2, 28.6%	0.0	n=1, 50%	n=2, 100%
Itraconazole	**MIC µg/ml**	0.5 - > 16	0.01 – 0.06	0.25 – 0.5	> 16
	AC MIC µg/ml	2*	0.06	1*	0.125
	S (n, %)	n=5, 71.4%	n=3, 100%	n=2, 100%	0.0
	R (n, %)	n=2, 28.6%	0.0.	0.0	n=2, 100%
Isavuconazole	**MIC µg/ml**	0.03 – 0.75	0.01 – 0.03	0.19 – 0.25	0.38 - > 32
	AC MIC µg/ml	ND	ND	ND	ND
	S (n, %)	NA	NA	NA	NA
	R (n, %)	NA	NA	NA	NA
Flucytosine	**MIC µg/ml**	0.03 – 0.12	0.01 – 0.06	0.12 - 32	0.06 – 0.12
	AC MIC µg/ml	ND	ND	ND	ND
	S (n, %)	NA	NA	NA	NA
	R (n, %)	NA	NA	NA	NA
Caspofungin	**MIC**	0.03 – 0.06	0.01 – 0.03	0.03 - 1	0.06 – 0.25

	µg/ml				
	AC MIC µg/ml	0.12 – 0.5**	0.25 – 1**	0.25 – 1**	0.25 – 1**
	S (n, %)	n=7, 100%	n=3, 100%	n=2, 100%	n=2, 100%
	R (n, %)	0.0	0.0	0.0	0.0
Micafungin	**MIC µg/ml**	0.008 – 0.01	0.008 – 0.01	0.01 – 0.12	0.01
	AC MIC µg/ml	0.03	0.016	0.25*	0.06*
	S (n, %)	n=7, 100%	n=3, 100%	n=2, 100%	n=2, 100%
	R (n, %)	0.0	0.0	0.0	0.0
Anidulafungin	**MIC µg/ml**	0.02 – 0.09	0.01	0.04 – 0.12	0.04 – 0.12
	AC MIC µg/ml	0.06	0.03	0.06	0.06
	S (n, %)	n=5, 71.4%	n=3, 100%	n=1, 50%	n=1, 50%
	R (n, %)	n=2, 28.6%	0.0	n=1, 50%	n=1, 50%

AC – values by standard**; ND –** no specific MIC**; ***ECOFF values**; ****CLSI values**;** **NA,** not applicable**; S –** sensitive**; R –** resistant**; MIC –** minimum inhibitory concentration;

Table 12. Sensitivity to antimycotic drugs of the rarer representatives of the genus *Candida*.

Antimycotics		***C. kefyr*** (n=1)	***C. parapsilosis*** (n=1)	***C. dubliniensis*** (n=1)
Fluconazole	**MIC µg/ml**	0.5	1	> 256
	AC MIC µg/ml	1*	2 - 4	2 – 4*
	S (n, %)	n=1	n=1	0.0
	R (n, %)	0.0	0.0	n=1
Voriconazole	**MIC µg/ml**	0.01	0.01	32
	AC MIC µg/ml	ND	0.12 – 0.25	0.12 – 0.25*
	S (n, %)	NA	n=1	0.0
	R (n, %)	NA	0.0	n=1
Itraconazole	**MIC µg/ml**	0.12	0.03	> 16
	AC MIC µg/ml	ND	0.125	0.06*
	S (n, %)	NA	n=1	0.0
	R (n, %)	NA	0.0	n=1

Isavuconazole	**MIC µg/ml**	0.003	0.04	0.02
	AC MIC µg/ml	ND	ND	ND
	S (n, %)	NA	NA	NA
	R (n, %)	NA	NA	NA
Flucytosine	**MIC µg/ml**	8	0.06	0.12
	AC MIC µg/ml	ND	ND	ND
	S (n, %)	NA	NA	NA
	R (n, %)	NA	NA	NA
Caspofungin	**MIC µg/ml**	0.03	0.03	0.03
	AC MIC µg/ml	ND	2 – 8**	ND
	S (n, %)	NA	n=1	NA
	R (n, %)	NA	0.0	NA
Micafungin	**MIC µg/ml**	0.06	0.06	< 0.008
	AC MIC µg/ml	ND	2	ND
	S (n, %)	NA	n=1	NA
	R (n, %)	NA	0.0	NA
Anidulafungin	**MIC µg/ml**	0.09	0.19	0.02
	AC MIC µg/ml	ND	4	ND
	S (n, %)	NA	n=1	NA
	R (n, %)	NA	0.0	NA

AC – values by standard**; ND** – no specific MIC**; ***ECOFF values**; ****CLSI values**; NA,** not applicable**; S –** sensitive**; R –** resistant**; MIC –** minimum inhibitory concentration**;**

4.6.2 Study on the mechanisms of resistance to strategic antimicrobial drugs (third generation cephalosporins, carbapenems, glycopeptides) by molecular-genetic methods

In the study collection of 48 bacterial isolates, obtained from fecal samples, 30 isolates - *E. coli*, n=10; *E. cloacae*, n=7; *K. pneumoniae*, n=4; *S. marcescens*, n=1; *P. putida*, n=4; *P. aeruginosa*, n=2; *P. mendocina*, n=1; *P. composti*, n=1, demonstrated resistance to third and fourth generation cephalosporins, and 9 were defined as CPR - *P. putida*, n=4; *P. aeruginosa*, n=2; *P. mendocina*, n=1; *P. composti*, n=1; *E. cloacae*, n=1. All 15 *E. faecium* isolates were identified as vancomycin-resistant enterococci (VRE).

With all 48 problematic in terms of antibiotic resistance, fecal screening isolates we performed PCR experiments to detect carriers of genes, mediating the relevant type of resistance: the most common in Gram - negative bacteria, genes encoding broad-spectrum β-lactamases (CTX-M, SHV, TEM type) and carbapenemases (VIM, IMP, KPC, NDM, OXA-48), as well as *vanA*, *vanB* and *vanD* genes, associated with glycopepteptide resistance in isolates *Enterococcus* spp. were tested.

