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PHENOTYPIC AND GENOTYPIC STUDIES ON VANCOMYCIN-RESISTANT ENTEROCOCCI IN PATIENTS AT HIGH RISK OF COLONIZATION AND INFECTION WITH PROBLEMATIC MICROORGANISMS

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LIST OF ABBREVIATIONS

ace/acm – gene, encoding collagen-binding protein
ADPKD – Autosomal dominant polycystic kidney disease
agg/asa1 – gene, encoding aggregation substance
AMEs – aminoglycoside modifying enzymes
AMP – ampicillin
ARA – arabinose
BEAV – bile aesculin azide vancomycin
CAKUT – Congenital anomalies of the kidney and urinary tract
CFH – chronic heart failure
CI – confidence interval
CIP – ciprofloxacin
COPD –Chronic obstructive pulmonary disease
cyl – gene, encoding cytolysin
DAP – daptomycin
DDM – disk-diffusion method
efaA – gene, encoding endocarditis-specific antigen A
EUCAST – the European Committee on Antimicrobial Susceptibility Testing
esp – gene, encoding enterococcal surface protein
FN – false negative
FP – false positive
gelE – gene, encoding gelatinase
GEN – gentamicin
HAIs – hospital-acquired (nosocomial) infections
HD – hemodialysis
HLGR – high-level gentamicin resistance
HT – hematology
hyl – gene, encoding hyaluronidase
ICU – Intensive care unit
IPM – imipenem
LAP – leucine aminopeptidase
LVX – levofloxacin
LZD – linezolid
MIC – minimum inhibitory concentration
MAN – mannitol
MGP – methyl α-D-glucopyranoside
MLVA – multilocus variable-number tandem repeats analysis
NPV – negative predictive value
PCR – polymerase chain reaction
PFGE – pulsed-field gel electrophoresis
PPV – positive predictive value
PYR – L- pyrrolidonyl arylamidase
Q/D – quinupristin/dalfopristin
RAF – raffinose
SBL – sorbitol
STM – streptomycin
TEC – teicoplanin
TGC – tigecycline
TN – true negative
TP – true positive
VAN – vancomycin
vanC – enterococci with intrinsic low-level resistance to vancomycin
VR – vancomycin-resistant
VRE – vancomycin-resistant enterococci
VS – vancomycin-susceptible
VSE – vancomycin-susceptible enterococci

I. INTRODUCTION

Enterococci are widespread in the nature and can be found in soil, water, food, and various animals, including mammals, birds, insects, and reptiles. In humans, they are part of the normal microflora of the small and large intestines and are less commonly found in other areas, such as the genitourinary tract, oral cavity and perineal area.

Under certain circumstances, enterococci colonizing the intestinal tract, can spread to other areas and to cause infections. In the recent years, hospital-acquired infections (HAIs) associated with these microorganisms have been increasing. Enterococci are the second most frequent causative agents of HAIs in the USA and the third in Europe. Their established role as leading nosocomial pathogens is largely due to their innate resistance to major classes of antimicrobial drugs and their ability to acquire new genes encoding resistance to almost all antibiotics with activity against enterococci. Commonly used antibiotics such as penicillins, aminoglycosides and fluoroquinolones have poor activity, and cephalosporins have no effect against enterococci.

Since the appearance of the first strains of vancomycin-resistant enterococci (VRE) in the late 1980s, vancomycin resistance in *Enterococcus* spp. has grown significantly. Nosocomial clones of vancomycin-resistant (VR) *Enterococcus faecium* and *Enterococcus faecalis* with multiple resistance determinants and pathogenicity factors has been spread in the hospitals. Currently, VRE are described everywhere around the world, with widely varying incidence between the countries. Adults and seriously ill patients with comorbid diseases, treated in hematology and oncology departments, dialysis centers,

anesthesiology and intensive care departments, and etc., are the most commonly colonized and/or infected. The endemicity of VRE in these units is the highest, with varying frequency between different groups of patients.

Until 2012, invasive VRE have not been registered in Bulgaria, despite the wide spread of enterococci with high resistance to vancomycin around the world. Subsequently, VRE and in particular VR *E. faecium* have been isolated at an increasing rate, and they are now present in all major hospitals in the country.

Regardless of the large number of publications about VRE worldwide, in our country the studies are quite limited. The data about the phenotypic and genotypic properties of circulating VRE isolates, the genes encoding their antimicrobial resistance and virulence determinants, the risk factors and frequency of fecal colonization with VRE in hospitalized patients, the capabilities of different culture media in fecal screening for VRE and etc. are extremely insufficient. This determines the need for in-depth research in that direction, some of which are the subject of this dissertation.

II. AIM AND TASKS

The **aim** of the present dissertation is to study the phenotypic and genotypic characteristics of clinical and intestinal vancomycin-resistant enterococci (VRE) and the risk factors for colonization and infection with these bacteria in hospitalized patients.

The following **tasks** were set to achieve this goal:

1. To evaluate the capabilities of different types of selective culture media for the primary isolation and identification of intestinal VRE.

2. To study the intestinal colonization with VRE in groups of patients at high risk for colonization and infection with problematic microorganisms.

3. To analyze the spread of clinical VRE in UMHAT "Dr. G. Stranski", Pleven.

4. To determine the antimicrobial susceptibility and the genes encoding resistance to glycopeptides and aminoglycosides in the isolated clinical and intestinal VRE.

5. To study the genes encoding virulence factors in the isolated clinical and intestinal VRE.

6. To perform epidemiological typing of the isolated clinical and intestinal VRE.

III.MATERIALS AND METHODS

1. Bacterial isolates

A total of **186 VRE** were studied, of which:

- **117** clinical isolates **– 94 VRE** from UMHAT "Dr. G. Stranski", Pleven (2016 – 2020); **23 VRE** from Acibadem City Clinic UMNAT "Tokuda", Sofia (2020); **5 VRE** from "Heart and Brain" Hospital (2019) and **4 VRE** from UMHAT "St. Marina", Pleven (2017 – 2020).
- **69** intestinal enterococci, all isolated during fecal screening for VRE of immunocompromised patients from UMHAT "Dr. G. Stranski", Pleven for the period 2017 – 2019.

2. Fecal samples

A total of **506 samples** (250 rectal swabs and 256 feces)

- **159** rectal swabs from 97 patients undergoing hemodialysis;
- **256** feces from **119** patients with hematologic malignancies;
- **91** rectal swabs from **91** patients treated in intensive care units.

3. Patients

The demographic and clinical risk factors of a total of **392** patients treated at the UMHAT "Dr. G. Stranski", Pleven for the period 01.01.2016 – 31.12.2020 were studied.

3.1. **307** patients screened for intestinal VRE carriage;

3.2. **85** patients with detected VR *E. faecium* isolates.

4. Culture media and cultivation conditions

4.1. Culture media for primary isolation of clinical VRE.

4.2. Culture media for primary isolation of intestinal VRE – Brilliance™ VRE Agar (Oxoid, UK); chromID™ VRE Agar (bioMerieux, France); HiCrome™ VRE Modified Agar (HIMEDIA, India); Bile Aesculin Azide Vancomycin Broth (Liofilchem, Italy).

4.3. Culture media used for subculturing positive BAEV broths or primary isolated intestinal VRE from chromogenic media, etc.

5. Antimicrobial agents

6. Methods for phenotypic identification of enterococci

6.1. Conventional methods.

6.2. Identification by RapID™ STR System (Oxoid, UK).

6.3. Identification by automated systems.

7. Methods for testing the antimicrobial susceptibility

7.1. Kirby-Bauer disk-diffusion method.

7.2. Gradient-diffusion method (*E*-test).

8. Genetic methods

8.1. Methods for isolation and quantitation of DNA.

8.2. Multiplex PCR for detection of species-specific genes, genes encoding antibiotic resistance, and virulence factors.

8.3. Methods for epidemiological typing of VRE

8.3.1. Multilocus variable-number tandem repeats analysis (MLVA)

8.3.2. Pulsed-field gel electrophoresis (PFGE)

9. Statistical methods

9.1. GraphPad Prism version 7.00

9.2. SPSS Software version 7.00

IV. RESULTS AND DISCUSSION

1. EVALUATION OF SELECTIVE CULTURE MEDIA FOR THE PRIMARY ISOLATION AND IDENTIFICATION OF INTESTINAL VRE

1.1. Results from the evaluation of four selective culture media

When 159 rectal swabs were cultured on four selective culture media, 101 samples showed bacterial growth. A total of 211 bacterial agents were isolated: 33 VRE, 7 vancomycin-susceptible enterococci (VSE), 11 Gram-positive, catalase-negative cocci, which did not belong to genus *Enterococcus*, 34 Gram-positive rods, and 126 Gram-negative enteric bacteria **(*Table 1*)**. Among the isolated 33 VRE, there were 7 VR *E. faecium* (3 non-repeated and 2 repeated isolates), 1 *E. gallinarum* with VanA phenotype and 25 enterococci with VanC phenotype, from which 15 were *E. gallinarum* (11 non-repeated and 2 repeated isolates) and 10 were *E. casseliflavus* (6 non-repeated and 2 repeated isolates). The total number of non-repeated VR enterococcal isolates was 27 – 5 *E. faecium*, 14 *E. gallinarum* and 8 *E. casseliflavus*.

Six VR *E. faecium* (2 non-repeated and 2 repeated isolates) were detected from the three chromogenic media and BEAV broth, and one VR *E. faecium* – only from the broth. One *E. gallinarum* and one *E. casseliflavus* were cultured simultaneously from HiCrome VRE Modified and BEAV broth. In addition, one *E. gallinarum* with VanA phenotype, 23 enterococci with VanC phenotype, 2 VSE (1 *E. faecium* and 1 *E. faecalis*), 11 Gram-positive, catalase-negative cocci and 34 Gram-positive rods were isolated only from the BEAV broth.

Distinct growth of the six VR *E. faecium* with purple colony color was found on Brilliance VRE and chromID VRE after 24 hours of

incubation (***Figure 1***). New VRE were not detected on these media at the 48th hour of incubation. One VS *E. faecium* with purple colony color characteristic for VR *E. faecium* was cultured on these culture media. In addition, 1 *E. hirae* with light blue colony color was isolated on Brilliance VRE and 1 VS *E. faecalis* with blue-green colony color was isolated on chromID VRE. These two strains should be presumptively identified as VR *E. faecalis* according to the manufacturer's instructions. The possibility of VSE to grow on chromID VRE and Brilliance VRE has been described in several publications (Delmas et al. 2007; Grabsch et al. 2008; Asir et al. 2009; Ongut et al. 2013; Soares et al. 2017). The presence of false-positive VR enterococcal isolates is a prerequisite for unreliable VRE identification and decreased specificity of the chromogenic medium.

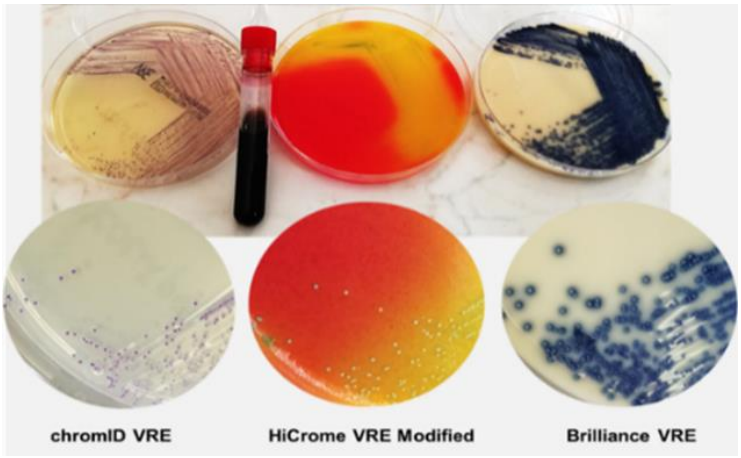


Figure 1. Colonies of VR *E. faecium* №17/HD on the three chromogenic media and positive BEAV broth.

All BEAV broths were positive after 24 hours of incubation. When subculturing the positive BAEV broths on the non-selective chromogenic agar chromID CPS Elite, the seven VR *E. faecium* grew with violet-colored colonies. The *E. gallinarum* strain with high level of

vancomycin resistance demonstrated a dark-blue colony color, while the colonies of all enterococci with VanC phenotype were green.

The results of the samples cultivation allowed the calculation of the sensitivity, specificity, positive predictive value, and negative predictive value of each of the chromogenic media and the BEAV broth (***Table 2***). It was found that the highest number of true-positive and false-positive VR enterococcal isolates were detected from the BEAV broth – 7 and 28, respectively. The sensitivity of the three tested chromogenic agars was 85.7% (CI=48.7 – 99.3), and that of the BEAV broth was 100% (CI=64.6 – 100). Brilliance VRE and chromID VRE demonstrated identical specificity of 98.7% (CI=95.3 – 99.8), while the specificity of HiCrome VRE Modified and BEAV broth was 96.7% (CI=92.5 – 98.6) and 81.6% (CI=74.7 – 86.9), respectively. The positive predictive value was 20% (CI=10 – 35.9) for BEAV broth, 54.6% (CI=28 – 78.7) for HiCrome VRE Modified and identical for Brilliance VRE and chromID VRE – 75% (CI=40.9 – 95.6). The negative predictive value was 100% (CI=97 – 100) for BEAV broth and 99.3% (CI=96.3 – 100) for the three chromogenic media. Suwantarat et al. (Suwantarat et al. 2014) tested five different chromogenic agars for the isolation of VRE from fecal samples and reported a sensitivity of 89.9% – 94.9% and a specificity of 98.3% – 99.7%. These results are comparable to the sensitivity (85.7%) and specificity (96.7% - 98.7%) calculated in the current study for the three tested chromogenic media.

Table 1. Number and identification of bacteria isolated from culturing of 159 rectal swabs on selective media

Medium	Number of enterococci with VanA phenotype[a]		Number of enterococci with VanC phenotype[b]		Number of VSE isolates[c]			Number of non-enterococcal isolates
	E. faecium	*E. gallinarum*	*E. gallinarum*	*E. casseliflavus*	*E. faecium*	*E. faecalis*	*E. hirae*	
BAEV broth	7	1	15	10	1	1	NA[g]	Gram-positive, catalase negative cocci[d] (*S. mutans* (1), *S. gallolyticus* (1), *S. sanguinis* (1), *L. garvieae* (2), *L. mesenteroides* (2), *L. pseudomesenteroides* (3), *P. pentosaceus* (1), Gram-positive rods (34)[e]
Brilliance VRE	6	NA	NA	NA	1	NA	1	NA
chromID VRE	6	NA	NA	NA	1	1	NA	NA
Himedia VRE	6	NA	1	1	2	1	NA	Enteric bacteria, part of normal intestinal flora (126)[f]

[a] Number of enterococci with VanA phenotype isolated from the chromogenic medium and the BEAV broth, identified by VITEK 2 Compact and VITEK MS, resistant to vancomycin (MIC > 4µg/ml) and susceptible to teicoplanin (MIC > 2µg/ml).

