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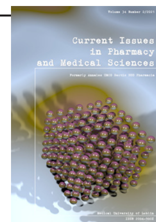

Analysis of the phenotypic and genotypic antimicrobial resistance profiles of clinically significant enterococci isolated in the Provincial Specialist Hospital in Lublin, Poland

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ABSTRACT

The increasing significance of enterococci as healthcare-associated pathogens can be linked to their limited susceptibility to antibiotics.

In this study, phenotypic and genotypic resistance profiles of 35 [n=18 *E. faecium* (*Efm*); n=17 *E. faecalis (Efs)*] invasive isolates cultured from hospitalized patients were analysed. Phenotypic identification was verified by the multiplex PCR targeting the 16S rDNA and the *ddl* genes encoding for the *Efs* and *Efm* – specific ligases. Antimicrobial susceptibility was determined using the disc diffusion method and E-tests. The high-level streptomycin resistance (HLSR), high-level gentamicin resistance (HLGR) and glycopeptide resistance was verified by amplification of the *ant(6)-Ia, aac(6')-Ie-aph(2")-Ia,* as well as *vanA* and *vanB genes,* respectively.

More than 70% of all isolates were cultured from patients in the Intensive Care and Internal Medicine Units. Blood was the predominant (77%) site of isolation. All *Efm* isolates were resistant to ampicillin, imipenem, and norfloxacin; 17 isolates demonstrated high-level aminoglycoside resistance (HLAR), including 27.7% with HLSR, 38.8% with HLGR and 27.7% with both phenotypes. HLAR was also common in *Efs* (HLSR>70%, HLGR>50%), followed by norfloxacin (64.7%) and ampicillin (11.7%) resistance. The *ant(6)-Ia and aac(6')-Ie-aph(2")-Ia* genes were detected in >90% of the HLSR and HLGR isolates, respectively. Glycopeptide resistance was detected in 4 (22.2%) *Efm* isolates and mediated by the *vanA* gene. 19 (54.3%) isolates were multidrug resistant, including 17 (89.5%) *Efm*. All isolates were susceptible to linezolid.

The study constitutes a contribution to the analysis of enterococcal antimicrobial resistance in Polish hospitals. The monitoring of enterococcal prevalence and antimicrobial resistance is crucial to control and prevent infections.

INTRODUCTION

Enterococci are common members of the human gastrointestinal microbiota. They represent a group of Gram-positive facultative anaerobes capable of survival in the presence of unfavourable environmental conditions, including high salt concentrations and elevated temperatures [1-3]. These bacteria are also able to resist chemical stress induced by chlorine and alcohol-based disinfectants [4] and quickly adapt to a changing environment [5]. The intrinsic properties of enterococci confer the ability to resist host defences and to compete in the intestinal tract, which subsequently leads to persistence and spread in the environment, hence favouring colonization of new hosts [6].

In spite of the fact that the inherent virulence potential of enterococci lags behind that of the more pathogenic streptococci, during the past few decades, they have emerged as important healthcare-associated pathogens [1,4]. Indeed, enterococci have gained the position of the leading opportunistic pathogens, especially in elderly, multimorbid, critically ill and immunocompromised populations [1,2,5].

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According to Miller *et al.* [2], in the timeframe between 2015 and 2017, enterococci were reported as the second leading cause of healthcare-associated infections (HAI) overall, including their leading role in the etiology of central line-associated bloodstream infections in long-term acute care hospitals and in oncology units. Infections of enterococcal etiology typically manifest as urinary tract infections, bacteremia, intra-abdominal infections, endocarditis as well as skin, soft tissue infections and device infections [2,6,7]. Their increasing clinical significance can be ascribed to their durable and commensal nature [2,7], but the real challenge that overshadows the treatment is a limited susceptibility to antibiotics, due to both intrinsic and acquired antibiotic resistance [6].

Enterococcus faecalis and *Enterococcus faecium* represent two most clinically relevant enterococcal species [7,8]. They demonstrate intrinsic resistance to cephalosporins, low levels of aminoglycosides, clindamycin and trimethoprim-sulfamethoxazole. In addition, due to a remarkably plastic genome, the two species have been able to readily acquire resistance to further antimicrobial agents, including high-level aminoglycoside resistance (HLAR), high-level ampicillin resistance and vancomycin resistance (vancomycin-resistant enterococci, VRE). The acquired resistance phenotypes develop as a result of mutation or a horizontal transfer of genetic elements harboring resistance determinants [7,9]. Moreover, resistance to newer antibiotics, such as daptomycin and oxazolidinones continues to emerge [2,10], with the latest threat and therapeutic dilemma being the multidrug-resistant (MDR) *E. faecium* [4,5]. *E. faecium* has been classified as one of the microbes that "escape" antibiotic treatment, hence, it has been included in the acronym ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa, Enterobacter* spp.) among other important MDR bacteria involved in HAI [8,11].