- **PCR experiments to prove genes, encoding CTX-M, SHV and TEM ESBLs in *Enterobacterales* order isolates:**

In 22 (33.8%) bacterial isolates, demonstrating resistance to third-generation cephalosporins (*E. coli*, n=10; *E. cloacae*, n=7; *K. pneumoniae*, n=4; *S. marcescens*, n=1) the following genes were demonstrated: $bla_{\text{SHV-like}}$ (n=4, *K. pneumoniae*), $bla_{\text{CTX-M-like}}$ (n=20; *E. coli*, n=9*; K. pneumoniae*, n=3; *E. cloacae* complex, n=7; *S. marcescens*, n=1), $bla_{\text{TEM-like}}$ (n=14; *E. coli*, n=5; *K. pneumoniae*, n=2; *E. cloacae*, n=6; *S. marcescens*, n=1). In 13 (46.7%) of isolates, more than one gene was identified as follows: $bla_{\text{CTX-M-like}}$ + $bla_{\text{TEM-like}}$ (n=13; *E. coli*, n=4; *K. pneumoniae*, n=2; *E. cloacae*, n=6; *S. marcescens*, n=1); $bla_{\text{CTX-M-like}}$ + $bla_{\text{SHV-like}}$ (*K. pneumoniae*, n=3); $bla_{\text{CTX-M-like}}$ + $bla_{\text{TEM-like}}$ + $bla_{\text{SHV-like}}$ (n=2, *K. pneumoniae*) (Figures 8 and 9).

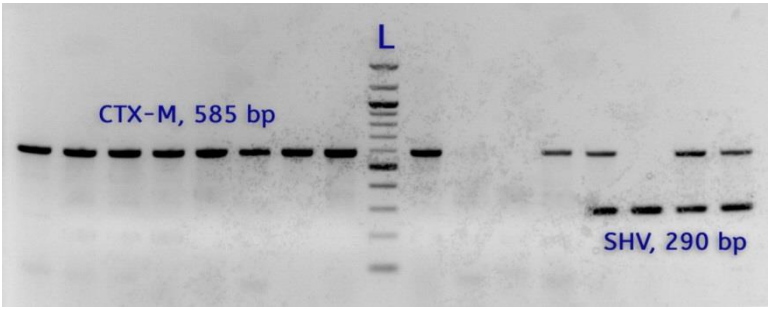

Figure 8. Multiplex PCR to demonstrate CTX-M and SHV in isolates *E. coli* and *K. pneumoniae*. L, marker 100 bp

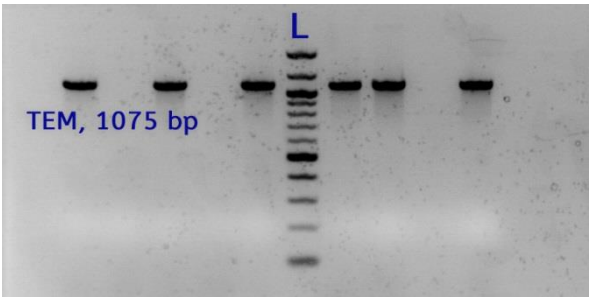

Figure 9. PCR for detection TEM ESBL in *E. coli* isolates. L, marker 100 bp

- **PCR experiments to detect genes, encoding KPC, VIM, IMP, NDM and OXA-48 carbapenemas in isolates of the genus *Pseudomonas* and *Enterobacter cloacae***

Out of a total of 9 CPR fecal screening isolates (*P. putida*, n=4; *P. aeruginosa*, n=2; *P. mendocina*, n=1; *P. composti*, n=1; *E. cloacae*, n=1), 7 resulted in amplification products of 390 bp corresponding to $bla_{\text{VIM-like}}$ and, in the case of two isolates (*P. aeruginosa* and *P. putida*), no amplification product was obtained with any of the primers used, therefore other mechanisms, mediating resistance to carbapenems (Figure 10) were assumed.

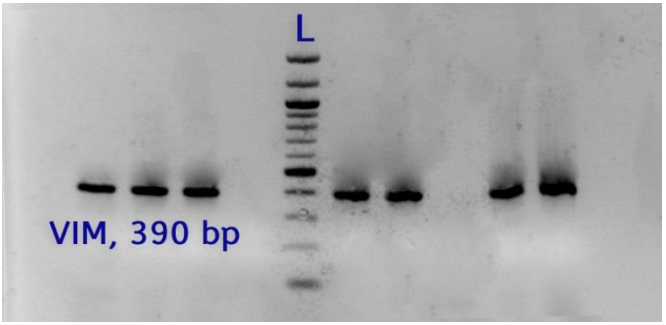

Figure 10. PCR to demonstrate VIM gene in *Pseudomonas* spp. L, marker 100 bp

- **<u>Sequencing of genes, encoding ESBLs and carbapenemases</u>**

Sixteen representative isolates, according to the phenotype of antibiotic resistance and the results of epidemiological typing (*E. coli*, n=4;

K. pneumoniae, n=2; *E. cloacae*, n=2; *S. marcescens*, n=1; *Pseudomonas* spp., n=7) were tested with CTX-M-P1/P2 and TEM A/B, SHV and VIM primers and gave a positive reaction, which confirmed the presence of CTX-M 1-group, SHV-like, TEM-like and VIM-like genes. By nucleotide sequencing, the presence of $bla_{\text{CTX-M-15}}$ (n=5), $bla_{\text{CTX-M-3}}$ (n=3), $bla_{\text{TEM-1}}$ (n=1), $bla_{\text{SHV-1}}$ (n=1), $bla_{\text{SHV-12}}$ (n=1), $bla_{\text{VIM-1}}$ (n=1) and $bla_{\text{VIM-2}}$ (n=6) in the respective isolates is identified.

- **PCR experiments to demonstrate glycopeptide resistance in *Enterococcus faecium* isolates**

All 15 *E. faecium* isolates underwent a screening PCR proving *vanA/vanD* genes (Figure 11). All isolates amplified a product of 610 bp size corresponding to *vanA/vanD*, and the additional multiplex PCR used for *vanA* and *vanB* genes confirmed the presence of *vanA* (Figure 12).

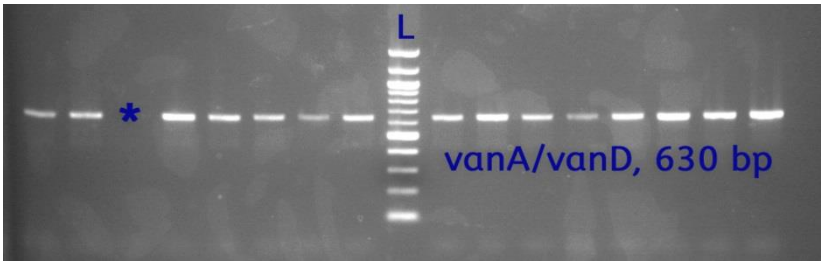

Figure 11. Screening PCR to detect *vanA/vanD* genes. L, marker 100 bp**; *** Negative control *(E. faecalis).*

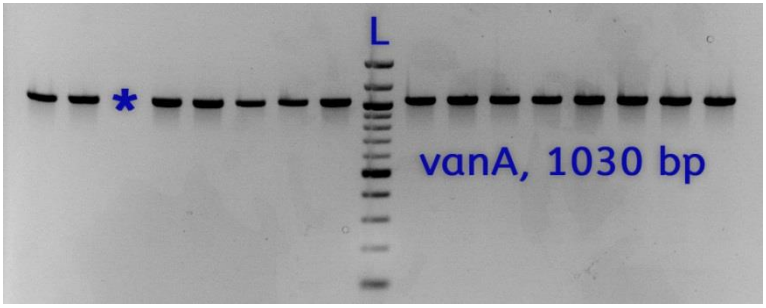

Figure 12. Multiplex PCR for the detection of *vanA* and *vanB* genes. L, marker 100 bp**; *** Negative control (*E. faecalis*).