[b] Number of enterococci with VanC phenotype isolated from the chromogenic medium and the BEAV broth, identified by VITEK 2 Compact and VITEK MS, resistant to vancomycin (MIC ≥ 4µg/ml) and susceptible to teicoplanin.

[c] Number of VSE isolated from the chromogenic medium and the BEAV broth, identified by VITEK 2 Compact and VITEK MS, susceptible to vancomycin and teicoplanin.

[d] Number of Gram-positive, catalase-negative cocci, identified by VITEK 2 Compact.

[e] Number of Gram-positive rods. They are not fully identified, but are grouped based on colony morphology and Gram staining.

[f] Number of intestinal bacteria, part of the normal intestinal flora. They are grouped based on the colony morphology and the Gram staining.

[g] Not applicable (NA).

Table 2. Analysis of the chromogenic media and BEAV broth for the detection of VRE from 159 rectal swabs

Medium	**Number of isolates[a]**				**Performance % (95% CI)[b]**			
	TP	FP	TN	FN	Sensitivity	Specificity	PPV	NPV
BEAV broth	7	28	124	0	100 % (64.6 - 100)	81.6 % (74.7 - 86.9)	20 % (10 - 35.9)	100 % (97 - 100)
Brilliance VRE	6	2	150	1	85.7 % (48.7 - 99.3)	98.7 % (95.3 - 99.8)	75 % (40.9 - 95.6)	99.3 % (96.3 - 100)
chromID VRE	6	2	150	1	85.7 % (48.7 - 99.3)	98.7 % (95.3 - 99.8)	75 % (40.9 - 95.6)	99.3 % (96.3 - 100)
HiCrome VRE Modified	6	5	147	1	85.7 % (48.7 - 99.3)	96.7 % (92.5 - 98.6)	54.6 % (28 - 78.7)	99.3 % (96.3 - 100)

[a] True-positive (TP) – isolates with an appropriately colored colonies growing directly on chromogenic medium or the subculture from BAEV broth that was identified by supplemental tests, RapID STREPT Panel, VITEK 2 and VITEK MS as *E. faecium* and resistant to vancomycin (MIC > 4µg/ml) and teicoplanin (MIC > 2µg/ml). False positive (FP) – isolates with typically colored colonies on chromogenic medium or on a BAEV broth subculture that are not identified as VR *E. faecium*. True negative (TN) – no growth on chromogenic medium or on a BAEV broth subculture or growth of colonies without the typical coloration and morphology for VR *E. faecium* on the respective medium. False negative (FN) - absence of VR *E. faecium* isolate on all chromogenic media, but isolation from BEAV broth.

[b] Confidence interval (CI); positive predictive value (PPV); negative predictive value (NPV);

1.2. Results of identification with basic phenotypic tests

Forty-five non-repetitive suspicious for VRE isolates were subjected to biochemical identification. They showed a morphology of Gram-positive cocci and did not produce the enzyme catalase. A total of 36 isolates were positive to the classic phenotypic tests, which are typical for the genus *Enterococcus*: growth in medium with 40% bile salts and esculin hydrolysis, growth in 6.5% NaCl, positive PYR and LAP tests. Subsequently, 34 of them were identified as enterococcal species and two of them as *L. garvieae*. The rest 9 Gram-positive, catalase-negative cocci demonstrated variable results regarding the indicated phenotypic tests and were grouped as *Enterococcus*-like microorganisms.

The results of presumptive species identification with phenotypic tests of all non-repetitive intestinal isolates of the *Enterococcus* genus showed that *E. faecium* were MGP negative, fermented mannitol and arabinose, hydrolyzed arginine, and sorbitol and raffinose fermentation was demonstrated in 2 and 3 of them, respectively. Biochemical tests of *E. faecalis* isolates were similar to those of *E. faecium* with exception for arabinose fermentation. MIC data for glycopeptides showed that only 5 *E. faecium* were resistant to vancomycin (MIC ≥ 256 μg/ml) and teicoplanin (MIC 4 – 6 μg/ml). The rest 3 *E. faecium*, 3 *E. faecalis* and *E. hirae* were susceptible to both glycopeptides and therefore dropped out from the study.

E. gallinarum and *E. casseliflavus* isolates were motile, MGP positive, ferment mannitol, arabinose and raffinose, and all *E. casseliflavus* revealed a yellow pigment. Two *E. casseliflavus* fermented sorbitol, and arginine hydrolysis was demonstrated in 20 out

of 22 VanC strains. Sorbose was not fermented by any of the enterococcal isolates. Regarding glycopeptide resistance, one *E. gallinarum* had a high-level resistance to vancomycin (MIC ≥ 256 μg/ml) and teicoplanin (MIC ≥ 256 μg/ml) – VanA phenotype, and the rest *E. gallinarum* and *E. casseliflavus* had low-levels resistance to vancomycin (MIC 4 – 12 μg/ml) and susceptibility to teicoplanin (MIC 0.50 – 0.75 μg/ml) – VanC phenotype.

1.3. Results of identification with manual and automated systems, and detection of genes for species identification

All 27 non-repetitive VR enterococcal isolates were identified to species using the RapID STR manual test, VITEK 2 Compact and VITEK MS automated systems, and PCR for detection of species-specific *ddl* genes.

RapID STR correctly determined the species level of 20 (74.1%) enterococcal isolates, of which 5 *E. faecium* (probability > 99.9%), 10 *E. gallinarum* (probability: 89.45 – > 99.9%) and 5 *E. casseliflavus* (probability: 98.5% – > 99.9%). Seven vanC enterococci were misidentified despite of the high confidence rate (98.59 – > 99.9%). Four *E. gallinarum* were initially identified as *E. casseliflavus*, while 3 *E. casseliflavus* were identified as *E. gallinarum*. Appelbaum et al. (Appelbaum et al. 1986) have used RapID STR to determine the species level of 48 isolates, of which 46 (93.7%) were correctly identified as *E. faecalis* (28), *E. faecium* (9) and *E. durans* (9).

The automated system Vitek 2 Compact gave a definitive biochemical identification of 21 out of 27 VRE (77.8%), and in 6 (22.2%) there was a low level of species discrimination between *E. casseliflavus* and *E. gallinarum*. The results from Vitek 2 Compact results showed a

high degree of similarity in the substrate profile of *E. faecium* isolates and a probability level of 90% (Good identification) to 97% (Excellent identification). The Vitek MS system determined accurately the species level of all VRE isolates, which completely correlated with the detected species-specific *ddl* genes. Ramotar et al. (Ramotar et al. 2000) reported that from 115 *vanC1* isolates, 13 were correctly identified as *E. gallinarum*, 45 were identified as *E. gallinarum*/*E. casseliflavus*, 27 were misidentified as *E. faecalis* or *E. faecium*, and 29 VRE have been confirmed only to the genus level. Due to the reduced capabilities of VITEK to identify non-*faecalis* and non-*faecium* species, the use of the MALDI-TOF system has been recommended in the recent years (Matrix-assisted Laser Desorption Ionization-Time-of-Flight) (Teixeria et al. 2015).

2. STUDY OF THE INTESTINAL COLONIZATION WITH VRE IN PATIENTS AT HIGH RISK FOR COLONIZATION AND INFECTION WITH PROBLEMATIC MICROORGANISMS

2.1. Demographic and clinical characteristics of the studied patients

The demographic data collected from 307 patients (97 from the HD Clinic, 119 from the HT Clinic, and 92 from the ICU) screened for fecal VRE carriage showed that their age range was 18–89 years, the majority falling in the range from 70 to 79 years (a total of 90) and from 60 to 69 (a total of 82). These two age groups predominate in all three clinical units and make up a total of 56% of all patients.

The analysis of the gender revealed an approximately equivalent proportion between men – 158 (51.5%) and women – 149 (48.5%). However, in the different clinics the distribution is uneven. In the HD Clinic, men are almost twice as many as women - men:women 61:36, while in ICU it is the opposite - men:women 27:64.

The summary results from the study of the main demographic and clinical indicators of the screened patients are as follows:

- In the hemodialysis patients, the mean age was 61.95 ± 12.08 years, (range: 26-83 years), and the mean duration of dialysis treatment was 63 months (range: 1-337 months). In 94.9% of them, the weekly number of dialysis is three times, and in 71.1% the vascular access for carrying out the dialysis procedure is through an arterio-venous fistula. Antibiotics were taken by 86.6% of all patients in the last 30 days.
- In the patients with hematological malignancies, the mean age was 66.94 ± 12.69 years (range: 20-88 years), and the mean length of

hospital stay was 9 days (range 6-46 days). The most common underlying disease is myelodysplastic syndrome, found in 23.5% of the patients. About 10% of all patients took antibiotics during the hospital stay and/or in the last 30 days.

- In the intensive care patients, the mean age was 58.60 ± 16.25 years (range: 18-87 years), and the mean length of hospital stay was 15 days (range: 3-105 days). The leading reason for admission to the intensive care unit in 69.2% of patients was the need for postoperative care. Concomitant cardiovascular disease was recorded in about half of the patients (44%). During the hospital stay, 93.4% took antibiotics.

Tables 3, ***4***, and ***5*** show demographic and clinical data of VRE-colonized and non-colonized patients in the three risk groups.

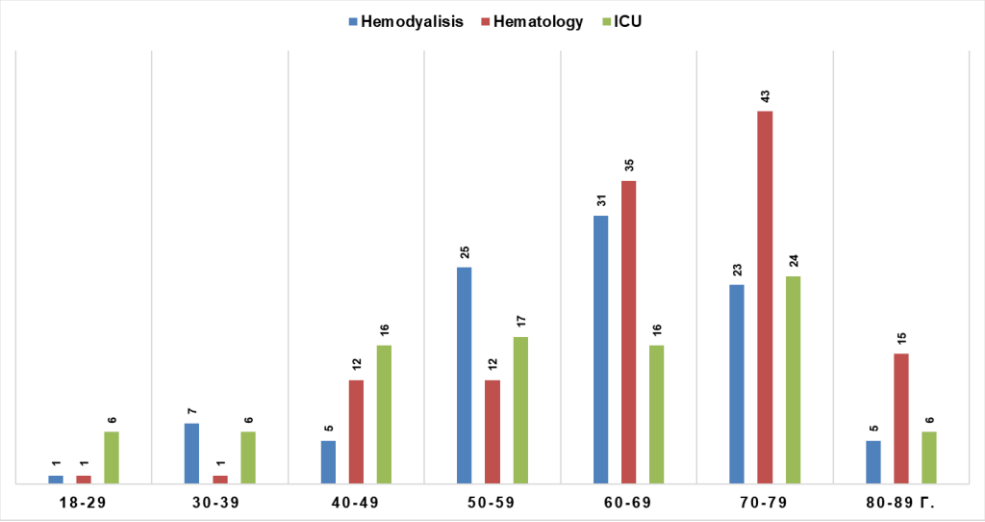


Figure 2. Age distribution of patients colonized with VRE.

Table 3. Demographic and clinical data of 97 hemodialysis patients tested for fecal VRE carriage

Characteristics	VRE colonized	non-VRE colonized	*p*-value
Demographics			
Age (years)	63.56 ± 2.3	61.33 ± 1.45	NS
Sex; male:female	16:11	45:25	NS
Primary disease; n (%)			
Chronic glomerulonephritis	9 (33.3)	14 (20)	NS
Arterial hypertension	9 (33.3)	22 (31.4)	NS
CAKUT	-	2 (2.9)	NS
Diabetes mellitus type II	3 (11.1)	12 (17.1)	NS
Chronic interstitial nephritis	1 (3.7)	2 (2.9)	NS
Chronic pyelonephritis	1 (3.7)	5 (7.1)	NS
Agenesis	-	4 (5.7)	NS
Balkan endemic nephropathy	-	1 (1.4)	NS
ADPKD	3 (11.1)	7 (10)	NS
Diabetes mellitus type I	-	1 (1.4)	NS
Multiple myeloma	1 (3.7)	-	NS
Duration of hemodialysis (months), medium (range)	71.4 (2-252)	59.71 (1-337)	NS
0-12 months	7 (25.9)	12 (17.1)	NS
13-60 months	8 (29.6)	37 (52.9)	**0.040**
Over 60 months	12 (44.4)	21 (30)	NS
Frequency of hemodialysis (per week); **n (%)**			
Once	-	2 (2.9)	NS
Twice	2 (7.4)	1 (1.4)	NS
Tree times	25 (92.6)	67 (95.7)	NS
Vascular access			
Arteriovenous fistula	17 (63)	52 (74.3)	NS
Tunneled permanent catheter	13 (48.1)	19 (27.1)	**0.049**
Graft	-	1 (1.4)	NS
Underling diseases; n (%)			
Cardio-vascular	15 (55.6)	41 (58.6)	NS
- Arterial hypertension	7 (25.9)	27 (38.6)	NS
- Heart failure	8 (29.6)	14 (20)	NS
Endocrine	1 (3.7)	4 (5.7)	NS
- Diabetes mellitus type II	1 (3.7)	3 (4.3)	NS
- Hyperthyroidism	0	1 (1.4)	NS
Respiratory	2 (7.4)	4 (5.7)	NS
- COPD	1 (3.7)	3 (4.3)	NS
- Asthma	1 (3.7)	1 (1.4)	NS
Urogenital	2 (7.4)	7 (10)	NS
- Chronic glomerulonephritis	-	1 (1.4)	NS
- Kidney stone disease	1 (3.7)	5 (7.1)	NS
- Chronic pyelonephritis	1 (3.7)	1 (1.4)	NS
Hepatobiliary	3 (11.1)	4 (5.7)	NS
Malignancies	3 (11.1)	5 (7.1)	NS
Gout	1 (3.7)	3 (4.3)	NS

Legend CAKUT: Congenital anomalies of the kidney and urinary tract; ADPKD: Autosomal dominant polycystic kidney disease; COPD: Chronic obstructive pulmonary disease.