There have been relatively few literature data raising the problem of enterococcal antibiotic resistance in Poland, with the most recent reports being by Chmielarczyk *et al.* (2021) [12], Krawczyk *et al.* (2020) [9], Kamińska *et al.* (2019) [3], Talaga-Ćwiertnia & Bulanda (2018) [11], Talaga *et al.* (2018) [13], and Gawryszewska *et al.* (2017, 2016) [14,15].

AIM

The aim of the study was to characterize phenotypic and genotypic resistance profiles of 35 invasive enterococcal isolates cultured from patients hospitalized in the Provincial Specialist Hospital in Lublin, Poland.

MATERIALS AND METHODS

Phenotypic identification of the isolates, antimicrobial susceptibility testing

The study included 35 clinically significant enterococal isolates collected in the Stefan Kardynał Wyszyński Provincial Specialist Hospital in Lublin, Poland, in the period between October 2020 and March 2021 in passive surveillance. The isolates were cultured from 20 male and 15 female patients (one isolate per patient). The age of patients ranged from 45 to 97 years (mean age: 71.5 years). Information regarding the patients, as well as the types of clinical samples from which enterococci were cultured and hospital wards in which the infected patients were hospitalized are included in Table 1.

Identification of the isolates to the species level was performed in the hospital laboratory using standard microbiological methods, including the BD Phoenix™ (Becton Dickinson, USA) automatic system. Antimicrobial susceptibility of the isolates was determined using the disc diffusion method and E-tests, and interpreted according to recommendations of the European Committee on the Antibiotic Susceptibility Testing (EUCAST, v.11.0). The susceptibility testing included discs impregnated with ampicillin (2 μg), gentamicin (30 μg) (Oxoid, England), imipenem (10 μg), norfloxacin (10 μg), linezolid (10 μg), streptomycin (300 μg), vancomycin (5 μg) and teicoplanin (30 μg) (Becton, Dickinson and Company, USA), and E-tests containing teicoplanin (0,016-256 mg/L), streptomycin (0,064-1024 mg/L), gentamicin (0,016-256 mg/L) and vancomycin (0,016-256 mg/L) (Liofilchem, Italy).

The HLAR phenotype was subcategorized into the high-level gentamicin resistance (HLGR) and high-level streptomycin resistance (HLSR). The presence of HLGR and HLSR phenotypes was detected using the disc diffusion method, and verified using E-tests. According to the EUCAST recommendations, HLGR is defined if the gentamicin MIC value is >128 mg/L or the zone of inhibition around the disc with gentamicin is <8 mm, while HLSR is defined if the streptomycin MIC value is >512 mg/L or the zone of inhibition around the disc with streptomycin is <14 mm. The HLGR phenotype is consistent with bacterial resistance to all aminoglycosides with the exception of streptomycin and is tantamount to the loss of the synergy between aminoglycosides and beta-lactams or glycopeptides in the therapy. HLSR phenotype is consistent with bacterial resistance to streptomycin and is tantamount to the loss of the synergy between the drug and beta-lactams or glycopeptides in the therapy.

Reference strains represented by *E. faecalis* ATCC 29212 (wild type, fully susceptible) and *E. faecalis* ATCC 51299 [HLAR, VRE phenotypes; genotype: *ant*(*6*)*-I, aac*(*6'*) *aph*(*2"*)*, vanB*] were used as quality controls.

Molecular identification of the isolates, detection of antimicrobial resistance genes

Phenotypic identification of the isolates was verified by the multiplex PCR reaction targeting the 16S rDNA region (to serve as a positive control for the amplification of the DNA sample in the PCR reaction) and the *ddl* genes encoding for the D-Ala:D-Ala ligases specific for *E. faecalis* and *E. faecium* ($ddl_{E. faecalis}$ and $ddl_{E. faecium}$, respectively). HLGR was detected by amplification of the *aac*(*6'*)-*Ie-aph*(*2"*)*-Ia* gene which encodes a bifunctional aminoglycoside modifying enzyme/AME designated AAC(6')-APH(2")-Ia. HLSR was ascertained by amplification of the *ant*(*6*)*-Ia* gene encoding for the monofunctional AME, 6-nucleotidylotransferase I (ANT(6)-I). Vancomycin resistance was established using primers designed to amplify the *vanA* and *vanB* genes involved in the modification of the

Table 1. Characterization of enterococcal isolates analysed in the study, including their phenotypic and genotypic antibiotic resistance profiles