Discussion

In order to monitor the colonization status of patients included in this dissertation, fecal screening for fungi and for bacteria, demonstrating a profile of multiple resistance was conducted. The results obtained prove a high proportion of patients with intestinal colonization with this type of monitored microorganisms (49%). Similar results were reported by Scheich et al., who found that 53.8% of their patients after allogeneic HSCT had intestinal colonization by microorganisms, exhibiting multiple resistance (*Scheich S, 2018).* Other authors tracking the colonization status of patients after HSCT found a much lower incidence of fecal colonization – 31% (*Bilinski J, 2016).*

Among all the tested bacterial isolates, the dominance of Gram–negative bacteria was found, with the representatives of order *Enterobacterales* being the most numerous (66.7%), with a leading type of *E. coli*. Gram – positive bacteria are represented exclusively by *E. faecium* (23%). Our results differ significantly from those of Scheich and Bilinski, who reported a higher incidence of intestinal colonization with Gram – positive bacteria represented mainly by *E. faecium* - 85.9% and 55% respectively (*Bilinski J, 2016; Scheich S, 2018*).

In addition to bacterial isolates, fungi are also isolated from the fecal samples, all of which are representatives of the genus *Candida*. The largest numbers are representatives of nonalbicans species (82.4%), by pre-raining *C. glabrata* (50%). In a study involving 77 patients after autologous and allogeneic HSCT and tracking mycotic intestinal carrier, colonization was found in 66% of the patients studied, most often isolating *C. albicans* (*Zollner-Schwetz I, 2008*). The incidence of nonalbicans species in the same study was significantly lower (24%), with the dominance of *C. glabrata* (*Zollner-Schwetz I, 2008*). Hamzavi et al. conducted a two-year study among pediatric patients with HM and documented 15.4% intestinal mycotic carriers. With the greatest frequency of *C. albicans* (72%), followed by *C. krusei* and *C. kefyr* (*Hamzavi SS, 2019*). These data differ significantly from the results obtained in our study.

Of all the isolated bacteria twenty-two Gram - negative isolates (46%) were defined as ESBL producers. A three-year study tracking 107 patients after allogeneic HSCT showed 20% intestinal colonization with ESBL producers (*Bilinski J, 2016*). Another survey for the period 2006 – 2016 in Germany, a 20.4% intestinal carrier of ESBL – producing *Enterobacteriaceae* was found among patients with acute myeloid leukemia and subsequent allogeneic HSCT (*Scheich S, 2018*). Among ESBL producers, our study also documented reduced sensitivity to other

antibacterial agents, such as aminoglycosides, sulfonamides and quinolones. Alarming is colonization with bacteria, demonstrating a lack of sensitivity to trimethoprim/sulfamethoxazole (41%), ciprofloxacin/levofloxacin (54.5% - 41%) and piperacillin/tazobactam (45.4%), since these antibacterial agents are commonly used for prevention and empirical therapy in patients with febrile neutropenia. Among aminoglycosides, preserved activity of amikacin (4.5%) was noted, as opposed to that of gentamicin (68.1%). A study of Scheich et al. reported a complete lack of sensitivity to quinolones among all ESBL producers, a phenomenon most likely due to the combination of genes responsible for quinolone and β-lactam resistance in one common plasmid (*Scheich S, 2018*).

Of all the isolated bacteria, nine (18.8%) demonstrated resistance to carbapenems (*Pseudomonas* spp., n=8; *E. cloacae*, n=1). For low incidence of CPR bacteria reported Scheich et al. (8.5%), as observed dominance of *P. aeruginosa* (*Scheich S, 2018*). Bilinski et al. (6%) reported similar results (*Bilinski J, 2016*). For 8 years Giannella et al. screened patients after transplantation of a solid organ and document a frequency of 26.6% intestinal colonization with CPR enerobacteria (*Giannella M, 2019*). Among CR isolates in our study 8 demonstrated resistance to ceftazidime/avibactam and all were sensitive to colistin.

Three isolates have been identified as *S. maltophilia*, falling into the group of polyresistent bacteria due to their lack of sensitivity to all groups of antibiotics (incl. thrimethoprim/sulfamethoxazole) with the exception of colistin.

All isolated Gram – positive bacteria are defined as *E. faecium*, exhibiting glycopeptide resistance. After testing with a microdiluation method and determination the MIC of vancomycin and teicoplanin, they were confirmed as VRE. The incidence of VRE in our study as colonizing agents in patients after HSCT was 23%. Close results were also reported in a survey conducted in Poland between 2010 and 2013 – 21% VRE (*Bilinski J, 2016*). A significantly higher proportion of VRE was reported in a German study over a ten-year period among transplanted patients – 85.9%. Differences in the incidence of VRE colonization can be explained by the type of underlying disease, the pre-transplant therapeutic plan, and the administration of antibiotics for the purpose of prevention such as quinolones, which are known to be a risk factor for colonization with VRE (*Scheich S, 2018*). In all 15 VRE isolates, linezolid demonstrated preserved activity.

Patients with HM are at risk of occurrence of infectious complications due to immunosuppression caused by the underlying disease and chemotherapy. Infections caused by polyresistant strains lead to higher

mortality compared to those caused by bacteria with preserved sensitivity. In recent years, infectious complications associated with polyresistant microorganisms are increasing. It is believed that this is due to the widespread use of antibiotics in humans and animals. One of the main risk factors for the occurrence of infection caused by MDR bacteria is pre-colonization with such bacteria. Due to the fact that a large proportion of patients with HM use prolonged antimicrobial therapy, those undergoing HSCT are at higher risk of being colonized by MDR bacteria before transplantation (*Scheich S, 2018*).

A number of studies prove the role of intestinal microflora in HSCT in the occurrence of invasive bloodstream infections (*Bilinski J, 2016; Ford CD, 2017b; Scheich S, 2017; Giannella M, 2019*). Transplantation strongly affects the intestinal microbiota. The human intestinal microflora interacts with the host's immune system and less diversity leads to an inadequate immune response. In patients who are still with suppressed immune systems after HSCT and after exposure to antibiotics, altered intestinal flora can affect immune recovery and explain the high levels of deaths, associated with infectious in colonized patients (*Scheich S, 2018*).