Table 3. continued from previous page

Antibiotic use in last 30 days; n (%)			
Penicillins	1 (3.7)	3 (4.3)	NS
1st generation cephalosporins	-	3 (4.3)	NS
2nd generation cephalosporins	3 (11.1)	6 (8.6)	NS
3rd generation cephalosporins	21 (77.8)	55 (78.6)	NS
4rd generation cephalosporins	7 (25.9)	9 (12.9)	NS
Carbapenems	-	1 (1.4)	NS
Monobactams	-	4 (5.7)	NS
Aminoglycosides	9 (33.3)	26 (37.1)	NS
Fluoroquinolones	4 (14.8)	10 (14.3)	NS
Antifungal drugs	1 (3.7)	4 (5.7)	NS
Vancomycin	7 (25.9)	5 (7.1)	**0.033**
Metronidazole	2 (7.4)	6 (8.6)	NS

Table 4. Demographic and clinical data of 119 patients with hematological malignancies tested for fecal carriage of VRE

Characteristics	**VRE colonized**	**non-VRE colonized**	***p*-value**
Demographics			
Age (years)	74.31±6.58	65.52±13.37	**0.025**
Gender; male:female	12:6	57:44	NS
Mean length of stay (days)	11.15±4.705	8.57± 5.11	NS
Primary hematology disease; n (%)			
Myelodysplastic syndrome	3 (23.08)	20 (20.83)	NS
Non-Hodgkin Lymphoma	0	25 (26.04)	NS
Multiple myeloma	7 (53.85)	13 (13.54)	**0.001**
Chronic lymphocytic leukemia	2 (15.38)	13 (13.54)	NS
Hodgkin Lymphoma	0	9 (9.38)	NS
Chronic erythremia	0	4 (4.17)	NS
Acute myeloblastic leukemia	1 (7.69)	4 (4.17)	NS
Acute lymphoblastic leukemia	0	3 (3.13)	NS
Acute myelomonocytic leukemia	0	2 (2.08)	NS
Acute promyelocytic leukemia	0	1 (1.04)	NS
Hairy-cell Leukemia	0	1 (1.04)	NS
Chronic monocytic leukemia	0	1 (1.04)	NS
Risk factors; n (%)			
Chronic heart failure	9 (69.23)	48 (50)	NS
Chronic obstructive pulmonary disease	1 (7.96)	10 (10.42)	NS
Chronic renal failure	2 (15.38)	7 (7.29)	NS
Diabetes mellitus type II	5 (38.46)	18 (18.75)	NS
Obesity	2 (15.38)	14 (14.58)	NS
Chemotherapy	8 (61.54)	45 (46.88)	NS
Corticosteroids use	7 (53.85)	39 (40.63)	NS
Antibiotic use in last 30 days and during the current hospital stay; n (%)			
3rd generation cephalosporins	1 (7.96)	3 (3.13)	NS
Fluroquinolones	1 (7.96)	2 (2.08)	NS
Macrolides	0	4 (4.17)	NS
Antifungal drugs	0	2 (2.08)	NS

Table 5. Demographic and clinical data of 91 intensive care patients tested for fecal carriage of VRE

Characteristics	VRE colonized	non-VRE colonized	*p*-value
Demographics			
Age (years)	62.5 ± 14.83	57.36 ± 16.59	0.299
Gender; male:female	7:15	20:49	0.800
Mean length of stay (days)	16.86 (3-55)	14 (3-105)	0.365
Reason for admission in ICU; n (%)			
Septic shock	2 (9.1)	5 (7.3)	0.247
Postoperative cares	8 (36.4)	55 (79.7)	**< 0.001**
Respiratory failure	3 (13.6)	3 (4.3)	0.150
Trauma	2 (9.1)	2 (2.9)	0.245
Others*	7 (31.8)	4 (5.8)	**0.003**
Underling diseases; n (%)			
Cardiovascular	16 (72.7)	28 (40.6)	**0.009**
Respiratory	2 (9.1)	5 (7.2)	0.674
Hepatobiliary	1 (4.5)	4 (5.8)	0.651
Gastrointestinal	1 (4.5)	3 (4.3)	0.969
Genitourinary	1 (4.5)	3 (4.3)	0.969
Endocrine	5 (22.7)	14 (20.3)	0.772
Malignancies	1 (4.5)	8 (11.6)	0.446
Recent abdominal operations	5 (22.7)	21 (30.4)	0.486
Use of invasive devices; n (%)			
Central venous catheter	21 (95.5)	57 (82.6)	0.176
Drainage tube	16 (72.7)	57 (82.6)	0.360
Nasogastric tube	4 (18.2)	17 (24.6)	0.531
Urinary catheter	12 (54.5)	42 (60.9)	0.599
Endotracheal tube	15 (68.2)	65 (94.2)	**0.003**
Antibiotic use during the current hospital stay; n (%)			
Penicillins	1 (4.5)	4 (5.8)	0.822
1st generation cephalosporins	1 (4.5)	1 (1.4)	0.427
3rd generation cephalosporins	13 (65)	54(76.1)	0.322
4rd generation cephalosporins	1 (4.5)	1 (1.4)	0.427
Carbapenems	3 (13.6)	4 (5.8)	0.353
Aminoglycosides	-	7 (10.1)	0.189
Metronidazole	7 (31.8)	31 (44.9)	0.278
Fluoroquinolones	1 (4.5)	3 (4.3)	0.969
Tigecycline	-	3 (4.3)	0.610
Vancomycin	1 (4.5)	3 (4.3)	0.969
Antifungal drugs	5 (22.7)	10 (14.5)	0.509
Colistin	2 (9.1)	2 (2.9)	0.245
Clindamycin	1 (4.5)	2 (2.9)	0.569
Linezolid	-	3 (4.3)	0.320

*__Others__: Traffic accidents (n=5); Intoxications (n=4); Suicide injuries (n=2).

Figure 2 shows the age of patients colonized with VRE in the three clinical units. It is clear that people aged 70 to 79 (25 out of 67 – 37.3%) and 60 to 69 (17 out of 67 – 25.4%) were the most commonly affected, while patients aged 18 to 29 (1 from 67 – 1.5%) were the least

affected. These data corresponded to the demographic characteristics of the studied patient groups. In all three clinical units, persons in the age groups of 70 to 79 years and 60 to 69 years were predominating.

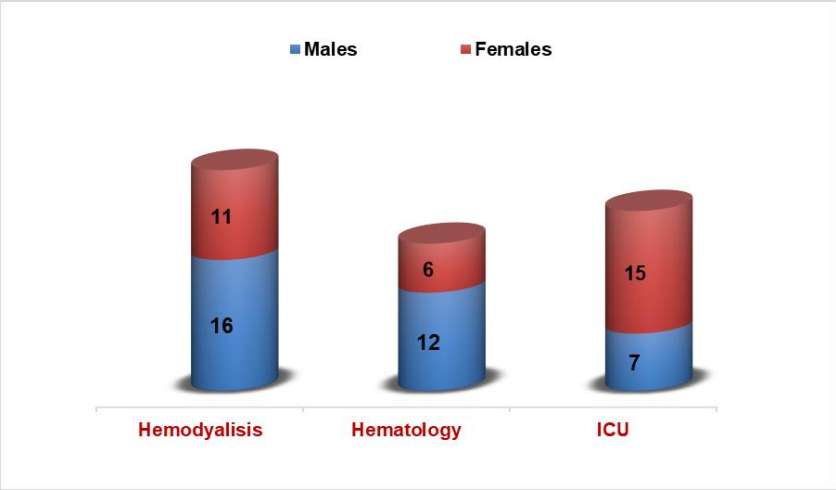


Figure 3. Gender distribution of the patients colonized with VRE.

Figure 3 demonstrates the proportion of males and females colonized with VRE in the three clinical units. It can be seen that fecal carriage predominates in men in the HT Clinic (male:female, 12:6), while in ICU the colonized females were twice as many as males (male:female, 7:15).

The summary results of the study of the main demographic and clinical parameters of the patients colonized with VRE are as follows:

- In the colonized hemodialysis patients, the average age was 63.56 ± 2.3 with a predominance of men (n=16; 59.3%), and the average duration of hemodialysis was 71.4 months (range: 2-252 months). The leading underlying disease in 18 (66.6%) of the VRE positive individuals was chronic glomerulonephritis or arterial hypertension, and in 15 (55.6%) diseases of the cardiovascular system were confirmed as accompanying (arterial hypertension or heart failure).

- In the colonized patients with hematological malignancies, the average age was 74.31 ± 6.58 years with a predominance of men (n=12, 66.7%). The mean length of hospital stay was 11.15 ± 4.705 days. The most common underlying disease was multiple myeloma, found in 53.9% of colonized individuals, and CHF was confirmed as a concomitant disease in 69.2%.
- In the colonized intensive care patients, the average age was 62.5 ± 14.83 years with a predominance of women (n=15, 68.2%). The mean length of hospital stay was 17 days. The leading reason for admission to the intensive care unit in 36.4% of the colonized patients was the need for postoperative care, and in 72.2% of the individuals it was accompanied by a disease of the cardiovascular system.

2.2. Risk factors for intestinal colonization with VRE

The patients undergoing hemodialysis included in the statistical analysis were divided into two groups - case and control. The case group included 27 patients, who were colonized with VRE. The other 70 individuals had no evidence of colonization/infections with VRE or another microorganism. They were included in the control group.

The univariate analysis of risk factors for intestinal colonization with VRE in hemodialysis patients found a statistically significant association between colonization and the following variables: duration of hemodialysis between 13-60 months (p=0.040), vascular access through an indwelling tunneled catheter (p=0.049) and use of vancomycin (p=0.033). The use of vancomycin increased the risk of VRE colonization 4.550-fold [OR (95% CI) = 4.550 (1.301-15.918)]. In addition, multivariate analysis confirmed only vancomycin

administration as an independent risk factor for VRE colonization [*p*=0.027, OR (95% CI) = 0.236 (0.065-0.848)] in the patients undergoing hemodialysis. D'Agata et al. (D'Agata et al. 2001) also reported that the use of vancomycin promoted fecal colonization with VRE in hemodialysis patients.

Among patients with hematological malignancies, 18 were VRE positive, and 13 patents were assigned to the case group because they had hospital-acquired VRE colonization. The remaining 5 patients had positive cultures for vanC enterococci during the first 48 hours of hospitalization and were excluded from the analysis. Colonization/infection with VRE or another microorganism was not detected in 96 out of 101 non-VRE colonized patients and they were included in the control group.

The results of univariate analysis of risk factors for fecal carriage of VRE in patients with hematologic malignancies confirmed a statistically significant association between colonization and the following variables: age 70 to 79 years (*p*=0.025) and multiple myeloma (*p*=0.001). In patients aged 70 to 79 years, the risk of acquiring VRE was on average 4 times higher [OR (95% CI) = 3.697 (1.114-12.264)], when compared to the other age groups. Also, the probability of acquiring VRE in patients with multiple myeloma was on average 7 times higher [OR (95% CI) = 7.449 (2.161-25.669)]. Multivariate analysis confirmed age 70 to 79 years as an independent risk factor for fecal VRE colonization in patients with hematological malignancies [*p*=0.019, OR (95% CI) = 4.328 (1.395-14.737)]. Metallidis et al. (Metallidis et al. 2006) also found a correlation between patients' age and VRE colonization, while other authors found no relationship

between these two variables (Suntharam et al. 2002; Batistão et al. 2012; Pan et al. 2012; Ford et al. 2015).

VRE colonization was found in 22 intensive care patients assigned to the case group. The remaining 69 individuals without colonization/infection with VRE or another microorganism were included in the control group.

The data from univariate analysis of risk factors for VRE intestinal colonization in intensive care patients demonstrated a statistically significant association between colonization and the following four variables: postoperative care (p<0.001), other reason for admission to ICU (p=0.003), cardiac vascular disease (p=0.009) and presence of an endotracheal tube (p=0.003). Multivariate analysis demonstrated that postoperative cares [p=0.021, OR (95% CI) = 0.223 (0.063-0.798)] and cardiovascular diseases [p=0.018, OR (95% CI) = 4.128 (1.272-13.398)] are independent risk factors for intestinal colonization with VRE in the intensive care patients. Amberpet et al. (Amberpet et al. 2016) found that the prolonged hospitalization (p=0.00), the younger patient age (p=0.030), use of ceftriaxone (p=0.25) and vancomycin (p=0.048) were associated with the acquisition of VRE in the intensive care units. A study by Pan et al. (Pan et al. 2012) showed that the length of hospitalization in an intensive care unit was an independent risk factor for VRE colonization (p=0.03).

2.3. Incidence of intestinal colonization with VRE

The results of the testing of the three groups of high-risk patients indicated that intestinal colonization with VRE was detected:

- In 27 (27.8%) out of 97 patients on hemodialysis, and in 24 of them the VRE colonization was detected during the initial screening, and

in 3 during the second. When screening 1318 patients from 56 Belgian dialysis centers, 437 (33.2%) of them were found to be VRE carriers, with colonization rates between 14.3% to 61.1% among different centers (Descheemaeker et al. 2000).

- In 18 (15.1%) out of 119 patients with hematological malignancies, and VRE colonization was detected in 5 of them during the first 48 hours of hospitalization, in 12 – on the 5th day, and in 1 – on the 18th day. In addition, two types of VRE were isolated from one patient. Gedik et al. (Gedik et al. 2014) found that 50 (39.7%) from 126 studied patients with hematological malignancies were colonized with VRE.
- In 22 (24.2%) out of 91 intensive care patients, and two types of VRE were isolated from one patient. These findings were in correlation with the data presented in a study by Amberpet et al. (Amberpet et al. 2016), who reported 29% incidence of VRE colonization in an intensive care unit.

Intestinal colonization with VRE was demonstrated in 67 (21.8%) out of 307 screened patients. Carriage of one type of VRE was found in 65 patients, and two types of VRE were isolated from two patients. The frequency of colonization is highest in the HD Clinic (27.8%), followed by ICU (24.2%) and the HT Clinic (15.1%).

A total of 69 intestinal VRE – 1 (1.5%) *E. faecalis*, 14 (20.3%) *E. faecium*, 21 (30.4%) *E. casseliflavus* and 33 (47.8%) *E. gallinarum* were isolated from all 67 colonized patients. None of the VRE colonized patients in the three risk groups developed VRE infection during the hospital stay.

The species distribution of VR enterococcal isolates by patients' groups is presented in ***Figure 4***. The data showed that out of 14 *E. faecium*, 7 were found in patients from the intensive care units, 5 in patients undergoing hemodialysis and 2 in patients with hematological malignancies. Two patients from the HT Clinic were colonized with *vanA* or *vanB* enterococci. The number of patients carrying *E. casseliflavus* in the HD and ICU Clinics is approximately the same, 8 and 9, respectively, while in the HT Clinic this number is twice lower – 4. The number of the colonized persons with *E. gallinarum* was the highest in the HD Clinic– 14, and the lowest in ICU – 7 (***Figure 4***).