Patient no. (gender, age)	Strain no.	Site of isolation	Hospital ward	Molecular identification	Phenotypic resistance profile	Genotypic resistance profile
1. (M, 80)	E1	Blood	Intensive Care Unit	*E. faecium*	AMP, IPM, NOR, HLSR *	ant(6)-Ia
2. (F,79)	E2	Blood	Allergology and Lung Diseases Unit	*E. faecalis*	NOR, HLSR	ant(6)-Ia
3. (F, 68)	E3	Urine	Internal Medicine Unit	*E. faecalis*	NOR, HLSR, HLGR	ant(6)-Ia, aac(6')-Ie-aph(2'')-Ia
4. (F, 78)	E4	Blood	Internal Medicine Unit	*E. faecium*	AMP, IPM, NOR, HLGR *	aac(6')-Ie-aph(2'')-Ia
5. (F, 61)	E5	Blood	Intensive Care Unit	*E. faecium*	AMP, IPM, NOR, HLSR *	ant(6)-Ia
6. (F, 68)	E6	Blood	Intensive Care Unit	*E. faecium*	AMP, IPM, NOR, HLSR *	ant(6)-Ia
7. (M, 69)	E7	Urine	Internal Medicine Unit	*E. faecalis*	AMP, NOR, HLSR, HLGR *	ant(6)-Ia, aac(6')-Ie-aph(2'')-Ia
8. (M, 63)	E8	Blood	Nephrology Unit	*E. faecium*	AMP, IPM, NOR, HLSR *	ant(6)-Ia
9. (F, 63)	E9	Wound	Surgery Unit	*E. faecalis*	fully susceptible	–
10. (M, 45)	E10	Blood	Cardiology Unit	*E. faecalis*	fully susceptible	–
11. (M, 40)	E11	Wound	Intensive Care Unit	*E. faecium*	AMP, IPM, NOR, HLSR, HLGR, VA, TEC *	ant(6)-Ia, aac(6')-Ie-aph(2'')-Ia, vanA
12. (M, 47)	E12	Blood	Intensive Care Unit	*E. faecium*	AMP, IPM, NOR, HLSR *	–
13. (M, 48)	E13	Blood	Intensive Care Unit	*E. faecalis*	NOR, HLSR, HLGR	ant(6)-Ia, aac(6')-Ie-aph(2'')-Ia
14. (F, 76)	E14	Blood	Intensive Care Unit	*E. faecium*	AMP, IPM, NOR, HLGR, VA, TEC *	aac(6')-Ie-aph(2'')-Ia, vanA
15. (M, 65)	E15	Blood	Internal Medicine Unit	*E. faecium*	AMP, IPM, NOR, HLGR *	aac(6')-Ie-aph(2'')-Ia
16. (F, 85)	E16	Blood	Internal Medicine Unit	*E. faecium*	AMP, IPM, NOR, HLGR *	aac(6')-Ie-aph(2'')-Ia
17. (F, 86)	E17	Blood	Internal Medicine Unit	*E. faecium*	AMP, IPM, NOR, HLSR, HLGR, VA, TEC *	ant(6)-Ia, vanA
18. (F, 77)	E18	Blood	Intensive Care Unit	*E. faecalis*	NOR, HLSR, HLGR	ant(6)-Ia, aac(6')-Ie-aph(2'')-Ia
19. (F, 76)	E19	Urine	Internal Medicine Unit	*E. faecium*	AMP, IPM, NOR, HLSR, HLGR *	ant(6)-Ia, aac(6')-Ie-aph(2'')-Ia
20. (M, 74)	E20	Urine	Allergology and Lung Diseases Unit	*E. faecium*	AMP, IPM, NOR, HLGR *	aac(6')-Ie-aph(2'')-Ia
21. (F, 56)	E21	Blood	Intensive Care Unit	*E. faecalis*	AMP, IPM, NOR, HLSR, HLGR *	ant(6)-Ia, aac(6')-Ie-aph(2'')-Ia
22. (M, 81)	E22	Blood	Intensive Care Unit	*E. faecium*	AMP, IPM, NOR	–
23. (M, 97)	E23	Blood	Internal Medicine Unit	*E. faecium*	AMP, IPM, NOR, HLSR, HLGR *	aac(6')-Ie-aph(2'')-Ia
24. (M, 75)	E24	Blood	Allergology and Lung Diseases Unit	*E. faecalis*	NOR, HLSR	ant(6)-Ia
25. (F, 78)	E25	Blood	Internal Medicine Unit	*E. faecium*	AMP, IPM, NOR, HLGR *	aac(6')-Ie-aph(2'')-Ia
26. (M, 91)	E26	Blood	Cardiology Unit	*E. faecalis*	NOR, HLSR, HLGR	ant(6)-Ia, aac(6')-Ie-aph(2'')-Ia
27. (M, 73)	E27	Blood	Internal Medicine Unit	*E. faecalis*	fully susceptible	–
28. (M, 66)	E28	Blood	Allergology and Lung Diseases Unit	*E. faecalis*	NOR, HLSR, HLGR	ant(6)-Ia, aac(6')-Ie-aph(2'')-Ia
29. (M, 75)	E29	Blood	Allergology and Lung Diseases Unit	*E. faecalis*	NOR, HLSR	ant(6)-Ia
30. (M, 86)	E30	Urine	Internal Medicine Unit	*E. faecalis*	NOR, HLSR, HLGR	ant(6)-Ia, aac(6')-Ie-aph(2'')-Ia
31. (F, 85)	E31	Blood	Internal Medicine Unit	*E. faecium*	AMP, IPM, NOR, HLGR *	aac(6')-Ie-aph(2'')-Ia
32. (M, 67)	E32	Blood	Intensive Care Unit	*E. faecalis*	fully susceptible	–
33. (F, 80)	E33	Urine	Internal Medicine Unit	*E. faecium*	AMP, IPM, NOR, HLSR, HLGR, VA, TEC *	ant(6)-Ia aac(6')-Ie-aph(2'')-Ia, vanA
34. (M, 81)	E34	Blood	Nephrology Unit	*E. faecalis*	HLSR, HLGR	ant(6)-Ia
35. (M, 66)	E35	Blood	Intensive Care Unit	*E. faecalis*	fully susceptible	–