In patients with previous colonization, selective pressure can lead to a selection of VRE, which dominate over the other representatives of the intestinal flora. It is known that the intestinal carrier of VRE is associated with loss of microbial diversity. This phenomenon is associated with a higher incidence of GVHD and its more severe course (*Scheich S, 2018*). In addition to VRE, colonization with *Candida* spp. is associated with an increased risk of acute GVHD (*Malard F, 2021*).

Seventeen colonizing mycotic isolates, of which 82.5% representatives of *C.* nonalbicans species, were subjected to microdilution and E-test MIC methods to study their sensitivity to a set of antimycotic agents. Among *Candida* nonalbicans species, the reported sensitivity is varied – from completely preserved to completely absent. The highest resistance among the isolates tested is documented to azole antimycotics and in particular, fluconazole (70.6%). In our transplant center, fluconazole is the most commonly used and it is the first choice drug at the start of empirical antimycotic therapy in patients suspicious for mycotic infection. The results we obtained support the theory of selective pressure and the appearance of resistant isolates. An alternative to this agent in our transplant center is the newer agent from the group of triazoles – voriconazole, exhibiting activity, both against fluconazole-resistant yeasts and against some molds. Resistant isolates (*C. dubliniensis, C. glabrata, C. krusei, C. tropicalis*) were also documented among the studied collection (37.5%).

Invasive mycotic infections are a serious complication in patients after HSCT. *Candida* spp. are considered the most common causative agents of these complications. It is assumed that invasive infection is preceded by colonization of the macroorganism and it is of endogenous origin, with a large number of studies pointing to the digestive system for the most frequent source (*Nucci A, 2001; Zollner-Schwetz I, 2008*). When an invasive infection occurs due to *Candida* spp. (candidaemia), echinocandines are recommended as first-choice agents, according to ECIL 6 (*Tissot F, 2017*). In our study, no isolates resistant to caspofungin and micafungin were found, but 4 *Candia* nonalbicans isolates demonstrated resistance to anidulafungin. In a study of Zollner-Schwetz among isolates *C. glabrata*, colonizing patients after HSCT, highly reduced sensitivity to azole preparations fluconazole (MIC90 ≥ 64 mg/l) and voriconazole (MIC90 = 4 mg/l) was reported, data similar to ours (*Zollner-Schwetz I, 2008*).

This study also examined the sensitivity to one of the newest azole antimycotics isavuconazole, for which there is not yet a generally accepted standard for sensitivity reporting. The isolates tested demonstrated MIC values from 0.003 to ≥ 32 μg/ml. Similar results have been reported in a two-year study of the sensitivity of 29 different mycotic agents to isavuconazole (*Desnos-Ollivier M, 2019*).

The tested *C. albicans* isolates demonstrated a preserved sensitivity to all antimycotics studied. Close to our results reported Zollner-Schwetz et al. after examining 177 *C. albicans* from fecal samples of patients after HSCT (*Zollner-Schwetz I, 2008*).

Among the studied collection of fecal *Candida* spp. stood out 4 nonalbicans species, which can be defined as polyresistant due to the documented lack of sensitivity to both azoles and echinocandins. It is believed that this phenomenon is due to selective pressure. Due to severe neutropenia, subsequent HSCT, antimycotic prophylaxis is often used in patients at doses that differ from therapeutic ones in order to prevent invasive infection. This approach can lead to a lack of the necessary concentration in the digestive tract and adaptation of the fungus to the corresponding concentration and even the occurrence of mutants not affected by high (therapeutic) doses (*Healey KR, 2017*).

One of the goals of the current dissertation was to identify the mechanisms, associated with the emergence of resistance to broad-spectrum cephalosporins by molecular-genetic methods. The tested bacterial isolates demonstrating resistance to the third and fourth generation cephalosporins were subjected to molecular genetic testing to detect the genes encoding this resistance. The widest prevalence was shown to be $bla_{\text{CTX-M-like}}$ (91%), with

bla$_{\text{CTX-M-15}}$ being the dominant variant, followed by *bla*$_{\text{TEM-like}}$ (63.6%) and *bla*$_{\text{SHV-like}}$ (18.2%). In the past SHV and TEM beta– lactamases were the dominant type of enzymes, with SHV being proven in many centers (*Markovska R, 2008*). In 2001, the first CTX-M enzymes were detected in Bulgaria and their prevalence rapidly increased – from 16.7% (2001) to 97% of all ESBL enzymes, mainly dominated by CTX-M-15, data reported in many other geographic regions (*R. Markovska, 2018).* In a large number of the isolates, more than one *bla* gene has been proven to confirm the mobility and plasticity of these genes, as well as the ability of bacteria to accumulate genes responsible for the production of various enzymes.

The carbapenem resistance demonstrated in 9 isolates in our study is mainly associated with the production of VIM-2 (66.7%) in 4 types of *Pseudomonas* spp. and less frequently with VIM-1 metal - carbapenemase, proven in single isolate *E. cloacae*. In Bulgaria there are described single cases of *P. aeruginosa*, carriers of VIM carbapenemases, the first case being reported in 2006. The *bla*$_{\text{VIM-15}}$ gene, which is a variant of *bla*$_{\text{VIM-2}}$ has been detected (*Schneider I, 2008*). Strateva et al. recently reported the emergence of VIM-2 producing *P. aeruginosa* belonging to the high-risk type 111 group (*Strateva T, 2021*). Isolates *E. cloacae*, the carriers of *bla*$_{\text{VIM-1}}$, are described in different parts of the world, mainly associated with clonal distribution in a hospital environment (*Falcone M, 2009; Heller I, 2012*). There is no previous data to document such isolate in Bulgaria.

In the current study, the spectrum of Gram - positive multipledrug-resistant isolates, associated with fecal colonization is dominated exclusively by vancomycin - resistant *E. faecium*, all carriers of the *vanA* gene. In the period 2017 – 2018 Hitkova et al. investigated hospitalized patients for fecal carrier of glycopeptide – resistant enterococci and reported an incidence of 29.4% with *vanC* gene dominance. The authors detect *vanA* only in 5.5% of isolates (*Hitkova H, 2019*). In line with our results, a study of Strateva et al. involving isolates *E. faecium* from three major hospitals in Bulgaria obtained 51 VRE, all carriers of the *vanA* gene (*Strateva T, 2018*).