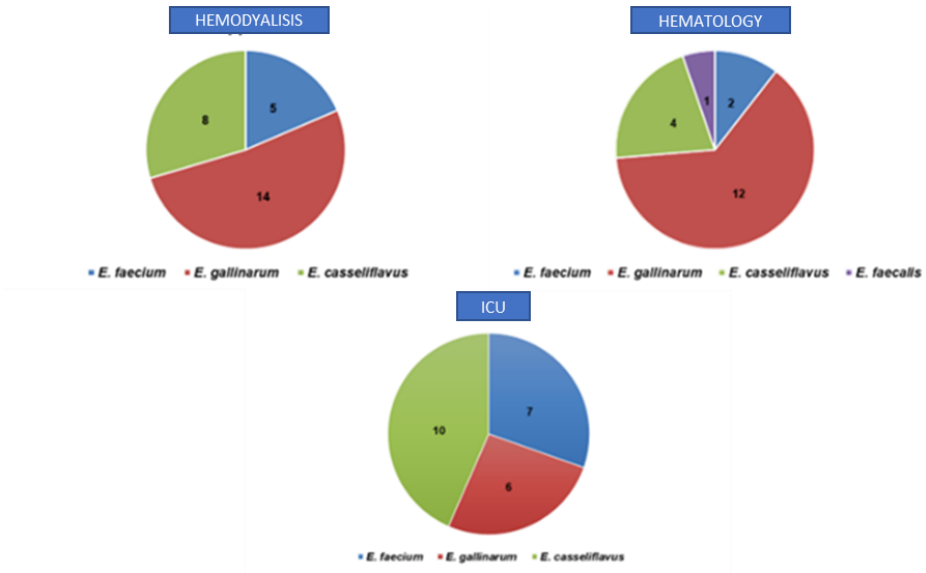


Figure 4. Species distribution of intestinal VRE in the patients from the three clinical units.

The species distribution of VRE isolates showed that in all three clinical units *E. gallinarum* and *E. casseliflavus* (total 54 or 78.3%) predominated, when compared to *E. faecium* and *E. faecalis* isolates (a total of 15 or 21.7%).

3. CHARACTERISTICS OF VRE, ISOLATED IN FECAL SCREENING OF PATIENTS AT HIGH RISK FOR COLONIZATION AND INFECTION WITH PROBLEMATIC MICROORGANISMS

3.1. Antimicrobial susceptibility and detection of *van* genes in intestinal VRE

Table 6 presents the antimicrobial susceptibility of 14 intestinal VR *E. faecium* and 1 VR *E. faecalis* determined by *E*-test and the detected *van* genes in the same isolates.

***Table 6*.** MIC values and *van* genes in 15 intestinal *E. faecium* and *E. faecalis*

Isolate №/ Clinical unit	Species	MIC (μg/ml)									*van* gene
		AMP	GEN	CIP	VAN	TEC	TGC	LZD	Q/D	DAP	
56/HT	*E. faecalis*	**0.25**	≥1024	**2**	**12**	**0.50**	0.125	3	3	0.75	*vanB*
56/HT	*E. faecium*	≥256	≥1024	≥32	≥256	**96**	0.047	3	**≥32**	2	*vanA*
58/HT	*E. faecium*	≥256	**8**	≥32	**8**	**0.50**	0.064	2	2	1	*vanB*
11/HD	*E. faecium*	≥256	≥1024	≥32	≥256	**6**	0.064	1.5	**≥32**	1	*vanA*
16/HD	*E. faecium*	≥256	≥1024	≥32	≥256	**6**	0.064	2	**≥32**	1	*vanA*
36/HD	*E. faecium*	≥256	≥1024	≥32	≥256	**6**	0.064	2	**≥32**	1	*vanA*
47/HD	*E. faecium*	≥256	≥1024	≥32	≥256	**6**	0.064	2	**≥32**	1	*vanA*
75/HD	*E. faecium*	≥256	≥1024	≥32	≥256	**6**	0.064	1.5	**≥32**	1	*vanA*
3/ICU	*E. faecium*	≥256	≥1024	≥32	≥256	**≥256**	0.032	2	0.50	0.38	*vanA*
4/ICU	*E. faecium*	≥256	≥1024	≥32	≥256	**128**	0.064	3	3	0.75	*vanA*
5/ICU	*E. faecium*	≥256	≥1024	≥32	≥256	**48**	0.125	3	0.50	0.50	*vanA*
6/ICU	*E. faecium*	≥256	≥1024	≥32	≥256	**16**	0.094	2	0.75	1	*vanA*
10/ICU	*E. faecium*	≥256	≥1024	≥32	≥256	**16**	0.125	2	0.50	1	*vanA*
26/ICU	*E. faecium*	≥256	**12**	≥32	≥256	**≥256**	0.094	2	0.75	0.75	*vanA*
64/ICU	*E. faecium*	≥256	≥1024	≥32	≥256	**6**	0.094	2	0.25	1	*vanA*

Legend AMP: ampicillin; GEN: gentamicin; CIP: ciprofloxacin; VAN: vancomycin; TEC: teicoplanin; TGC: tigecycline; LZD: linezolid; Q/D: quinupristin/dalfopristin; DAP: daptomycin.

The results of DDM and *E*-test showed that *E. faecalis* №56/HT and *E. faecium* №58/HT from the Clinic of Hematology had a low level of resistance to vancomycin (MIC: 8 – 12 μg/ml) and sensitivity to teicoplanin (MIC = 0.50 μg/m), which corresponded to the classical VanB phenotype. The *vanB* gene was confirmed in the two isolates. *E. faecalis* №56/HT had a high level of resistance to gentamicin (MIC ≥ 1024 μg/ml), while *E. faecium* №58/HT to ampicillin (MIC ≥ 256 μg/ml) and ciprofloxacin (MIC ≥ 32 μg /ml).

High-level resistance to ampicillin (MIC ≥ 256 µg/ml), gentamicin (MIC ≥ 1024 µg/ml), ciprofloxacin (MIC ≥ 32 µg/ml) and vancomycin (MIC ≥ 256 µg/ml), together with varying levels of resistance to teicoplanin (MIC: 6 – ≥ 256 µg/ml) and sensitivity to tigecycline, linezolid and daptomycin were found in 12 *E. faecium*. One *E. faecium* (№26/ICU) showed similar multiple resistance patterns, except for gentamicin values (MIC = 12 µg/ml). In all 13 VR *E. faecium*, the *vanA* gene was found. Only 5 (38.5%) of the 13 *vanA* isolates had the usual high level of vancomycin (MIC ≥ 256 µg/ml) and teicoplanin (MIC: 48 – ≥ 256 µg/ml) resistance. The other 8 (61.5%) showed low to moderate resistance to teicoplanin (MIC: 6 – 16 µg/ml), resembling the VanD phenotype (VanD-*vanA*). In a study of 20 VanD-*vanA E. faecium* isolated from intestinal screening of intensive care patients, Song et al. (Song et al. 2013) found that these isolates were a heterogeneous, unstable population that could transform to the VanA phenotype after exposure to glycopeptides. These results led the authors to the conclusion that teicoplanin was not effective for the treatment of VanD-*vanA* enterococci.

Regarding the susceptibility to quinupristin/dalfopristin, the two *vanB* and half of the *vanA* isolates had retained their susceptibility to this agent. The five *E. faecium* from the Hemodialysis Clinic and *E. faecium* №56/HT from the Hematology Clinic were resistant to quinupristin/dalfopristin (MIC ≥ 32 µg/ml).

Table 7 and ***Table 8*** present the MIC_{50} and MIC_{90} values for 32 intestinal *E. gallinarum* and 21 *E. casseliflavus*.

Table 7. MIC_{50} and MIC_{90} in 32 intestinal *E. gallinarum* with VanC phenotype

Antimicrobial agent	MIC range (µg/ml)	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	Susceptibility (%)
Ampicillin	0.75 - ≥ 256	1.5	2	96.9
Gentamicin	0.75 - ≥ 1024	1.5	4	96.9
Ciprofloxacin	1 - ≥ 32	3	4	93.8
Vancomycin	2 - 16	8	12	0
Teicoplanin	0.50 - 1	0.50	1	100
Linezolid	1 - 3	1.5	2	100
Tigecycline	0.038 - 0.19	0.064	0.125	100
Daptomycin	0.38 - 2	0.50	0.75	100

Table 8. MIC_{50} and MIC_{90} in 21 intestinal *E. casseliflavus* with VanC phenotype

Antimicrobial agent	MIC range (µg/ml)	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	Susceptibility (%)
Ampicillin	0.38 - 2	1	1	100
Gentamicin	0.50 - 64	2	3	95.2
Ciprofloxacin	1 - 32	2	4	95.2
Vancomycin	2 - 12	4	6	0
Teicoplanin	0.5 - 1	0.50	1	100
Linezolid	1 - 3	2	2	100
Tigecycline	0.32 - 0.125	0.064	0.125	100
Daptomycin	0.38 - 2	0.50	1	100

E. gallinarum №31/HD showed multiple resistance by using the DDM, including resistance to vancomycin and teicoplanin, and susceptibility only to tigecycline and linezolid (***Figure 5A***). The same isolate demonstrated high levels of resistance to vancomycin and teicoplanin (MIC ≥ 256 µg/ml) (***Figure 5B***), characteristic of the VanA phenotype, and *vanA* gene was confirmed in it. Various studies have reported the acquisition of *van* genes in non-*faecium* non-*faecalis* intestinal enterococci (Corso et al. 2005; Neves et al. 2009; Shirano et al. 2011; Eshaghi et al. 2015).

The MIC values for the other tested antibiotics were as follows: ampicillin (MIC ≥ 256 µg/ml), gentamicin (MIC ≥ 1024 µg/ml), ciprofloxacin (MIC ≥ 32 µg/ml), quinupristin/dalfopristin (MIC ≥ 32 µg/ml ml), linezolid (MIC = 2 µg/ml), tigecycline (MIC = 0.064 µg/ml) and daptomycin (MIC = 2 µg/ml).

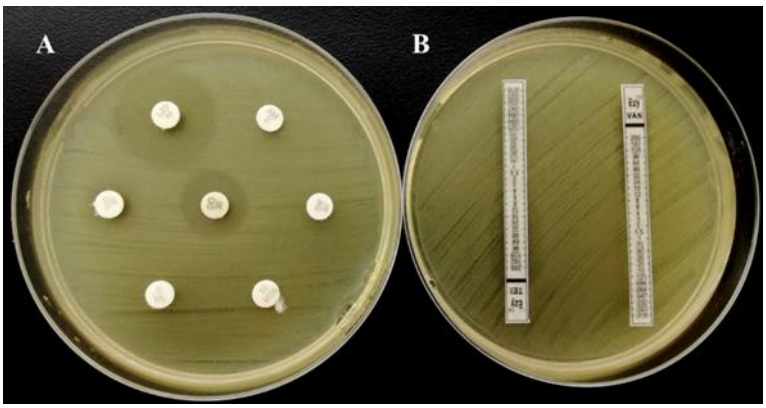


Figure 5. Antibiotic profile of E. gallinarum № 31/HD with VanA phenotype, determined by (**A**) Disc-diffusion method; (**B**) *E*-test method.

The rest *E. gallinarum* and *E. casseliflavus* isolates, except the *E. gallinarum* №31/HD, had low levels of resistance to vancomycin, (MIC_{50} = 8 μg/ml / 4 μg/ml and MIC_{90} = 12 μg/ml / 6 μg/ml, respectively) and were susceptible to teicoplanin (***Table 7*** and ***Table 8***), which corresponded to the VanC phenotype. The *vanC1* gene was confirmed in 32 *E. gallinarum*, and the *vanC2* in all *E. casseliflavus*.

MIC values showed that VanC isolates were susceptible to linezolid and tigecycline. The susceptibility of *E. gallinarum* and *E. casseliflavus* to ampicillin was 96.9%/100%, respectively; to gentamicin – 96.9%/95.2%, to ciprofloxacin – 93.8%/95.2% and to daptomycin – 100%/100%.

3.2. Detection of genes encoding aminoglycoside resistance in intestinal VRE

Resistance to aminoglycosides was demonstrated in 18 out of 69 studied intestinal VRE – 14 *E. faecium*, 2 *E. gallinarum*, 1 *E. faecalis* and 1 *E. casseliflavus*. High-level resistance to gentamicin (MIC ≥ 1024 μg/ml) was found in 15 of the isolates, whereas 3 isolates (2 *E. faecium* and 1 *E. casseliflavus*) had MIC values ranging from 8 to 64 μg/ml.

The results from the detection of genes encoding aminoglycoside-modifying enzymes and MIC values to gentamicin showed that the

bifunctional *aac(6')-Ie-aph(2")-Ia* gene was detected in 17 VRE – in all 15 enterococci with high-level of aminoglycoside resistance and in two *E. faecium* with low MIC values (8 μg/ml and 12 μg/ml). In 2021, Chen et al. (Chen et al. 2021) first described 15 *E. faecium* and 2 *E. faecalis* with non-HLGR phenotype in which *aac(6')-Ie-aph(2")-Ia* gene was detected. These data indicate the ability of *E. faecium* isolates to alter the HLGR phenotype, which may help the pathogen adapt to the nosocomial environment (Chen et al. 2021).

In 15 of the isolates *aac(6')-Ie-aph(2")-Ia* was expressed alone, and in two *E. faecium* it was in combination with *aph(3')-IIIa*. In the *E. casseliflavus* isolate, the *ant(3')-Ia* gene was found.

3.3. Detection of genes encoding virulence factors in intestinal VRE

In 21 isolates (30.4%) – 1 *E. faecalis*, 14 *E. faecium* and 6 *E. gallinarum* one or more genes (*ace/acm*, *asa1*, *cylA*, *efaA*, *esp*, *gelE* and *hyl*) were identified. The genes *esp* (n=18), *acm* (n=17) and *hyl* (n=4) were most frequently detected. Genetic determinants for virulence factors were not revealed in 27 *E. gallinarum* and 21 *E. casseliflavus*.

The results of the PCR analysis illustrating the positive genetic determinants in the tested VRE are shown in ***Figure 6***. The distribution of the virulence factor genes in the intestinal VRE was as follows:

- The *E. faecalis* isolate carried four virulence genes (*gelE* + *asa1* + *efaA* + *ace*).
- The 14 VR *E. faecium* isolates were positive for virulence factors, 12 of them carrying two genes (*acm* + *esp*) and 2 carrying three genes (*hyl* + *acm* + *esp*).

- Six (18.2%) out of 33 *E. gallinarum* isolates were positive for virulence factors, 2 were carriers of one gene (1 with *hyl* and 1 with *esp*), 3 were with two genes (2 with *acm* + *esp* and 1 with *hyl* + *acm*), and 1 had five genes (*asa1* + *efaA* + *esp* + *ace* + *cylA*).
- The 21 E. casseliflavus isolates were negative for virulence determinants.