Abbreviations: M – male; F – female; AMP – ampicillin; IPM – imipenem; NOR – norfloxacin; HLSR – high level streptomycin resistance; HLGR – high level gentamycin resistance; VA – vancomycin; TEC – teicoplanin; * – MDR isolate

antibiotic binding site and linked to high-level vancomycin and teicoplanin resistance and vancomycin resistance only, respectively.

Primers used in the molecular analysis were synthesized by Genomed (Poland). Total bacterial DNA was prepared by a rapid lysis method. Briefly, enterococcal colonies grown on the blood agar medium were collected with an inoculation loop and suspended in 150 μl of water. The solution was incubated at 95°C for 10 minutes, followed by 10 min. incubation in an ultrasonic bath. The solution was then centrifuged for 5 min. at a maximum speed and the supernatant was transferred to a new tube. Primer sequences and amplification conditions used in the PCR assays are shown in Table 2.

The multiplex PCR assay for the identification of the species was performed in a final volume of 25 μl containing DNA as template, 0.1 μM of each primer, 0.1 mM of dNTPs, 0.625 U of the Taq DNA polymerase, 2 μl of the buffer, and 1.5 mM of $MgCl_2$.

The PCR assays for the *vanA*, *vanB*, *aac*(*6'*)*-Ie-aph*(*2''*)*-Ia* and *ant*(*6*)*-Ia* genes were performed in a final volume of 20 μl containing DNA as template, 0.2 μM of each primer, 0.2 mM of dNTPs, 0.5 U of the Taq DNA polymerase, 2 μl of the buffer and 1.5 mM of $MgCl_2$.

Amplification products were analyzed by 2% agarose gel electrophoresis.

RESULTS

Phenotypic identification of enterococcal isolates to the species level using the BD Phoenix system was verified by the multiplex PCR reaction and confirmed in 34 (97.1%) out of the 35 analysed isolates, as phenotypic and molecular identification gave discrepant results for the isolate no. E21. The BD Phoenix system identified the isolate as *E. faecium,* whereas the PCR reaction gave a positive result for the *E. faecalis* – specific ligase ($ddl_{E.\ faecalis}$). Hence, the overall number of enterococcal isolates investigated in the study included 18 isolates of *E. faecium* and 17 isolates of *E. faecalis*. The representative results of the molecular identification of the isolates are provided in Table 1 and in Figure 1.

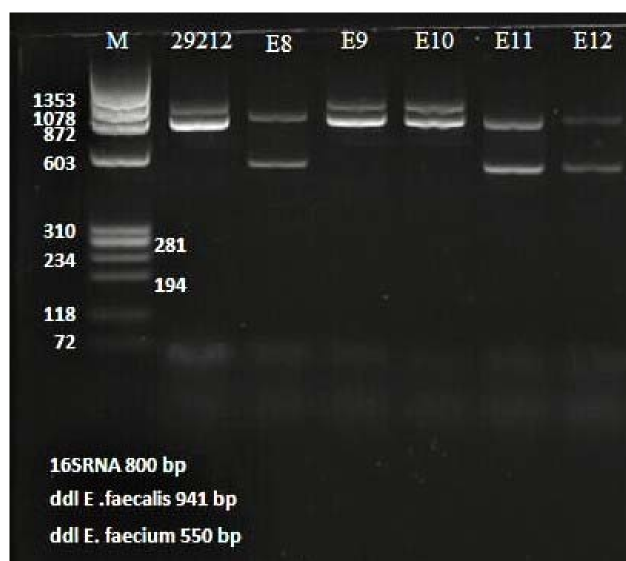
The majority of enterococcal isolates were isolated from patients hospitalized in the Intensive Care Unit/ICU (12 isolates/34.3%) and the Internal Medicine Unit/IMU (13 isolates/37.1%). We observed a greater prevalence of *E. faecium* compared to *E. faecalis* in these hospital wards (7 vs. 5 isolates and 9 vs. 4 isolates, respectively). *E. faecalis* was isolated more frequently in other hospital wards, including the Alergology and Lung Diseases, Cardiology, Nephrology and Surgery Units (Table 1).