In the period 2017 – 2018 Stankova et al. conducted a study on fecal isolates of patients hospitalized in 6 major hospitals across the country, comparing them with results obtained from healthy individuals. The authors reported a higher incidence of MDR bacteria, especially ESBL and carbapenemase producers among the group of hospitalized patients compared to that in the healthy group. The authors explain the difference with the intra-hospital clonal spread of resistant bacteria, as well as with antibiotic selective pressure (*Stankova P, 2020*).

Patients with HSCT have frequent hospitalizations for treatment or follow-up, and this is an important risk factor for colonization with

polyresistant strains. In addition, a large proportion of these patients take antibacterial agents for the purpose of prevention or therapy, which additionally leads to the appearance of resistance among microorganisms. According to literature data, colonization with MDR bacteria has different durations, and can persist for a long time, which risks disseminating these microorganisms in the society (*Stankova P, 2020*).

Conclusion

In the current dissertation, a high incidence of intestinal colonization with fungi and polyresistant bacterial isolates (49%) was found. ESBL production in third-generation-cephalosporin resistant Gram - negative isolates is associated with the CTX-M type enzyme, the most common variant being CTX-M-15. The incidence of carbapenem-resistant bacteria (*Pseudomonas* spp., *E. cloacae*) is over 18%, mediated by VIM-1 and VIM-2 metal-carbapenemases. VRE's share was 23%, with the resistance being fully associated with the *vanA* gene. The mycotic isolates are dominated by the representatives of *Candida* nonalbicans species, detecting species with polyresistance.

4.7. STUDY ON THE EPIDEMIOLOGICAL RELATIONSHIP BETWEEN FECAL ISOLATES AND BLOOD ISOLATES, DEMONSTRATING RESISTANCE TO 3RD GENERATION CEPHALOSPORINS, CARBAPENEMS AND GLYCOPEPTTIDE ANTIBIOTICS

For epidemiological typing purposes, all isolates, representatives of the *Enterobacteriaceae* family, demonstrating resistance to the third generation cephalosporins and carbapenems (n=21) and all vancomycin-resistant *Enterococcus faecium* (n=15) were studied. Thirty-six are the isolates, associated with fecal screening (*E. faecium*, n=15; *E. coli*, n=10; *E. cloacae*, n=7; *K. pneumoniae*, n=4) and 3 are blood isolates (*E. coli*, n=2; *E. cloacae*, n=1).

The isolates of the *Enterobacteriaceae* family were typed by ERIC – PCR, and RAPD – PCR was used in *E. faecium*. For each isolate, recognizable ERIC or RAPD profiles composed of 6 to 12 or 4 to 6 bands were generated, respectively. Isolates with a similarity index of ≥ 0.9 (ERIC – PCR) or ≥ 0.8 (RAPD – PCR) were accepted as a clonal group.

Among *E. coli* isolates (n=12) 10 ERIC types have been identified, 8 of them unique. Types A and B are represented by 2 isolates each. No epidemiological link is found between blood and faeces isolates (Figure 13).

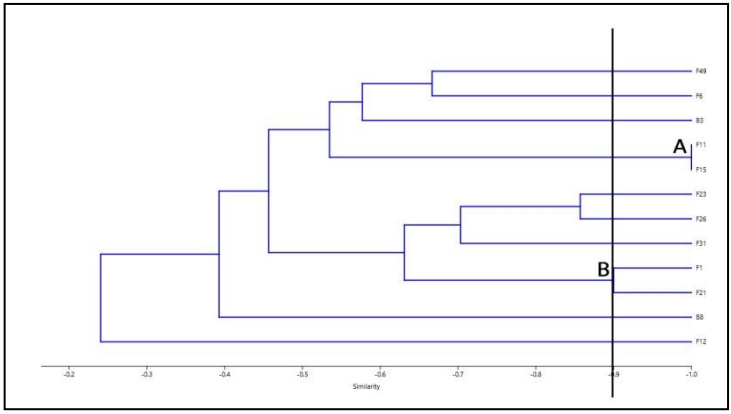

Figure 13. Dendropgram, reflecting the degree of similarity between the individual ERIC types established in *E. coli* isolates. Types A (F11, F15) and B (F1, F21).

In *E. cloacae* isolates (n=8), two ERIC types (A, B) have been identified, of which type A dominant, represented by one blood isolate and 5 by fecal ones (Figures 14 and 15). An epidemiological relation between blood and fecal isolates has been established, with B33 and F52 isolates derived from the blood and fecal samples of the same patient, respectively.

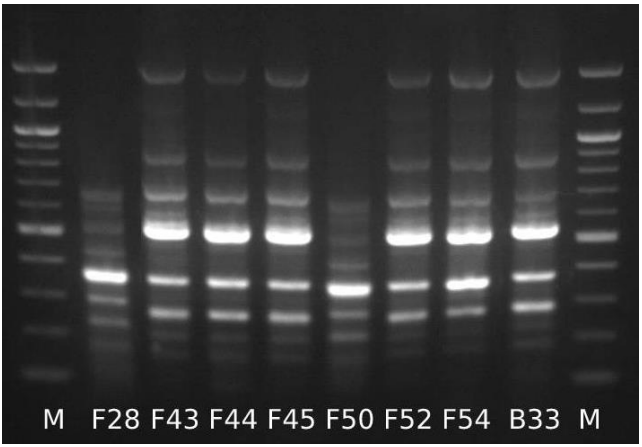

Figure 14. ERIC profiles of isolates *E. cloacae*. M, marker 100 bp; Fecal isolates: F28, F43, F44, F45, F50, F52, F54; Blood isolate: B33.

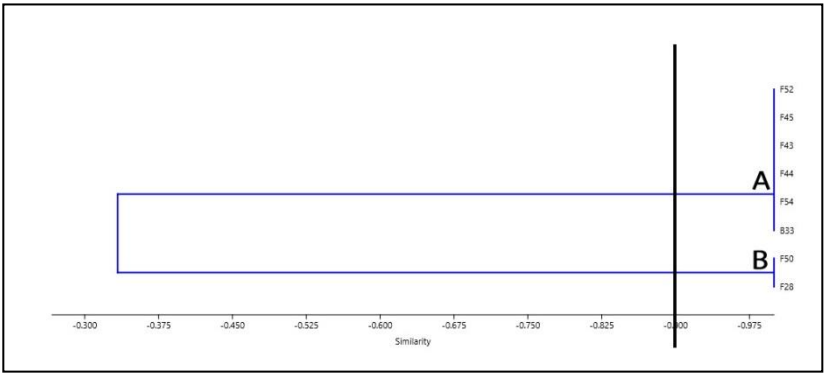

Figure 15. Dendropgram, reflecting the degree of similarity between the individual ERIC types established in *E. cloacae* isolates. Type A (F52, F45, F43, F44, F54, B33); type B (F50, F28).