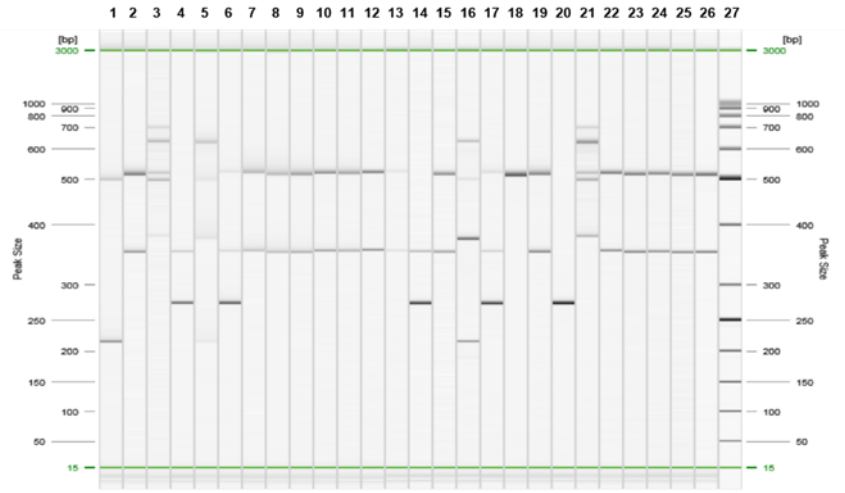


Figure 6. **Detection of *gelE*, *hyl*, *acm*, *asa1*, *efaA*, *esp*, and *cylA* genes in 14 *E. faecium*, 6 *E. gallinarum*, and 1 *E. faecalis* isolates by multiplex PCR**. Line 1 - Control strain for *gelE* and *efaA*; Lane 2 - Control strain № 5 for *acm* and *esp*; Lane 3 - Control strain № 17 for *asa1*, *efaA*, *esp* and *cylA*; Lane 4 - Control strain for *hyl* and *acm*; Lane 5 - Control strain for *gelE* and *asa1*; Lane 6 - №. 4 ICU; Lane 7 - № 5 ICU; Lane 8 - № 6 ICU; Lane 9 - № 10 ICU; Lane 10l - № 26 ICU; Lane 11 - № 64 ICU; Lane 12 - № 3 ICU.; Lane 13 - № 66 ICU; Lane 14 - № 79 ICU; Lane 15 - № 56 HT; Lane 16 - № 56' HT ; Lane 17 - № 58 HT; Lane 18 - № 88 HT; Lane 19 - № 11 HD; Lane 20 - № 60 HD; Lane 21 - № 17 HD; Lane 22 - № 31 HD; Lane 23 - № 16 HD; Lane 24 - № 36 HD; Lane 25 - № 47 HD; Lane 26 - № 75 HD; Lane 27 - marker.

3.4. Epidemiological typing of intestinal VRE

3.4.1. Results of typing of intestinal vanC enterococci in hemodialysis patients

The PFGE profile of 14 *E. gallinarum* is shown in ***Figure 7***. It can be seen that a total of 13 pulsotypes were formed among the tested enterococci, while two isolates (№81/HD and №91/HD) belonged to one type (PT11). The genetic relatedness in the other *E. gallinarum* varied from 18% to 74%.

The data from the PFGE analysis of 8 *E. casseliflavus* are presented in ***Figure 8***. It can be seen that each of the studied isolates

belonged to a different pulsotype, forming a total of 8 pulsotypes – PT1, PT2, PT3, PT4, PT5, PT6, PT7 and PT8. The highest percentage of genetic relatedness is observed between isolates №33/HD and №70/HD – 62%, while for the other strains the percentage varied from 34% to 54%.

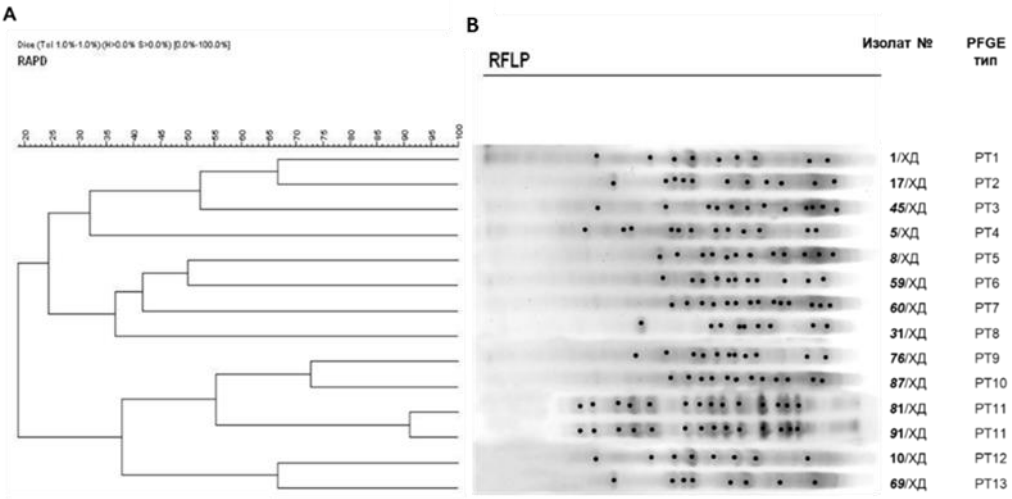


***Figure 7*. PFGE profile of 14 *E. gallinarum* isolates after DNA restriction by S*mal* enzyme.** Dendrogram (**A**) obtained as a result of automated analysis of the chromosomally fragmented DNA (**B**) by PFGE.

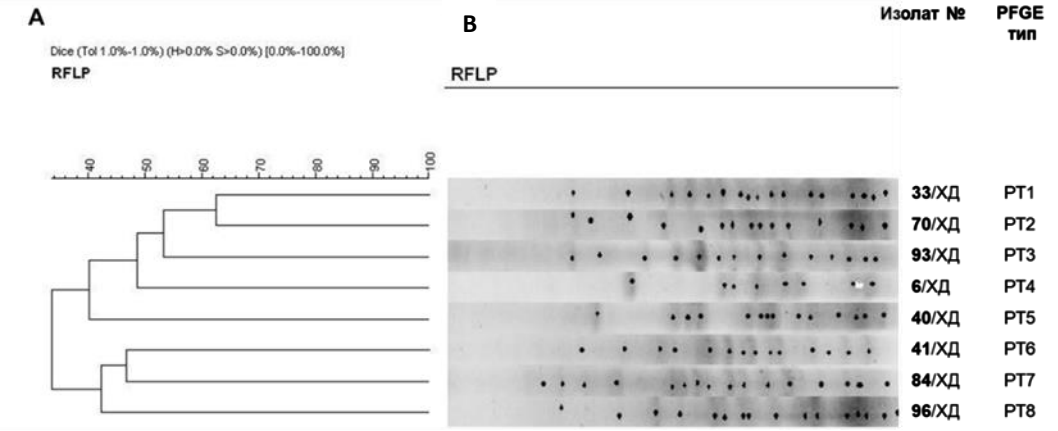


***Figure 8.* PFGE profile of 8 *E. casseliflavus* isolates after DNA restriction by S*mal* enzyme.** Dendrogram (**A**) obtained as a result of automated analysis of the chromosomally fragmented DNA (**B**) by PFGE.

3.4.2. Results of typing of intestinal *vanA E. faecium* from hemodialysis patients

The PFGE profile of 5 *E. faecium* isolated from hemodialysis patients are presented in ***Figure 9***. It was demonstrated that all enterococci belong to one major pulsotype 1 (PT1).

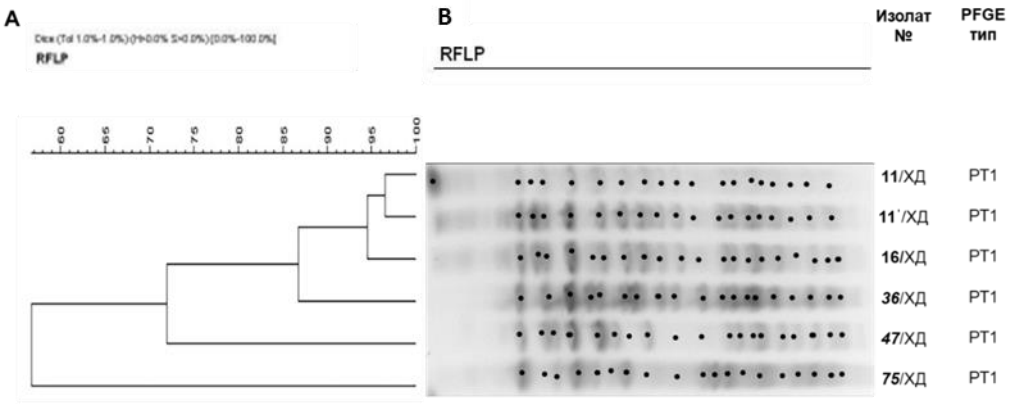


Figure 9. **PFGE profile of 5 *E. faecium* isolates after DNA restriction by S*mal* enzyme.** Dendrogram (**A**) obtained as a result of automated analysis of the chromosomally fragmented DNA (**B**) by PFGE.
Note: Strains №11/HT and №11'/HD are the same isolate tested twice.

3.4.3. Results of typing of all intestinal *vanA E. faecium*

The results of MLVA, reflecting the genetic relationship between 13 intestinal *vanA E. faecium* isolated from high-risk patients in three clinical units of UMHAT "Dr. G. Stranski", Pleven are presented in Figure 9. It can be seen that 10 (76.9%) of the isolates (4 from the Department of Hemodialysis, 1 from the Clinic of Hematology and 5 from ICU) belonged to MT11 (***Figure 10A, B*** and ***C***) and by one isolate belonged to each of the following MLVA types – MT76 (***Figure 10B***), MT77 (***Figure 10C***) and MT84 (***Figure 10A***) belonged to one isolate each. Two of the *E. faecium* isolates that differed from the main detected type MT11, originated from the ICU (MT76 and MT77) and one was from the Hemodialysis Unit (MT84).

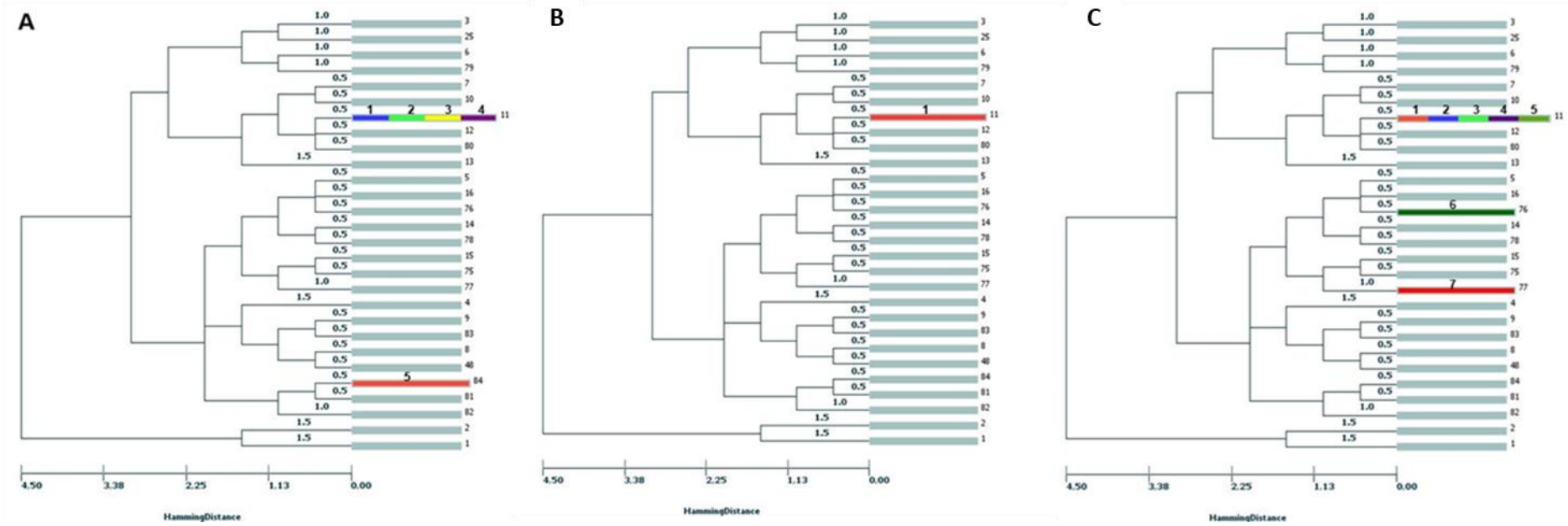


***Figure 10*. Dendrograms showing the genetic relationship between intestinal *vanA E. faecium* genotyped with MLVA10.**

The dendrogram was created using the specialized software PHYLOViZ 2.0. using hierarchical clustering with Hamming distance and Complete-Linkage method clustering algorithm. The distances between the individual clusters are marked with numbers. The following MLVA types (MT) were detected: (**A**) Hemodialysis Unit – MT11 and MT84; (**B**) Hematology Clinic - MT11; (**C**) Intensive care and resuscitation clinic - MT11, MT76 and MT77. The distribution of isolates to each type was as follows: MT11: 10 *E. faecium*; MT 76: 1 *E. faecium*; MT77: 1 *E. faecium*; MT84: 1 *E. faecium*.

4. SPREAD OF CLINICAL VRE IN UMHAT "Dr. G. STRANSKI", PLEVEN

4.1. Results for incidence and species prevalence of clinical VRE

For the period 2016 – 2020, a total of 3134 enterococci were isolated from patients treated at the UMHAT "Dr. G. Stranski", Pleven of which 2697 (86.1%) were VS *E. faecalis*, 334 (10.7%) were VS *E. faecium*, 94 (3%) were VRE and 9 (0.3%) were other VSE species. The species distribution of enterococcal isolates by year is presented in ***Figure 11***. The number of VS *E. faecalis* and VS *E. faecium* over the years remained constant with a relatively preserved ratio between VSE and VRE.

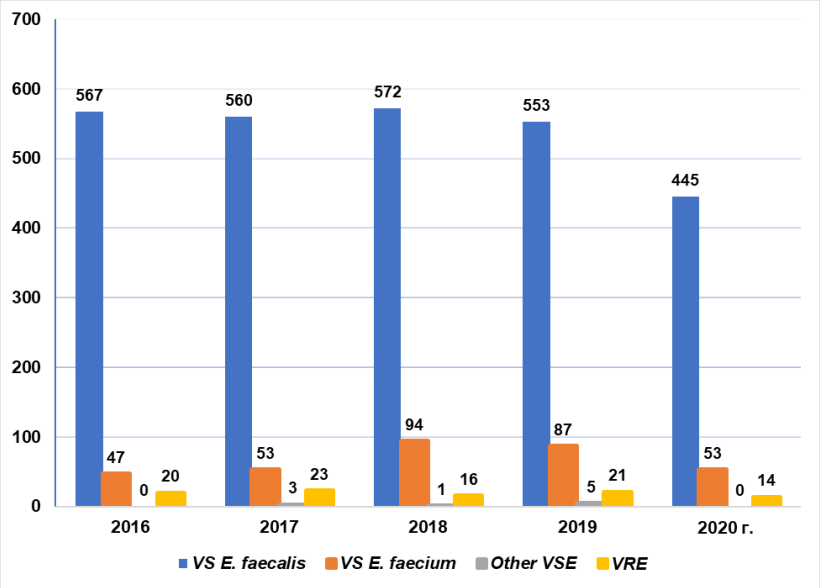


Figure 11. Distribution by year of enterococcal species isolated from patients in UMHAT "Dr. G. Stranski", Pleven for the period 2016 – 2020.