Table 2. Primer sequences, amplification conditions and sizes of the relevant PCR products used in the study

Amplified gene	Primer sequence	Amplification conditions (no. of cycles)	PCR product size	Reference
ddl E. faecalis	**F:** 5′-ATCAAGTACAGTTAGTCT-3′ **R:** 5′-ACGATTCAAAGCTAACTG-3′	PD: 94°C – 5 min (1) D: 94°C – 30 sec; A: 50°C – 90 sec; E: 72°C – 60 sec (30) FE: 72°C – 10 min. (1)	941 bp	[16]
ddl E .faecium	**F:** 5′-TAGAGACATTGAATATGCC-3′ **R:** 5′-TCGAATGTGCTACAATC-3′		550 bp	[16]
16S	**F:** 5′-GACTACCNGGGTATCTAATCC-3′ **R:** 5′-AGAGTTTGATCCTGGCTNAG-3′		800 bp	[17]
vanA	**F:** 5′-GGGAAAACGACAATTGC-3′ **R:** 5′-GTACAATGCGGCCGTTA-3′	PD: 94°C – 2 min (1) D: 94°C – 1 min; A: 50°C – 1 min; E: 72°C – 1 min (30) FE: 72°C – 10 min. (1)	732 bp	[16]
vanB	**F:** 5′-ATGGGAAGCCGATAGTC-3′ **R:** 5′-GATTTCGTTCCTCGACC-3′	PD: 94°C – 3 min (1) D: 94°C – 30 sec; A: 54°C – 40 sec; E: 72°C – 1 min (40) FE: 72°C – 10 min. (1)	635 bp	[16]
aac(6′)-Ie-aph(2″)-Ia	**F:**5′-CAGGAATTTATCGAAAATGGTAGAAAAG-3′ **R:**5′-CACAATCGACTAAAGAGTACCAATC-3′	PD: 95°C – 7 min (1) D: 95°C – 1 min; A: 55°C – 1 min; E: 72°C – 1 min (33) FE: 72°C – 10 min. (1)	369 bp	[18]
ant(6)-Ia	**F:**5′-CGGGAGAATGGGAGACTTTG-3′ **R:**5′-CTGTGGCTCCACAATCTGAT-3′		563 bp	[19]

Abbreviations: PD – preliminary denaturation; D – denaturation; A – annealing; E – extension; FE – final extension

The predominant site of isolation of the bacteria was blood (27 isolates/77.1%), followed by urine (6 isolates/17.1%) and wounds (2 isolates/5.7%) (Table 1). The frequency of *E. faecium* and *E. faecalis* isolation from clinical specimens did not reveal major differences or was even equal. The data is as follows: blood – 14 isolates *vs*. 12, urine – 3 isolates *vs*. 3, wounds – 1 isolate *vs*. 1, respectively.



M – ladder; 2912 – reference *E. faecalis* strain (ATCC 29212); E8, E11, E12 – clinical *E. faecium* isolates; E9, E10 – clinical *E. faecalis* isolates

Figure 1. Identification of enterococcal isolates analysed in the study using the multiplex PCR assay

Antimicrobial susceptibility testing results

Phenotypic and genotypic antimicrobial resistance profiles of the analysed isolates are shown in Table 1.

Enterococcal isolates demonstrated the highest rate of resistance against norfloxacin and, consequently, to related fluoroquinolones. As many as 29 (82.9%) isolates were norfloxacin-resistant, including 18 (100%) *E. faecium* isolates and 11 (64.7%) *E. faecalis*. According to the EUCAST recommendations, the disc diffusion test with norfloxacin can be used to screen for fluoroquinolone resistance. Ciprofloxacin and levofloxacin susceptibilities can be inferred from the norfloxacin susceptibility. However, there are no clinical breakpoints for *Enterococcus* spp. and moxifloxacin, which has been used for oral step-down treatment of endocarditis caused by *Enterococcus* spp. According to the EUCAST, the norfloxacin disk diffusion test or the moxifloxacin minimum inhibitory concentration (MIC) epidemiological cutoff value (ECOFF) (1 mg/L) can be used to screen for resistance mechanisms. When screen negative, the isolate should be reported "wild type" or "devoid of fluoroquinolone resistance mechanisms", but not as "susceptible to moxifloxacin".