The four typed *K. pneumoniae* isolates, all from fecal screening, demonstrated different ERIC profiles.

By RAPD PCR among 15 isolates *E. faecium*, all from fecal screening, 6 RAPD types have been identified. Type A is dominant, identified in 7 isolates and represented by 2 subtypes (Aa, Ab). RAPD type

B and C include 3 and 2 isolates, respectively. Three *E. faecium* isolates are demonstrated with unique RAPD profiles (Figure 16).

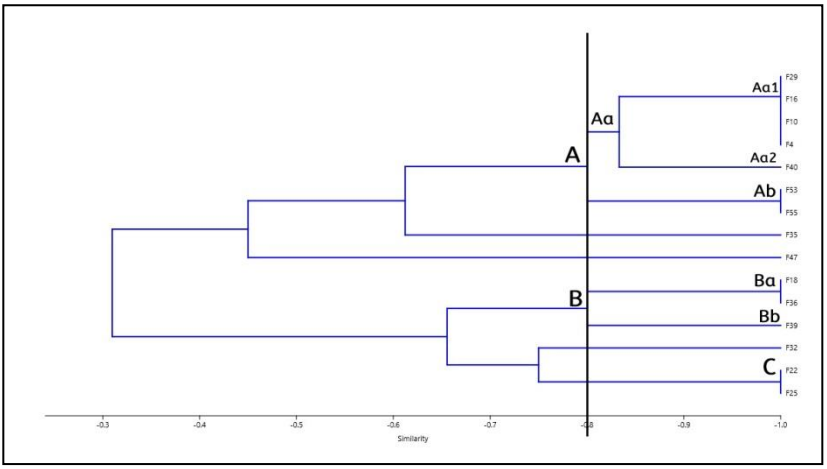

Figure 16. Dendropgram, reflecting the degree of similarity between the individual RAPD types established in *E. faecium* isolates. RAPD type A with subtypes Aa (F29, F16, F10, F4, F40) and Ab (F53, F55); RAPD types B with Subtypes Ba (F18, F36) and Bb (F39); type C (F22, F25).

Discussion

In recent years, polyresistant bacteria have become a major health problem worldwide, and hospitals are increasingly facing local outbreaks caused by these pathogens (*Mulliè C, 2022*). According to EARS-NET data for 2020, infections, caused by resistant microorganisms are steadily increasing (*EARS-NET, 2022*). In addition to acquiring resistance to a large number of antimicrobial agents, ESBL and carbapenemase producing enteric bacteria, GNGNF, VRE, add another pathogenicity factor to their arsenal – namely, their ability to disseminate clonally and cause intra-hospital outbreaks.

In the current dissertation by molecular – genetic methods the epidemiological relationship between 39 bacterial isolates derived from fecal samples and blood cultures was studied.

Among the 12 *E. coli* isolates, a relationship was only proven between isolates forming small groups of 2 with ERIC profiles A and B respectively, all carriers of the $bla_{\text{CTX-M-like}}$ gene. No relation between blood

and fecal isolates has been established. Close to our results are reported in a study of Kharrat et al., covering a 10-year period. The authors study antibacterial resistance and the epidemiological link between bacterial isolates, derived from patients after HSCT. They reported a high percentage of *E. coli* isolates (43%), the resistance of which is mainly due to the presence of $bla_{\text{CTX-M-like}}$ genes. After typing, the authors discovered a wide genetic diversity, highlighting 5 pulstypes (*Kharrat M, 2018*). Similar results were found by Uemura et al., who studied *E. coli* isolates obtained from patients in a oncohematology ward. Non-clonal distribution of isolates, associated with $bla_{\text{CTX-M-like}}$, $bla_{\text{SHV-like}}$ and $bla_{\text{TEM-like}}$ were documented *(Uemura M, 2017).*

Enterobacter cloacae is increasingly recognized as an opportunistic pathogen, responsible for a wide range of nosocomial infections (incl. intra-hospital outbreaks) such as bacteremia, pneumonia, wound and uroinfections, affecting patients in intensive care units and those with a suppressed immune system. Increased length of hospital stay, empirical administration of antibiotics (especially 3rd generation cephalosporins), the use of central venous and arterial catheters, intubation and other invasive manipulations, associated with disruption of natural mechanical barriers, underlying disease or conditions (diabetes, oncohematological diseases, solid tumors, transplantation, neutropenia) are documented risk factors for the development of infections caused by *E. cloacae*, especially bloodstream infections (*Sanders WE Jr, 1997; Lee, 2010*).

In our study, among the typed *E. cloacae* isolates, obtained from 7 patients, one dominant ERIC type (A) was identified, represented by 6 strains, demonstrating a 100% degree of similarity. Invasive (blood) isolate (B33) and one of the fecal isolates (F52) in this cluster are obtained from one patient. This fact proves the gastro-intestinal tract as an important source of microorganisms, causing infectious complications (incl. bloodstream infections) in patients after HSCT. In all cluster A isolates, resistance to third and fourth generation cephalosporins is mediated by the $bla_{\text{CTX-M}}$ gene, and the demonstrated resistance to carbapenems in one of the fecal isolates is associated with the carrier of the $bla_{\text{VIM-1}}$ gene. Our results confirm the potential of *E. cloacae* to cause invasive infections under certain conditions, acquire different genes, encoding resistance to antibacterial agents, and spread clonally. The data obtained by Dimitrova et al. fully support these results by demonstrating the clonal spread of *E. cloacae* isolates, carriers of $bla_{\text{CTX-M-15}}$ genes among patients hospitalized at the Clinic of Hematology of St. Marina University Hospital – Varna in the period 2014 - 2017. (*Dimitrova D, 2019*). In the course of the COVID-19

pandemic, the Mullié et al. followed-up an intra-hospital outbreak, occurring between March and October 2020 in an intensive care clinic of a university hospital in France with causative agent *E. cloacae* complex. All studied isolates - from patients (from fecal samples and other clinical materials) and from the environment are identified as clonaly-related and carriers of the $bla_{\text{VIM-4}}$ gene (*Mullié C, 2022*).