During the same period, 94 non-repeating VR enterococci were isolated from clinical materials of patients, which represented 3% of the total number of enterococci. Popova et al. (Popova V et al. 2014) report 13 VRE isolates in UMHAT "Dr. G. Stranski", Pleven for the period 01.03.2013 – 28.02.2014.

The species distribution of VRE isolated from patients in UMHAT "Dr. G. Stranski", Pleven for the period 2016 – 2020 showed that the proportion of VR *E. faecium* was highest – 85 isolates (90.4%). The isolation of other VRE was as follows: *E. gallinarum* – 5 (5.3%), *E. casseliflavus* – 3 (3.2%) and *E. durans* – 1 (1.1%).

The distribution of the isolates by years (***Figure 12***) shows that the number of VR *E. faecium* varied from 12 to 22. However, relative to the total number of isolated enterococci, the frequency of VR *E. faecium* remained relatively constant over the years. In 2017, 1 *E. casseliflavus* was detected, in 2018 and 2020 – 1 *E. gallinarum* each, and in 2019 the number of vanC enterococci isolated was a total of 5. Of particular interest is the appearance of 1 VR *E. durans* in 2020.

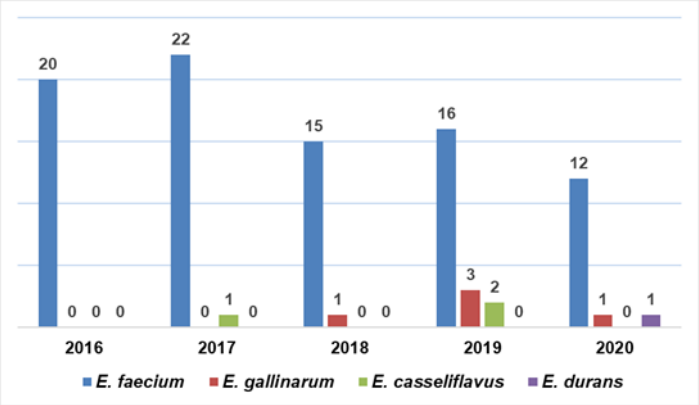


Figure 12. Prevalence by year of 94 VRE isolated from patients in UMHAT "Dr. G. Stranski", Pleven for the period 2016 – 2020.

The results regarding the circulation of VR *E. faecium* in the clinical units of the hospital are presented in ***Figure 13***. It was demonstrated that the ICU (Ist ICU, IInd ICU and IIIrd ICU), the Surgery Clinics (Ist Surgery, IInd Surgery, IIIrd Surgery and Surgical oncology Clinic), the Nephrology and Hemodialysis Clinic, the Urology Clinic and the Pulmonology Clinic were predominantly affected. The highest

number of isolates was from ICU (34) and the Surgical Clinics (31), constituting 76.5% of the total number.

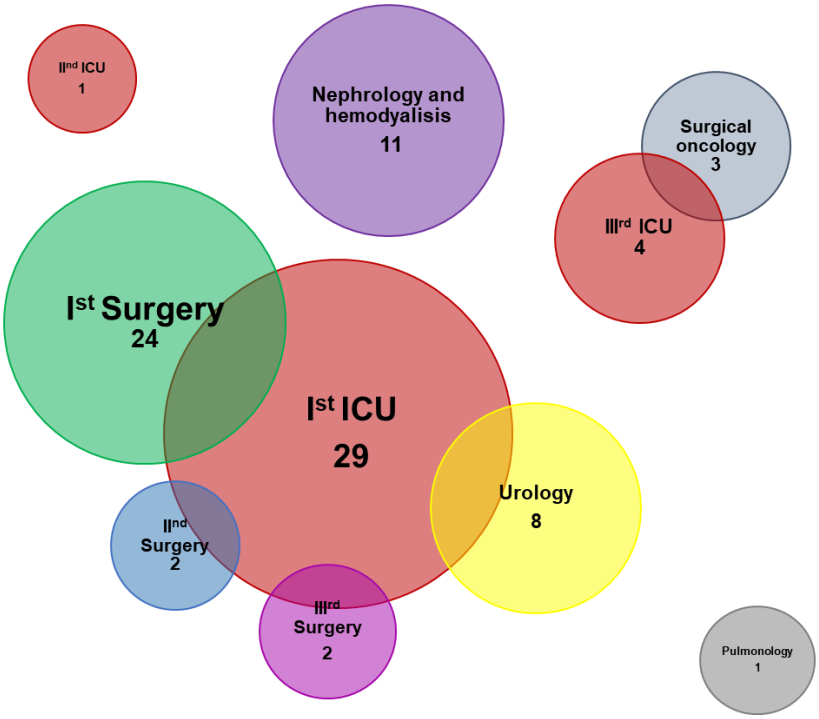


Figure 13. Distribution of VR *E. faecium* in the clinical units of the UMHAT, Pleven.

The distribution of isolates by clinical material from the respective clinics is shown in ***Figure 14***. It can be seen that the number of wound secretions with VR *E. faecium* were approximately the same in the ICU and the Clinics of Surgery, 18 and 21, respectively. The number of urines with VR *E. faecium* was the highest in the Nephrology and Hemodialysis Clinic – 9, the Urology Clinic – 8 and the ICU – 8, and the lowest in the Pulmonology Clinic – 1. Among the clinical materials, wound swabs (39) and urines (32) were predominating – a total of 71 (83.5%). The remaining 14 positive samples (16.5%) were distributed as follows: blood cultures – 6 (4 from the ICU and 2 from the Nephrology Clinic), punctates – 2, gastric aspirates – 2, drains – 2, ascite fluids – 2, (1 from the ICU and 1 from the Surgery Clinics).

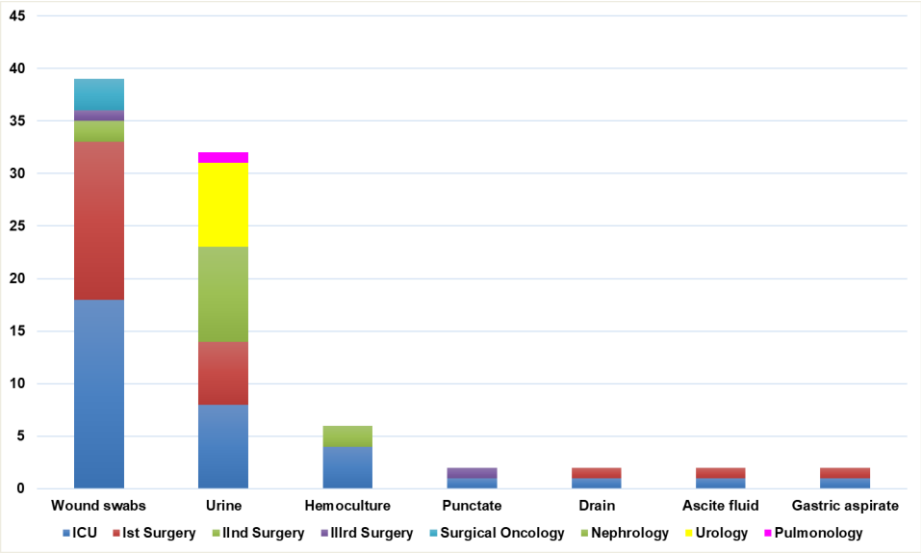


Figure 14. Distribution of VR *E. faecium* isolates by clinical materials and clinical units.

4.2. Demographic characteristics of patients with detected VRE

The data from the study of the demographic characteristics of all 85 patients with VR *E. faecium* were as follows: men – 55 (64.7%), women – 30 (35.3%). The mean age was 67±13.09 years (range: 22-87 years).

Table 9 presents the results of the analysis by age groups and the relevant clinical unit of hospitalization, and ***Table 10*** – by age and types of clinical materials, respectively. It was noteworthy that 72.9% of persons with VR *E. faecium* were ≥ 60 years old. In that age group the largest number of patients were treated in ICU and in the Ist Surgery. The established difference was statistically significant (p=0.005). In the age group 40-59 years, the patients in the ICU and in the Ist Surgery are again the most affected, when compared to those treated in the other clinical units, and the difference was statistically significant (p=0.039). In the youngest age group – 22-39 years, there was an even distribution of patients positive for VR *E. faecium* among the clinical units.

Table 9. Distribution of the patients with VR *E. faecium* by age and clinics

Group	Age (years)	% of the total	Number (%) of the total number in the respective age group				Ratio ICU and Ist Surgery/Other
			Total	ICU and Ist Surgery	Other*	*p*-value	
I	22 - 39	4.7	4	2	2	NS	1
II	40 - 59	22.4	19	14	5	**0.039**	0.36
III	≥ 60	72.9	62	42	20	**0.005**	0.48

Legend: Age, clinical unit (number, n) **1.** 22-39 years – ICU (n=2). **Other**: IInd Surgery (n=1), Surgical Oncology (n=1). **2.** 40-59 years – ICU (n=12), Ist Surgery (n=2). **Other**: IIIrd Surgery (n=1), Surgical Oncology (n=1); Nephrology (n=3). **3.** ≥ 60 years – ICU (n=20), Ist Surgery (n=22). **Other**: IInd Surgery (n=1), IIIrd Surgery (n=1), Surgical Oncology (n=1), Urology (n=8), Nephrology and Hemodialysis (n=8) and Pulmonology (n=1).

Table 10. Distribution of the patients with VR *E. faecium* by age and clinical samples

Group	Age (years)	% of the total	Number (%) of the total number in the respective age group						Ratio	
			wound swabs	other*	*p*-value	urine samples	other	*p*-value	wound swabs/ others *	urine sample/ other*
I	22 - 39	4.7	2	1	NS	1	1	NS	2	1
II	40 - 59	22.4	9	4	**0.033**	6	4	NS	2.25	1.5
III	≥ 60	72.9	28	9	**0.003**	25	9	**0.008**	3.11	2.78

*****Other= blood cultures, punctates, drainage fluids, ascitic fluids and gastric aspirates.

Table 10 shows that in the age range of 40-59 years, a statistically significant difference was present between the group of VR *E. faecium* positive wound secretions and the other clinical materials (*p*=0.033). In the age ≥ 60 years, statistically significant differences were also demonstrated between positive wound secretions and other materials (*p*=0.003), as well as between positive urines and other materials (*p*=0.008). Sievert et al. (Sievert et al. 2013) reported 3314 *E. faecium* isolates, of which 654 were causative of catheter-associated infections and 517 of surgical site infections.

5. CHARACTERISTICS OF VRE ISOLATED FROM CLINICAL MATERIALS OF HOSPITALIZED PATIENTS

5.1. Antimicrobial susceptibility and detection of *van* genes in clinical VRE

5.1.1. Results for UMHAT "Dr. G. Stranski", Pleven

Table 11 presents the MIC values and detected *van* genes for all 85 clinical VR *E. faecium* isolates.

The data showed that among the tested VRE, there were two main genotypes of glycopeptide resistance – *vanA* and *vanB*. The *vanB* gene was detected in only one *E. faecium* №3239/18, which had the usual VanB phenotype with low level of resistance to vancomycin (MIC = 8 μg/ml) and sensitivity to teicoplanin (MIC = 0.50 μg/ml). This isolate demonstrated MIC to gentamicin of 8 μg/ml, and it was resistant to ampicillin, gentamicin, ciprofloxacin, and susceptible to tigecycline, linezolid, quinupristin/dalfopristin, and daptomycin.

The *vanA* gene was confirmed in the rest 84 *E. faecium*. All of them had high level of vancomycin resistance (MIC ≥ 256 μg/ml), but only 7 (8.3%) of them had the typical for this phenotype high-level of resistance to teicoplanin (MIC: 32 – ≥ 256 μg/ml). The remaining 77 (91.7%) isolates showed low to moderate resistance to teicoplanin (MIC: 6 – 16 μg/ml), which resembled the VanD phenotype (VanD-*vanA*). Such unusual VREs have been circulating in UMHAT “Dr. G. Stranski”, Pleven since 2013 (Popova V 2018).

In general, the *vanA* isolates had high levels of resistance to ampicillin, gentamicin, ciprofloxacin and were susceptible to tigecycline, linezolid, quinupristin/dalfopristin and daptomycin. *E. faecium* №1179/17 demonstrating MIC to gentamicin of 6 μg/ml and *E. faecium*

№4234/16 with resistance to quinupristin/dalfopristin (MIC ≥ 32 μg/ml) were an exception.

All 8 vanC enterococci showed the usual VanC phenotype with low level of resistance to vancomycin (MIC: 4 – 8 μg/ml) and susceptibility to teicoplanin (MIC: 0.50 – 1 μg/ml). The *vanC1* gene was confirmed in 5 *E. gallinarum*, and *vanC2* in 3 *E. casseliflavus*. All vanC isolates were susceptible to the rest of the tested antimicrobial agents.

The single isolated *E. durans* showed a high-level resistance to vancomycin (MIC ≥ 256 μg/ml), moderate resistance to teicoplanin (MIC = 16 μg/ml) and the *vanA* gene was detected. This strain had high levels of resistance to ampicillin, gentamicin, ciprofloxacin and was susceptible to the other antibiotics.

5.1.2 Results for the other clinical centers

Among the tested 23 VRE, two main genotypes of glycopeptide resistance were found – *vanA* and *vanB*. The *vanB* gene was detected in only one *E. faecium* (№4760/20) from UMHAT “Tokuda”, which showed MIC to vancomycin of 8 μg/ml and MIC to teicoplanin of 0.50 μg/ml. This isolate was resistant to ampicillin, gentamicin, ciprofloxacin and susceptible to the other tested antibiotics.

The *vanA* gene was confirmed in the remaining 22 *E. faecium*. All of them have high-level resistance to vancomycin (MIC ≥ 256 μg/ml), but only 1 of them has the usual high-level resistance to teicoplanin (MIC = 32 μg/ml) typical for the VanA phenotype. The rest 21 isolates showed low to moderate resistance to teicoplanin (MIC: 6 – 16 μg/ml). Two *vanA E. faecium* demonstrated MICs to gentamicin of 12 μg/ml and 96 μg/ml, respectively, and in all other isolates the MIC to the same antibiotic was ≥ 1024 μg/ml.