Aminoglycoside resistance also occurred frequently among the analysed isolates. The HLSR phenotype was detected in 22 (62.9%) isolates, including 12 (70.5%) *E. faecalis* and 10 (58.8%) *E. faecium* isolates. In all HLSR-positive enterococal isolates, the MIC value for the antibiotic exceeded 1024 mg/l. The HLGR phenotype occurred in 21 (60%) isolates, including 12 (66.6%) *E. faecium* and 9 (52.9%) *E. faecalis.* In all HLGR-positive enterococal isolates, the MIC value for the antibiotic exceeded 256 mg/l. As many as 14 (40%) isolates were both HLSR and HLGR- positive, including 9 *E. faecalis* and 5 *E. faecium* isolates*.*

The HLAR phenotype detection was subsequently verified by the PCR detection of the *ant*(*6*)*-Ia and aac*(*6'*)*-Ie-aph*(*2"*)*-Ia* genes encoding for the enzymes involved in the enzymatic modification of streptomycin and gentamicin, respectively (Figure 2).

Twenty out of the 22 HLSR isolates (90.9%) and 19 out of the 21 HLGR isolates (90.4%) were the *ant(6)-Ia* and *aac*(*6*')*-Ie-aph*(*2"*)*-Ia* – positive, respectively (Table 1). As mentioned above, 40% of the HLAR isolates were both HLSR and HLGR – positive, and both resistance genes were detectable in 11 (78.5%) of them.

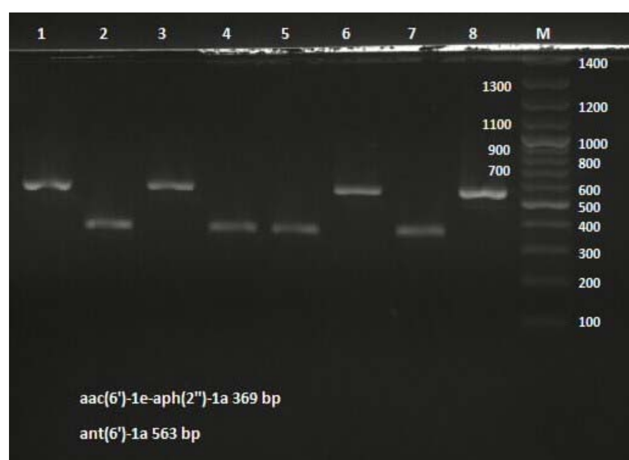
In the remaining isolates demonstrating HLAR phenotypically, but negative for the corresponding genes in the PCR assay (isolates no. E12, E17, E23, E34), other molecular mechanisms were most probably responsible for aminoglycoside resistance, including another type of enzyme capable of drug modification (HLSR/HLGR) or mutational modification of the 30S ribosomal subunit (HLSR).

Beta-lactam resistance in the analysed isolates was verified in terms of ampicillin- and imipenem resistance. It should be emphasized that 18 (100%) investigated *E. faecium* isolates were ampicillin resistant, whereas this type resistance was observed in only two (11.7%) out of the 17 *E. faecalis*. According to the EUCAST, ampicillin susceptibility result is tantamount to susceptibility to ampicillin, amoxicillin and piperacillin (with and without beta-lactamase inhibitor). Imipenem resistance was observed in all *E. faecium* isolates and only one *E. faecalis*. All isolates resistant to imipenem were simultaneously resistant to ampicillin.

Glycopeptide resistance was detected in a minority (n=4) of the isolates represented alone by *E. faecium*. The overall

resistance rate of VRE in *E. faecium* was 22.2%. The VRE isolates were resistant both to vancomycin and teicoplanin as assessed using the disc diffusion method and E-tests. The MIC values for vancomycin and teicoplanin were ≥256 mg/l and 12-64 mg/l, respectively. The isolates were positive for the *vanA* gene (Figure 3). Figure 4 summarizes the antibiotic resistance profiles of the analysed *E. faecium* and *E. faecalis* isolates.

We identified 19 (54.3%) MDR isolates, defined by their resistance to at least one drug belonging to at least three distinct groups of antibiotics (Table 1). Fourteen (73.7%) MDR isolates were cultured from blood, followed by 4 isolates (21.1%) cultured from urine and 1 isolate (5.3%) cultured from wound. As many as 17 (89.5%) out of the 19 MDR isolates were represented by *E. faecium*, and three (15.8%) among them remained susceptible to linezolid alone.