Among the representatives of the order *Enterobacterales*, *Klebsiella pneumoniae* is one of the most common causative agents of hard-to-treat infections. The presence of various factors of virulence and the ability to acquire multiple resistance, turn this microorganism into a super-bacterium. The presence of these characteristics enhances the potential of *K. pneumoniae* for colonizing patients who have been in medical institutions for a long time (*Fatima S, 2021*). In our study, a small number of *K. pneumoniae* isolates resistant to the 3rd generation of cephalosporins, all of which are carriers of different variants of *bla* genes and have no genetic link. A study by Kharrat et al. found similar data to ours. For 10 years, the authors studied ESBL – producers, colonizing and causing infections in patients after HSCT. After performing epidemiological typing by PFGE, among all 19 isolates *K. pneumoniae*, they identify 17 unique pulstypes (*Kharrat M, 2018*). Other researchers from Japan reported completely different data that supported claims about the ability for clonal spread of *K. pneumoniae* in hospital settings. Uemura et al. studied *K. pneumoniae* isolates, obtained from patients hospitalized in different wards of a university hospital. Through PFGE and PCR methods, the authors detect a cluster of strains, causing an intra-hospital outbreak of infections, affecting patients in several different clinics (*Uemura M, 2017*).

In line with the increasing global prevalence of bacteria with multiple resistance, the incidence of vancomycin-resistant enterococci also increased (*Weterings V, 2021*). Enterococci, demonstrating this type of resistance are associated with problematic infections and difficult-to-control intra-hospital outbreaks, turning in many hospital centers into a serious medical challenge (*Rangerberg A, 2019*). The current study examined the epidemiological link between 15 fecal vancomycin – resistant *E. faecium* isolates. A dominant RAPD type (A) was identified, represented by 7 strains, isolated from 7 patients, resulting in the potential of these bacteria for clonal spread. Resistance to vancomycin in all tested isolates is *vanA* associated. Between 2012 and 2013 in Norway, in the course of an intra-hospital outbreak in a surgical ward, 9454 fecal samples for VRE carrier have been tested, two cluster groups, represented by *E. faecium*, all *vanA* carriers, have been documented (*Rangberg A, 2019*).

For three years, Weterings at al. tracked an intra-hospital outbreak, caused by VRE. The authors identified intestinal colonization in 140 patients, and in some of them later bacteremia was seen. The epidemiological study through the AFLP proves the dissemination of one branch VRE, carriers of the *vanA* gene (*Weterings V, 2021*). In 2015 in Spain, de Artola et al. conducted active screening of fecal samples, obtained from 117 patients in a hematology clinic and detected intestinal colonization by *E. faecium* in 18.9%. In 9% of the colonized invasive infection, caused by the same strain, is documented. In addition, PFGE typing detected 3 closely related clusters, causing the intra-hospital outbreak, all isolates being carriers of the *vanA* gene (*de Artola DGM, 2017*). VRE, carriers of the *vanA* gene, in addition to the carrier of numerous factors of virulence and the potential for clonal dissemination, also possess the ability to displace other enterococci, as causative agents of infections, as Hughes et al reported. The authors document an emerging outbreak at a hospital in Australia that has endemic prevalence of the *vanB* gene among *E. faecium* isolates for years. The new outbreak was caused by *E. faecium*, a *vanA* carrier. After conducting further studies, they found that a large number of isolates fall into 5 cluster groups, and the representatives of one are carriers of both *vanA* and *vanB* genes (*Hughes A, 2019*).

In addition to clonal dissemination of MDR bacterial isolates among patients with HSCT, this study also found non-clonal prevalence. It is believed that the non-clonal or limited spread of these microorganisms is due to the effective hygiene measures that are applied from the patient's reception in the clinic to his discharge, leading to the minimization of the transmission of bacteria between different patients (*Kharrat M, 2018*).

Conclusion

The epidemiological study found the presence of clusters of isolates of the species *E. cloacae* and *E. faecium*, as well as those with unique ERIC or RAPD profiles (*E. coli, K. pneumoniae, E. cloacae, E. faecium*). *E. cloacae* isolate from fecal screening, genetically identical to blood isolate *E. cloacae*, obtained from the same patient has been documented. These results confirm the invasive potential and ability of these microorganisms for clonal dissemination in hospital environment among patients after HSCT. Conducting epidemiological typing by ERIC and RAPD PCR has been confirmed as useful and reliable method for detecting genetic link between representatives of different bacterial species.

5. CONCLUSIONS

After analyzing the obtained results, we can draw the following conclusions:

1. A high cumulative incidence of bloodstream infections was established in the study group of transplanted patients (32% - 38.5%), with an average period of occurrence of the infectious complication of 47 days after the procedure.
2. Fecal colonization and infection of the blood, preceding HSCT are independent risk factors for the occurrence of bloodstream infection in patients after HSCT.
3. A high 4-month survival rate was found among the entire group of patients (86.5%).
4. A statistically significant link was established between 4-month survival and the indicators - type of transplantation, underlying disease and lack or presence of a previous transplantation, with patients with allogeneic HSCT, previous HSCT and underlying leukaemia or lymphoma having lower chances of surviving the first 4 months after transplantation.
5. Gram – positive bacteria dominate the etiological spectrum of bloodstream infections in patients after HSCT, the most common causative agents being coagulase-negative staphylococci. The *E. coli* dominates among Gram - negative microorganisms. A low relative share of fungemia was documented.
6. Coagulase-negative staphylococci, associated with bloodstream infections in this study demonstrate very high levels of methicillin resistance, therefore, in cases of implemented CVC and suspected catheter – associated infection, it is advisable to start therapy with glycopeptides. Ampicillin, ciprofloxacin and trimethoprim/sulfamethoxazole have significantly reduced activity to Gram-negative bacteria isolated from blood. The share of ESBL producers among enteric bacteria, representatives of the *Enterobacteriaceae* family is 20%. Imipenem/meropenem, piperacillin/tazobactam and amikacin are the agents with best activity, making them suitable for initial empirical treatment in cases of febrile neutropenia and sepsis.
7. The main mechanism of resistance to 3rd generation cephalosporins in blood isolates of the *Enterobacteriaceae* family is the production of CTX-M-15 ESBL. Carbapenem resistance in *A. baumannii* is associated with the carriage of four *bla* genes

encoding carbapenemases of two different classes - class B (bla_{VIM}) and class D (bla_{OXA-48}, bla_{OXA-23}, $bla_{OXA-24/40}$). Methicillin resistance demonstrated in all CoNS is associated with the *mecA* gene.

8. The high relative proportion of slime-producing coagulase-negative blood staphylococci, among which the most common is *Staphylococcus epidermidis*, is associated with the presence of *ica* genes. A statistically significant link has been established between *ica* gene carriage and methicillin resistance.
9. In the studied group of patients, a low incidence of invasive pulmonary aspergillosis was found, but high mortality among the documented cases.
10. The studied group of transplanted patients is characterized by a high incidence of intestinal colonization with fungi and multidrug-resistant bacteria (49%): 33.8% of ESBL producers, with CTX-M-15 being the most common ESBL; 13.8% carbapenem-resistant bacteria (*Pseudomonas* spp., *E. cloacae*), associated with production of VIM-1 and VIM-2 metal-carbapenemases and 23% VRE, all carriers of the *vanA* gene. Among the mycotic isolates dominate the representatives of *Candida* nonalbicans species, reporting species with simultaneous resistance to echinocandins and the azole group of antimycotics.
11. Clusters of identical and/or closely-related isolates *E. cloacae* and *E. faecium* are documented, as well as the genetic relation between fecal and blood isolates *E. cloacae*, derived from the same patient, confirm the gastrointestinal tract as an important reservoir for infectious complications in patients after HSCT, and the invasive potential and the ability of these microorganisms for clonal dissemination in hospital environment.