Table 11. MIC values and *van* gene in 85 clinical VR *E. faecium*

No of isolates	MIC or MIC range (μg/ml)									***van*** gene
	AMP	**GEN**	**CIP**	**VAN**	**TEC**	**TGC**	**LZD**	**Q/D**	**DAP**	
	2016 year									
19	≥256	≥1024	≥32	**≥256**	**8-16**	0.047-0.125	1-2	0.50-0.75	1.5-≤8	*vanA*
1	≥256	≥1024	≥32	**≥256**	**16**	0.064	2	**≥32**	≤8	*vanA*
	2017 year									
19	≥256	≥1024	≥32	**≥256**	**12-16**	0.047-0.125	1-3	0.25-1	3-≤8	*vanA*
2	≥256	≥1024	≥32	**≥256**	**≥256**	0.094	1.5-2	0.38-0.50	1.5-6	*vanA*
1	≥256	**6**	≥32	**≥256**	**≥256**	0.094	2	0.50	6	*vanA*
	2018 year									
14	≥256	≥1024	≥32	**≥256**	**8-16**	0.047-0.125	1-3	0.38-1	3-≤8	*vanA*
1	≥256	**8**	≥32	**8**	**0.50**	0.125	1	1	3	*vanB*
	2019 year									
14	≥256	≥1024	≥32	**≥256**	**6-16**	0.047-0.125	1-3	0.25-3	4-≤8	*vanA*
2	≥256	≥1024	≥32	**≥256**	**32-128**	0.064-0.094	2	0.38-0.50	6	*vanA*
	2020 year									
10	≥256	≥1024	≥32	**≥256**	**12-16**	0.047-0.094	1-2	0.50-3	1.5-≤8	*vanA*
1	≥256	≥1024	≥32	**≥256**	**48**	0.064	2	0.50	≤8	*vanA*
1	≥256	≥1024	≥32	**≥256**	**≥256**	0.064	2	0.38	4	*vanA*

Legend AMP: ampicillin; GEN: gentamicin; CIP: ciprofloxacin; VAN: vancomycin; TEC: teicoplanin; TGC: tigecycline; LZD: linezolid; Q/D: quinupristin/dalfopristin; DAP: daptomycin.

All *vanA E. faecium* had high levels of resistance to ampicillin and ciprofloxacin and were susceptible to tigecycline, linezolid, quinupristin/dalfopristin and daptomycin.

5.2. Detection of genes encoding aminoglycoside resistance in clinical VRE

5.2.1. Results for UMHAT "Dr. G. Stranski", Pleven

All 85 investigated VR *E. faecium* were positive for *aac(6')-Ie-aph(2")-Ia*, and 3 of them have a second gene – *aph(3')-IIIa*. In 83 *vanA* isolates, the phenotype of high-level resistance to gentamicin (MIC ≥ 1024 μg/ml) completely corresponded to the established genotype – *aac(6')-Ie-aph(2")-Ia* gene. In two isolates - *vanB E. faecium* №3239/2018 and *vanA E. faecium* №1179/2017, demonstrating low level of resistance to gentamicin (MIC: 6 – 8 μg/ml), this bifunctional gene was also demonstrated.

According to SAID (ECDC-Net 2019), the incidence of HLGR *E. faecium* in Europe in 2020 ranged from 4.1% for Greece to 51.6% for Poland. The data for Bulgaria showed 47.9% HLGR among *E. faecium*.

5.2.2. Results for the other clinical centers

The *aac(6')-Ie-aph(2")-Ia* gene was confirmed in all 14 VR *E. faecium* isolated from UMHAT "Tokuda", Sofia and two of them were also positive for *aph(3')-IIIa*. In 12 *E. faecium*, the gentamicin resistance phenotype with MIC ≥ 1024 μg/ml corresponded to the detected *aac(6')-Ie-aph(2")-Ia* gene. In 2 *E. faecium*, however, the presence of this gene was associated with low-level gentamicin resistance (MIC: 12 μg/ml and 96 μg/ml).

The studied 9 VR*E. faecium* (4 from UMHAT "St. Marina", Pleven and 5 from MHAT "Heart and Brain", Pleven) had high-levels of resistance to gentamicin (MIC ≥ 1024 μg/ml) and in all of them *aac(6')-Ie-aph(2")-Ia* gene was detected. Two *E. faecium* from UMHAT "St. Marina" were also positive for the *aph(3')-IIIa* gene.

5.3. Prevalence of genes encoding virulence factors in clinical VRE

5.3.1. Results for UMHAT "Dr. G. Stranski", Pleven

Positive for *acm* and *esp* were 77 (90.6%) VR *E. faecium* from 85 tested clinical VRE. In 6 isolates (7.1%), a third virulence gene was also found, respectively in 3 – *hyl* and in 3 – *gelE*. One *E. faecium* carried only the *gelE* gene, while another has four virulence genes, *gelE*, *asa1*, *esp* and *ace*. Strateva et al. (Strateva et al. 2016) investigated the prevalence of virulence determinants among a total of 110 clinical VS *E. faecium* and found *efaA* in 88.5% of them, *acm* in 72.8%, *hyl* in 24.2%, *asa1* in 22.8%, *gelE* in 17.1% and *esp* in 4.3% of the isolates.

All tested clinical vanC enterococci did not showed the presence of virulence genes, while in *E. durans* a combination of 2 genes – *acm* and *hyl* was confirmed.

5.3.2. Results for the other clinical centers

The distribution of genes encoding virulence factors in clinical VRE from UMHAT “Tokuda”, Sofia showed that 12 out of 14 *E. faecium* were positive for *acm* and *esp* genes, and only *efaA* gene was detected in 1 isolate. In *vanB E. faecium* there was a combination of 3 genes – *acm*, *esp* and *hyl*. Among the isolates from UMHAT "St. Marina", Pleven three *E. faecium* were carriers of 3 genes – *acm*, *esp* and *hyl*, and one was positive for 2 genes - *acm* and *esp*. In the five isolates from the

MHAT "Heart and Brain", Pleven the same 2 genes – *acm* and *esp* were also detected. The genes *esp* (n=106), *acm* (n=105) and *hyl* (n=7) were most frequently identified in the clinical *E. faecium*.

5.4. Epidemiological typing of clinical VR *E. faecium* isolates

5.4.1. Results for UMHAT "Dr. G. Stranski", Pleven

Figure 15 shows the phylogenetic trees reflecting the genetic relationship between the *vanA E. faecium* isolates, and ***Figure 16*** illustrates their clustering in the form of dendrograms depending on the number of tandem repeats at each locus.

The results showed that the dominant multilocus type in 72 (85.7%) of the isolates was MT11. Four (4.8%) *E. faecium* belong to MT75 and MT83, 2 (2.4%) belong to MT81 and one (1.2%) – to MT77 and MT82, respectivly. Sattari-Maraji et al. (Sattari-Maraji et al. 2019) typed 108 *E. faecium* and found 27 multilocus types, of which MT1 (40%), MT2 (13.8%) and MT3 (12.9%) were the most common. When testing 167 *E. faecium*, Billstrom et al. (Billström et al. 2008) identifed 23 different MLVA types. Among them, the most common were MT1 (65%) and MT159 (13%). Another study demonstrated 8 multilocus types in 71 VR *E. faecium* (Wardal et al. 2014).

During the period of study, a certain dynamic in multilocus types was observed, related to the appearance of new ones. In 2016, all 20 *E. faecium* were referred to MT11. In 2017, from 22 isolates, 19 belonged to MT11, and in 3 cases a new type was detected – MT75. It was noteworthy that the two MT75 *E. faecium* isolates were from the Departments of Oncology (1 from the Oncology ICU and 1 from the Surgical Oncology), and the third is from the Pulmonology Clinic. These units are not part of the main building of UMHAT "Dr. G. Stranski",

Pleven. In 2018, from 14 isolates, 13 were related to MT11 and 1 to MT77. The latest was isolated from the Ist ICU. In 2019, from 16 isolates, 11 belonged to MT11, 4 to MT83 and 1 to MT75. Two MT83 and the single MT75 were isolated from the Ist ICU, while the other two other MT83 were isolated from the Nephrology and Hemodialysis Clinic. In 2020, from 12 isolates, 9 referred to MT11. The remaining three belonged to the newly emerged MT81 and MT82. Two *E. faecium* were assigned to MT81 and 1 *E. faecium* to MT82. MT81 were isolated from the Ist Surgery and the Ist ICU, respectively, whereas MT82 – from the Nephrology and Hemodialysis Clinic.

When comparing the phenotypic and genotypic characteristics of MT11 isolates, it was found that all 72 *E. faecium* with MT11 carried the *aac(6')-Ie-aph(2")-Ia* gene for aminoglycoside resistance and generally shared common genes for virulence factors (*acm* and *esp*). In 69 of them (95.8%), similar antibiotic profile with high resistance to vancomycin (MIC ≥ 256 μg/ml) and teicoplanin MIC values of 6 – 16 μg/ml was confirmed. Three isolates (4.2%) showing high-level resistance to teicoplanin (MIC: 48 – ≥ 256 μg/ml) were an exeption.

5.4.2. Results for the other clinical centers

The MLVA analysis, reflecting the genetic relationship between 13 *vanA E. faecium* from UMHAT “Tokuda”, Sofia confirmed that 8 of the isolates belong to MT11, 2 to MT80 and one each to each of MT25, MT78 and MT79.

The MLVA analysis of 4 *E. faecium* from UMHAT "St. Marina", Pleven and 5 from MHAT "Heart and Brain", Pleven showed that they all belong to one type – MT11.

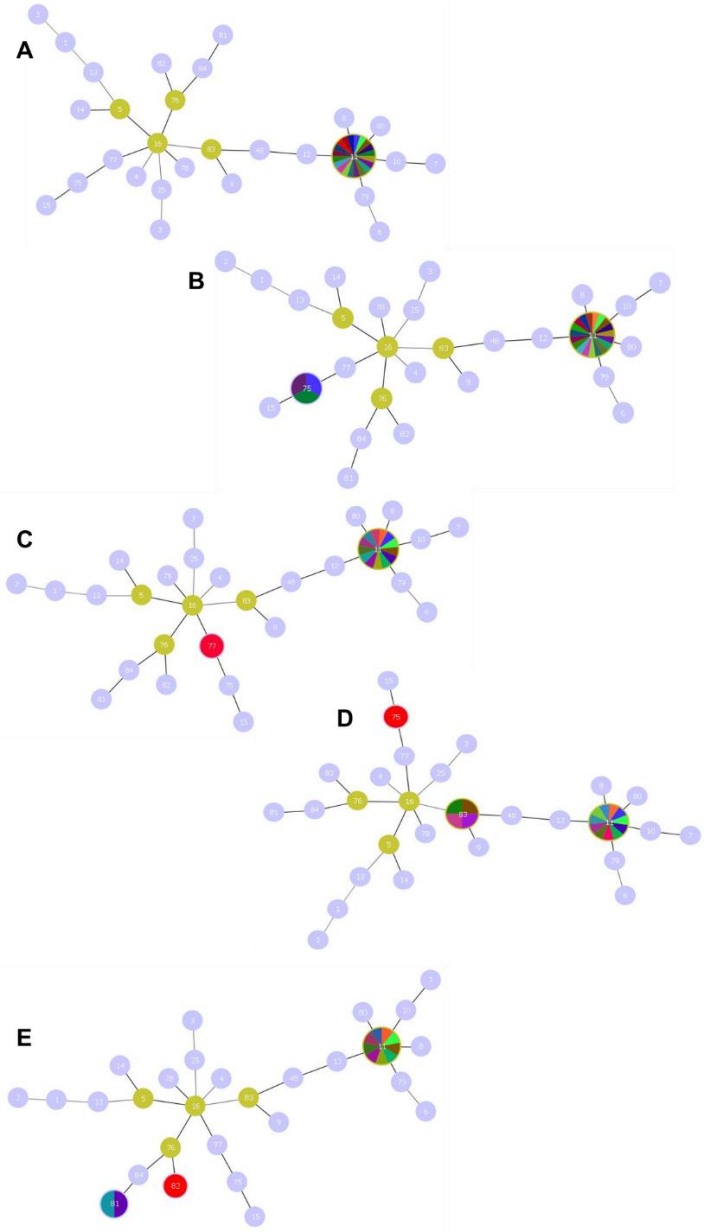


Figure 15. **Phylogenetic tree (Minimum spanning tree) of 84 *vanA E. faecium* genotyped with MLVA10.** The phylogenetic tree illustrates the grouping of strains according to the number of tandem repeats at each locus. Each circle represents one genotype, the size of the circle being proportional to the number of typed isolates. The following MLVA types (MT) were detected: (**A**) 2016 – MT11; (**B**) 2017 – MT11 and MT75; (**C**) 2018 – MT11 and MT77; (**D**) 2019 – MT11, MT75 and MT83; (**E**) 2020 – MT11, MT81 and MT82. The distribution of isolates to each type was as follows: MT11: **72** *E. faecium*; MT 75: 4 *E. faecium*; MT77: **1** *E. faecium*; MT81: **2** *E. faecium*; MT82: 1 *E. faecium*; MT83: 4 *E. faecium*. MLVA types are marked in yellow, which are genetically close to some of the types detected in the medical facility, but to which no isolates have been registered. The figure was created using the specialized software PHYLOViZ 2.0.