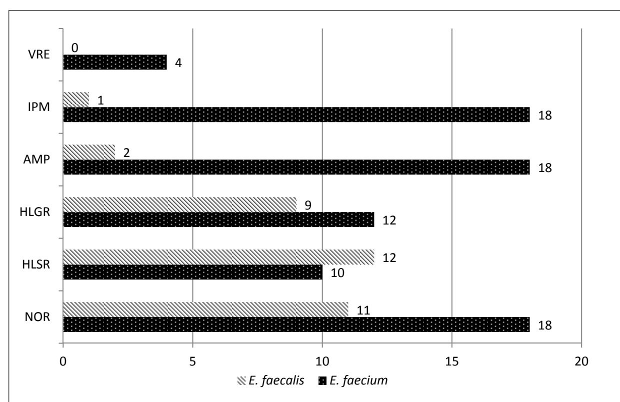


M – ladder; Lanes 1, 3, 6, 8 – *ant(6)-Ia* – positive isolates; Lanes 2, 4, 5, 7, – *aac(6')-aph(2")* – positive isolates

Figure 2. The PCR detection of the *ant(6)-Ia and aac(6')-aph(2")* genes mediating high-level streptomycin and gentamicin resistance, respectively

M – ladder; E11, E17, E14 – *vanA* – positive isolates; E1, E9 - *vanA* – negative isolates

Figure 3. Detection using the PCR of the *vanA* gene mediating glycopeptide resistance in enterococci



NOR – norfloxacin; HLSR –High Level Streptomycin Resistance; HLGR – High Level Gentamicin Resistance; AMP – ampicillin; IPM – imipenem; VRE – vancomycin resistant enterococci

Figure 4. The overall numbers of *E. faecalis* and *E. faecium* isolates demonstrating resistance to the tested antimicrobial agents

DISCUSSION

Our results add further evidence to an increasing problem of antibiotic resistance among enterococci in Polish hospitals, with particular emphasis on MDR *E. faecium*, which accounted for 94.4% of the overall number of the analysed isolates belonging to this species. More than 70% of the patients infected with enterococci included in the study were hospitalized in the ICU and the IMU, and the predominant site (77%) of enterococcal isolation was blood. Bacteremia and abdominal infections (which are frequent sources of bacteremia/septicaemia) represent clinical presentations typical for enterococci [14]. The ICU stay, in turn, is considered an important risk factor for enterococcal infections [13,14,20]. Although during a 5-month collection period nearly an equal number of *E. faecalis* (n=17; 48.5%) and *E. faecium* (n=18; 51.4%) was found, we have observed the important role of *E. faecium* as an etiologic agent of HAIs outcompeting *E. faecalis* in two hospital wards in which we simultaneously observed the highest rate of enterococcal isolation, namely in the ICU and IMU. In spite of *E. faecalis* predominance in hospitals a few decades ago, recent years have witnessed a significant change in the epidemiology of enterococal infections in the favour of *E. faecium* [14].

It should be emphasized that all *E. faecium* isolates included in the study were resistant to ampicillin, imipenem and norfloxacin, which, unfortunately, is not unusual, since according to the epidemiological data and reports of other authors, hospital-associated *E. faecium* is typically resistant to ampicillin and fluoroquinolones [1,13,14, 20-22]. Moreover, 17 out of the 18 isolates demonstrated HLAR, including 27.7% with the HLSR and 38.8% with the HLGR; an additional 27.7% of the isolates were both HLSR and HLGR. HLAR was also common in *E. faecalis* (HLSR>70%, HLGR>50%), which is in accordance with previous reports [14]. The average rate of HLAR in Poland (data from 2017) is 53% for invasive *E. faecium* and 41% for *E. faecalis [23]. The HLAR phenotype is usually mediated* by AMEs, among which the AAC(6')-APH(2')-Ia bifunctional enzyme (encoded by the *aac(6')-Ie-aph(2")*-Ia gene) with acetyltransferase and phosphotransferase activity is considered predominant [24]. This enzyme modifies

essentially all clinically available aminoglycosides, except streptomycin, which, in turn, eliminates the therapeutic use of all aminoglycosides other than streptomycin, including their combinations with beta-lactams/glycopeptides. The ANT(6)-I nucleotidyltransferase encoded by the *ant*(*6'*)-*Ia* frequently mediates HLSR [18,19]. The presence of the two genes was confirmed in the overwhelming majority (90%) of the HLAR isolates analysed in the study. Interestingly, 40% of the HLAR isolates (9/52.9% *E. faecalis* and 5/27.7% *E. faecium*) were both HLSR and HLGR – positive, with the two aforementioned resistance genes detectable in 11 (78.5%). The multiple aminoglycoside resistance genes were also frequently harboured by the enterococcal isolates studied by Kobayahi *et al.* [19], including 37.6% of *E. faecium* isolates carrying both the *ant(6)-Ia* and *aac*(*6'*)-*aph*(*2''*). For the HLAR isolates found in our study to be negative for the *ant*(*6*)-*Ia* or the *aac*(*6'*)-*aph*(*2''*) genes, two possible explanations should be taken into account. They include the possibility of mutation in the location of the primer annealing as reported previously by Vakulenko *et al.* [18] or the production of a different type of an enzyme capable of drug inactivation (HLSR/HLGR). Of note, alternative enzymes mediating aminoglycoside resistance have been described [18,19]. Finally, another plausible mechanism mediating HLSR could be a mutational modification of the 30S ribosomal subunit [19].