6. REFERENCE TO THE CONTRIBUTIONS OF THE DISSERTATION

Original contributions:

1. The frequency and risk factors for bacteremia and invasive mycotic infections were studied and analyzed in patients who underwent autologous and allogeneic HSCT, as well as 4-month survival in the entire patient group, analyzing the factors that affect it.
2. The etiological spectrum and susceptibility to antimicrobials of microbial causative agents of bloodstream infections in patients following autologous and allogeneic HSCT have been analyzed.
3. The colonizing status of the gastrointestinal tract with MDR bacteria (ESBL and carbapenemase – producing Gram – negative bacteria, vancomycin – resistant enteroococci and *S. maltophilia*) and fungi in patients after HSCT has been studied.
4. Carbapenem-resistant isolate *E. cloacae* complex has been identified from a fecal sample, carrier of $bla_{\text{VIM-1}}$.
5. Carbapenem-resistant isolates *Pseudomonas composti* and *Pseudomonas mendocina* from a fecal sample have been identified as carriers of $bla_{\text{VIM-2}}$.
6. Carbapenem-resistant isolate *A. baumannii* from blood culture, carrier of the $bla_{\text{OXA-48-like}}$ gene, has been identified. The same isolate is a carrier of 3 more genes ($bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24/40-like}}$, $bla_{\text{VIM-like}}$) encoding carbapenemases.
7. *Candida* nonalbicans isolates (*C. glabrata, C. krusei, C. tropicalis*) have been detected by fecal screening, demonstrating multiple resistance: simultaneous resistance to fluconazole, voriconazole, itraconazole and anidulafungin.

Confirmatory contributions:

1. The gastro-intestinal tract has been confirmed as an important source of infectious complications in patients after HSCT, and intestinal colonization with MDR bacteria is proven as a significant risk factor for the development of bacteremia in this group of immunocompromised patients.
2. Gram – positive bacteria, dominated by coagulase-negative staphylococci and less often *S. aureus*, are leading causative agents of bacteremia in patients after HSCT and placed CVC.

3. The widespread prevalence of methicillin resistance, mediated mainly by the *mec* gene, has been confirmed among CoNS isolates, including those associated with bloodstream infections.
4. The relationship between the carriage of *ica* operon and the production of slime is confirmed in coagulase-negative staphylococci isolated from blood, as well as the association between the carriage of *ica* genes in these isolates and methicillin resistance.
5. The widespread geographical spread of CTX-M types of extended-spectrum beta-lactamases, in particular CTX-M-15, which are also a major mechanism of resistance to third-generation cephalosporins in *Enterobacteriaceae* isolates derived from blood and feces in patients after HSCT.
6. Resistance to glycopeptides (vancomycin, teicoplanin) in vancomycin – resisted isolates *E. faecium* is most often mediated by the carrier of *vanA* gene.
7. The invasive potential of *E. cloacae* and the clonal dissemination of isolates of *E. faecium* and *E. cloacae* have been confirmed.
8. Invasive pulmonary aspergillosis in patients who underwent HSCT was associated with high mortality.

Scientifically applied contributions:

1. CRA test and Christensen's test were evaluated as phenotypic methods for detecting slime production by isolates *Staphylococcus* spp. from blood.
2. The ability of the immunoenzyme method Platelia *Aspergillus* Ag test (Bio-Rad, France) for the diagnosis of invasive pulmonary aspergillosis in patients after HSCT has been studied.

7. PUBLICATIONS AND REPORTS

Articles

1. **Niyazi D**, Micheva I, Stoeva T. Bacterial and fungal complications in patients undergoing hematopoietic stem cell transplantation. Hematology. 2020;56(1):27-31. (SJR_{2020} 0.101, Q4)
2. **Niyazi D**, Stoeva T, Atanasova S, Markovska R, Micheva I. Invasive Pulmonary Aspergillosis in Patients with Haematological Malignancies and Hematopoietic Stem Cell Transplantation: a Single-Center Study. Folia Medica. 2021;63(6):941-947. (SJR_{2021} 0.203, Q4)
3. **Niyazi D**, Micheva I, Markovska R, Stoeva T. Phenotypic and molecular detection of slime producing *Staphylococcus* spp. obtained from blood samples of patients undergoing hematopoietic stem-cell transplantation. Acta Medica Bulgarica. 2022 (SJR_{2021} 0.120, Q4) (*in press*)

Reports

1. **Niyazi D**, Stoeva T, Atanasova S, Resnick I, Micheva I. Evaluation of galactomannan test for the diagnosis of invasive aspergillosis in patients with hematological malignancies. 11th National congress of hematology, 13th Balkan day of hematology, 10 - 13 October 2019, Pravets, Bulgaria (oral presentation).
2. **Niyazi D**, Micheva I, Kaleva V, Stoeva T. Diversity and resistance profile of *Candida* spp. isolates recovered from clinical samples of patients after haematopoietic stem cell transplantation. National conference of hematology, 16 – 19 September 2021, Pravets, Bulgaria (poster).
3. **Niyazi D**, Savova D, Bozhkova M, Kamenova V, Bizheva S, Stoeva T. Phenotypic and molecular detection of biofilm production by *Staphylococcus* spp. obtained from blood samples of patients undergoing hematopoietic stem-cell transplantation. XIX National congress of clinical microbiology and infections, 14 – 16 September 2021, Sofia, Bulgaria (poster).
4. **Niyazi D**, Marinska B, Kaleva V, Stoeva T. A clinical case of invasive pulmonary aspergillosis in a paediatric patient with acute lymphoblastic leukemia after hematopoietic stem-cell transplantation. 11th South-east European conference of chemotherapy, infections, and cancer and 31st annual assembly of

international medical association Bulgaria (IMAB), 28 – 31 October 2021, Plovdiv, Bulgaria (oral presentation) – **1st place**

Projects and funding

1. Invasive bacterial infections in patients after autologous and allogeneic bone marrow transplantation: etiological spectrum and resistance to strategic beta-lactam and glycopeptide antibiotics. Science Fund, Medical University – Varna, No 19019, 2019 – 2022.

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