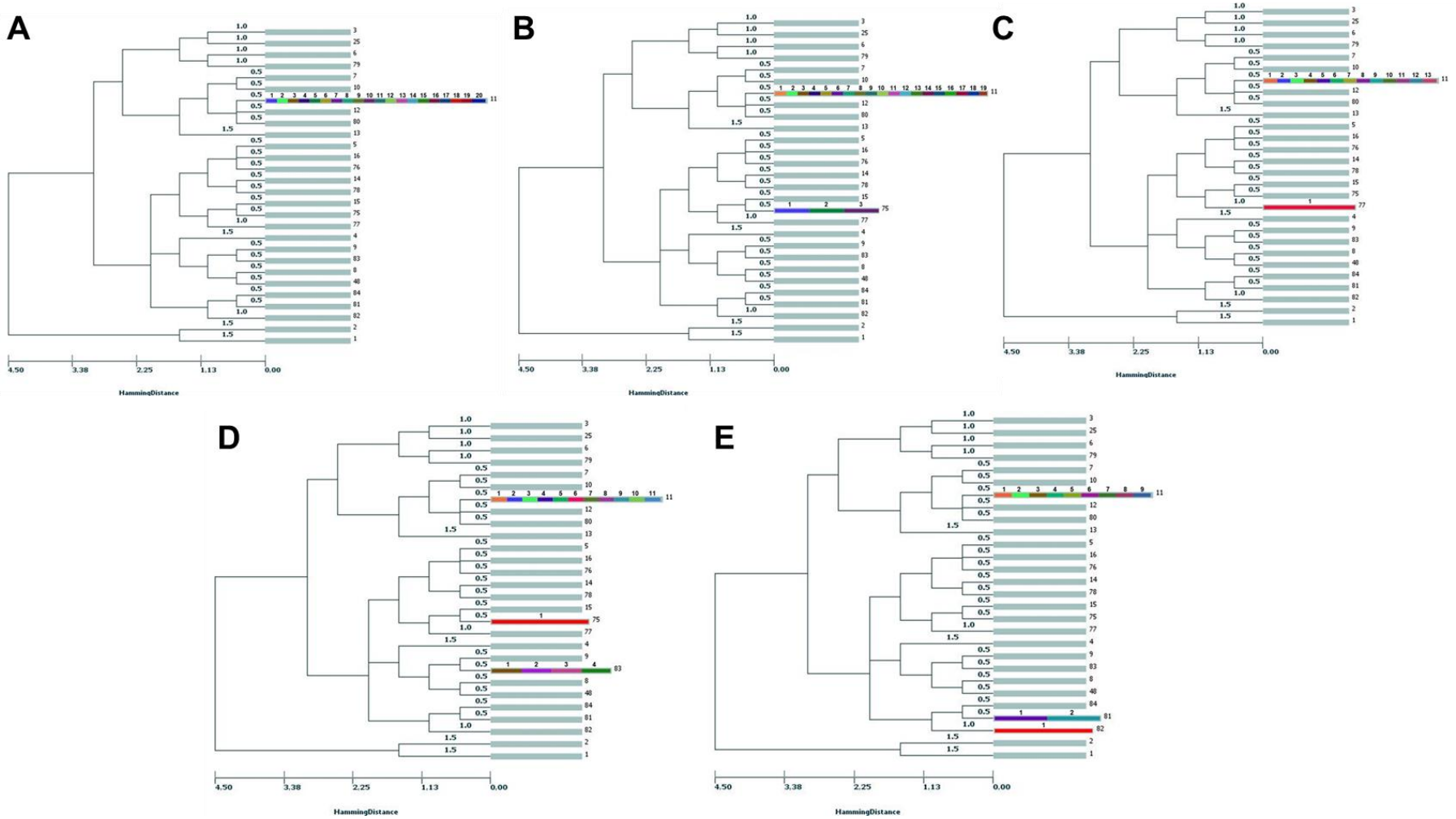


Figure 16. **Dendrograms showing the genetic relationship between clinical *vanA E. faecium* genotyped with MLVA10.** The dendrogram was created using the specialized software PHYLOViZ 2.0. using hierarchical clustering with Hamming distance and Complete-Linkage method clustering algorithm. The distances between the individual clusters are marked with numbers. The following MLVA types (MT) were detected: (**A**) 2016 – MT11; (**B**) 2017 – MT11 and MT75; (**C**) 2018 – MT11 and MT77; (**D**) 2019 – MT11, MT75 and MT83; (**E**) 2020 – MT11, MT81 and MT82. The distribution of isolates to each type was as follows: MT11: **72** *E. faecium*; MT 75: **4** *E. faecium*; MT77: **1** *E. faecium*; MT81: **2** *E. faecium*; MT82: **1** *E. faecium*; MT83: **4** *E. faecium*.

V. CONCLUSIONS

1. When testing Brilliance VRE, chromID VRE, HiCrome VRE Modified, and BEAV broth, it was demonstrated that the three chromogenic agars showed equal sensitivity (85.7%). Brilliance VRE and chromID VRE revealed identical specificity (98.7%), whereas the specificity of HiCrome VRE Modified was 96.7%. The sensitivity and specificity of BEAV broth were 100% and 81.6%, respectively.

2. The rate of intestinal colonization with VRE was highest at patients in the HD Clinic (27.8%), followed by the ICU (24.2%) and the HT Clinic (15.1%).

3. *E. gallinarum* and *E. casseliflavus* species accounted for 78.3% of all intestinal VRE and predominated in all three groups of high-risk patients.

4. Statistically significant risk factors for intestinal colonization with VRE in the patients undergoing hemodialysis were the duration of hemodialysis 13-60 months, the vascular access through an indwelling tunneled catheter and the vancomycin use; in the intensive care patients with the need for postoperative care, the cardiovascular diseases and the presence of an endotracheal tube; in the patients with hematological malignancies the age 70 - 79 years and the disease multiple myeloma.

5. VRE make up 3% of enterococci in UMHAT "Dr. G. Stranski", Pleven for the period 2016 – 2020. The most common isolates were VR *E. faecium* (90.4%), which circulates mainly in the wards for intensive care and their adjacent surgical units, where 76.5% of the isolates were found.

6. In VR *E. faecium*, the *vanA* genotype prevails, present in 98.2% of clinical and 92.9% of intestinal isolates.

7. Correlation between phenotype and genotype of glycopeptide resistance was found in all *E. gallinarum*, *E. casseliflavus*, *vanB E. faecium* isolates and in 13 *vanA E. faecium*, while VanD-*vanA* type was shown by 106 *E. faecium*.

8. The bifunctional aminoglycoside gene *aac(6')-Ie-aph(2'')-Ia* was demonstrated in all VRE with HLGR and in 6 VR *E. faecium* with low-level resistance to gentamicin.

9. Carriers of one or more virulence determinants were all tested VR *E. faecium* and VR *E. faecalis*, 6 intestinal *E. gallinarum* and 1 VR *E. durans* with the prevalence of the genes *acm* and *esp*.

10. A total of 85.7% of clinical and 76.9% of intestinal *vanA E. faecium* from UMHAT "Dr. G. Stranski", as well as 77.3% of *vanA E. faecium* from other hospitals belonged to MT11. They all expressed similar antibiotic profiles and shared common resistance and virulence genes.

VI. CONTRIBUTIONS OF THE DISSERTATION

Original contributions

1. For the first time in Bulgaria, the capabilities of different types of selective culture media for the primary isolation and identification of intestinal VRE have been evaluated.

2. For the first time in Bulgaria, the frequency of intestinal colonization with VRE was studied among three groups of patients at high risk for colonization and infection with problematic microorganisms (undergoing hemodialysis, suffering from hematological malignancies, and treated in intensive care units) and analyzed the relevant risk factors.

3. For the first time in Bulgaria, detailed microbiological and genetic studies together with genotyping of intestinal VRE were performed.

4. For the first time, an epidemiological study was conducted among patients with VR *E. faecium* isolates for a period of 5 years at the UMHAT "Dr. G. Stranski", Pleven.

5. For the first time in Bulgaria, the phenotypic and genotypic characteristics of clinical and intestinal vanC enterococci were studied.

Confirmative contributions

1. The selective and differentiating role of chromogenic media for the isolation of VR *E. faecium*/*E. faecalis* from fecal samples was confirmed.

2. The selective role of BEAV broth for the isolation of VR *E. faecium*/*E. faecalis* and vanC enterococci was confirmed.
3. The role of the following risk factors, which contribute to the VRE colonization, have been confirmed: advanced age of the patient, use of vancomycin, duration of hemodialysis, vascular access through an indwelling tunneled catheter, presence of an endotracheal tube, need for postoperative cares, cardiovascular diseases, and multiple myeloma.
4. The low prevalence of virulence factors in vanC enterococci and their high prevalence in VR *E. faecium* were confirmed.
5. The role of the *aac(6')-Ie-aph(2'')-Ia* gene for HLGR in VRE has been confirmed.
6. The circulation of *vanA* VR *E. faecium* with an unusual phenotype of glycopeptide resistance (VanD-*vanA*) has been confirmed in UMHAT "Dr. G. Stranski" and other clinical centers.

Scientific and applied contributions

1. An algorithm for the isolation and presumptive identification of intestinal VRE in screening of patients at high risk for colonization/infection with problematic microorganisms was developed.
2. The PCR protocols for detection of genes encoding resistance to aminoglycosides and virulence factors in vanC enterococci were modified.
3. The PFGE protocol for typing of vanC enterococci was modified.

VII. LIST OF SCIENTIFIC PUBLICATIONS AND REPORTS IN RELATION WITH THE DISSERTATION

Publications related to the dissertation

1. **Hristova PM**, Nankov VM, Stoikov I, Ivanov IN, Ouzounova-Raykova VV, Hitkova HY. Prevalence of genes encoding resistance to aminoglycosides and virulence factors among intestinal vancomycin-resistant enterococci. Jundishapur Journal of Microbiology. **2022**; 15(9):e128003. DOI: 10.5812/jjm-128003

   (**IF_{2021}–0.820)**

2. **Hristova PM**, Hitkova HY, Georgieva DS, Todorova GV, Sredkova MP. Evaluation of three chromogenic media and a selective broth for detection of vancomycin-resistant enterococci from rectal swab specimens. Comptes rendus de l'Academie bulgare des Sciences. **2021**;74(8):1146-1154. DOI: 10.7546/CRABS.2021.08.05.

   (**IF_{2021}–0.326)**

3. **Hristova P**, Hitkova H. Enterococcus and Enterococcus-like organisms recovered in faecal screening for vancomycin-resistance. Journal of IMAB. **2019**;25(3):2649-2654. DOI: 10.5272/jimab. 2019253.2649.

Reports related to the dissertation

1. **Hristova P**, Hitkova H, Szymon Walter de Walthoffen, Sredkova M. Phenotypic and genotypic characterization of vancomycin-resistant enterococci isolated from patients undergoing haemodialysis. 8th Congress of European Microbiologist (FEMS 2019), Glasgow, Scotland, July 7-11, **2019**. PM 287.
2. **Hristova P**, Nankov V, Georgieva D, Bulgaranova T, Walter de Walthoffen S, Ivanov I, Hitkova H. Prevalence of genes for virulence factors and glycopeptide resistance in intestinal isolates of *Enterococcus gallinarum* and *Enterococcus casseliflavus*. World Microbe Forum, 20-24 June, **2021**. Online Worldwide. Poster ID: WMF21-2002.
3. **Hristova P**, Nankov V, Stoikov I, Ivanov I, Hitkova H. Molecular characterization of vancomycin-resistant *Enterococcus faecium* isolated in a University hospital in Bulgaria. 32nd ECCMID, the European Congress of Clinical Microbiology and Infectious Diseases, Lisbon, Portugal, 23-26 April, **2022**. Abstract N: 3539.
4. **Hristova P**, Walter de Walthoffen S. Detection of vancomycin-resistant Enterococcus faecium in transplanted patients. XIV International medical scientific conference for students and young doctors, Medical University-Pleven, Bulgaria, 10-15 October, **2016**, P.134.
5. **Hristova P**, Hitkova H, Todorova G, Todorov V, Mitkov Y, Georgieva D, Edreva V, Dragoev K, Sredkova M. Detection of vancomycin-resistant enterococci in fecal samples by selective chromogenic agar media and selective bile esculin azid broth. XVI National congress of

clinical microbiology and infectiology, Sofia, May, 10-12, **2018**, Abstract book p. 50.

6. **Hristova P, Hitkova H,** Hristov I, Georgieva D, Sredkova M. Screening for intestinal colonization with vancomycin-resistant enterococci among patients with hematological malignancies. XVII National congress of clinical microbiology and infectiology, Sofia, May, 9-11, **2019**, Abstract book p. 47.
7. **Hristova P**, Hitkova H, Bogdanov S, Mihaylov I, Ivanov I, Georgieva D, Sredkova M. Faecal colonization with vancomycin-resistant enterococci among patients in intensive care units. Journal of Biomedical & Clinical Research. Volume 12, number 1, supplement 2, **2019**. PM 136.
8. **Hristova H**, Hitkova H, Walter de Walthoffen S, Georgieva D, Bulgaranova T, Todorova G, Nankov V, Edreva V, Sredkova M. Phenotypic and genotypic characteristics of intestinal vancomycin-resistant enterococci in patients on hemodialysis. XVIII 30- October 2, **2020**, Abstract book p. 59.
9. **Hristova P**, Nankov V, Georgieva D, Bulgaranova T, Stoikov I, Stamenov K, Ivanov I, Hitkova H. Detection of genes for antimicrobial resistance and virulence factors in intestinal vancomycin-resistant enterococci. XIX National congress of clinical microbiology and infectiology, Sofia, September, 14-16, **2021**, Abstract book p. 95-96.
10. **Hristova P**, Todorova G, Georgieva D, Hitkova H. Prevalence of intestinal vancomycin-resistant enterococci and risk factors for colonization in patients undergoing hemodialysis. XX Jubilee National congress of clinical microbiology and infectiology, Plovdiv, September, 16-18, **2022**, Abstract book.18-19.

SUMMARY

The **objectives** of the current work were to study the phenotypic and genotypic characteristics of 186 non-repetitive clinical (n=117) and intestinal (n=69) vancomycin-resistant enterococci (VRE) and the risk factors for colonization and infection with these bacteria in hospitalized patients.

Results: The three tested chromogenic agars for isolation and preliminary identification of intestinal VRE showed equal sensitivity (85.7%). Brilliance VRE and chromID VRE demonstrated identical specificity (98.7%), while the specificity of HiCrome VRE Modified was with minimal differences (96.7%). The sensitivity and specificity of BEAV broth were 100% and 81.6%, respectively.

The frequency of intestinal colonization with VRE was the highest in patients in the Hemodialysis ward (27.8%), followed by the ICU (24.2%) and the Hematology ward (15.1%). Among the three groups of high-risk patients *E. gallinarum* and *E. casseliflavus* species (78.3%) were the dominant intestinal isolates. Risk factors, statistically significant, for intestinal colonization with VRE in patients undergoing hemodialysis were duration of hemodialysis between 13-60 months, the vascular access through an indwelling tunneled catheter and the use of vancomycin; in intensive care patients the risk factors were the need for postoperative cares, the cardiovascular diseases and the presence of an endotracheal tube; in patients with hematological malignancies the risk factors for colonization with VRE were the aged 70-79 years and the multiple myeloma.

For the period 2016 – 2020 in UMHAT "Dr. G. Stranski", Pleven 3% of the enterococcal isolates were VRE with prevalence of VR *E. faecium*

(90.4%). The *VR E. faecium* was circulating mainly in the intensive care units and their adjacent surgical units.

Among the VR *E. faecium* isolates, the *vanA* genotype was prevalent and confirmed in 98.2% of clinical and 92.9% of intestinal isolates. A correlation between the phenotype and genotype of glycopeptide resistance was found in all *E. gallinarum*, *E. casseliflavus*, *vanB E. faecium* isolates and in 13 *vanA E. faecium*, whereas 106 *E. faecium* showed VanD-*vanA* type. The bifunctional aminoglycoside gene *aac(6')-Ie-aph(2")-Ia* was demonstrated in all VRE with HLGR and in 6 VR *E. faecium* with low-level gentamicin resistance. One or more virulence determinants were founded in all VR *E. faecium* and VR *E. faecalis*, 6 intestinal *E. gallinarum* and 1 VR *E. durans*. The predominant genes were *acm* and *esp*. 85.7% of the clinical and 76.9% of the intestinal *vanA E. faecium* from UMHAT "Dr. G. Stranski", as well as 77.3% of *vanA E. faecium* from other clinical centers belong to MT11. They all expressed similar antibiotic resistance profiles and share common virulence genes.

Conclusions: The current detailed phenotypic and genotypic study of large number of clinical and intestinal VRE might be useful for prediction of the type of circulating enterococci in the UMHAT "Dr. G. Stranski", Pleven. In addition, the evaluation of frequency of colonization/infection with VRE among the hospitalized patients and the contributing risk factors is an advanced approach to prevent spreading of these problematic microorganisms in hospitals.