The leading conclusion drawn from the results presented above is that there is a limited spectrum of available therapeutic options especially against *E. faecium*. This observation is in line with other reports. In the study of Talaga *et al.* [13] analysing enterococcal strains from four Polish hospitals, fluoroquinolone resistance ranged between 33.3% up to 96%. The authors also observed that ampicillin- and vancomycin resistance was demonstrated by all *E. faecium* isolates from the two hospitals, including 87.3% of the isolates presenting the HLAR phenotype. Most of the *E. faecium* isolates were MDR. All *E. faecalis* isolates, in turn, were susceptible to ampicillin, vancomycin, teicoplanin, linezolid and tygecycline. Data from Poland and other European countries indicate higher mortality rates due to infections caused by *E. faecium* than *E. faecalis,* which can be at least partially linked to higher resistance rates observed in this species [14].

Glycopeptides and oxazolidinones, in turn, seemed to be associated with the highest probability of therapeutic success against enterococci in the study presented here. All *E. faecium* and *E. faecalis* isolates remained sensitive to linezolid, whereas all *E. faecalis* and 77.8% of all *E. faecium* were susceptible to vancomycin and teicoplanin. Glycopeptide resistance was detected in four (22.2%) *E. faecium* isolates and was classified as the VanA phenotype due to the amplification of the *vanA* gene. Gawryszewska *et al.* [14] analysed 259 enterococcal isolates collected from 30 hospitals in Poland and also found that all isolates were linezolid sensitive, whereas vancomycin resistance mediated by *vanA* or *vanB* was detected in 7.1% of all *E. faecium.* Nevertheless, data provided by Talaga-Ćwiertnia *et al.* [11] indicate that resistance against vancomycin in *E. faecium* isolates has been on the systematic rise in Poland reaching the rate of 25.2% in 2016.

The average rate of invasive vancomycin resistant *E. faecium* in Poland (data from 2017) is 32% (28-37%) and is comparable to the rates observed in Lithuania (36%), Slovakia (32%), Hungary (28%), Romania (34%), Serbia (35%) and Greece (31%). In the United States and Australia, however, up to 50% or more of invasive *E. faecium* demonstrate resistance to vancomycin - which poses a severe public health threat [23]. The *vanA* gene cluster encoding enzymatic machinery altering the terminal peptydoglikan precursor's residues (D-alanyl-D-alanine) critical for antibiotic binding, remains the predominant gene mediating glycopeptide resistance in enterococci [4,9,11,20,25]. It is involved in inducible resistance to vancomycin and teicoplanin, and is typically located on transposones (eg, Tn1546) within plasmids [4,25].

It should be mentioned that continuous antibiotic pressure is a driving force towards the selection of new resistance phenotypes, among which one of the most recent threats in the context of enterococcal infections is the emergence of resistance against linezolid. All isolates reported here were linezolid sensitive. This is important information in view of the very disturbing phenomenon associated with a high rate of MDR *E. faecium* detection. The four vancomycin-resistant *E. faecium* isolates were simultaneously resistant to all other tested drugs, with the exception of linezolid. This indicates that oxazolidinones represent an important armamentarium and must be considered the drug of the last resort against many MDR enterococci. Unfortunately, resistance against this group of chemotherapeutics by the inhibition of the elongation of the polypeptide chain in bacterial cells has emerged. This can be mediated by mutations in the 23S rRNA gene region and ribosomal proteins (L3 and L4), as well as by the presence of transmissible, plasmid encoded determinants, including the *cfr* and *optrA* genes [4,10]. Cfr (chloramphenicol-florfenicol resistance) is a methylase able to modify the adenine nucleotide of the 23S rRNA, whereas the *optrA* gene encodes a putative ABC transporter mediating efflux and is associated with elevated MICs to oxazolidinones in the absence of known 23S rRNA mutations or the *cfr* gene [4,15].

A significant rise in the number of linezolid resistant enterococci (LRE) has been reported in Polish hospitals since 2012 [15]. Gawryszewska *et al.* [15] investigated 50 clinical LRE (including *E. faecium* – 82% and *E. faecalis* – 16%) collected between 2008-2015 (20 hospital settings located in 12 Polish cities) and found that the 23S rRNA mutation was the most frequent (94%) mechanism of linezolid resistance, whereas the *optrA* gene was identified in two *E. faecalis* isolates. Krawczyk *et al.* [9] identified, in addition to the 23S rRNA mutation, the *cftr* gene in 14 out of the 19 clinical linezolid-resistant *E. faecium* isolates. The detection of transmissible genetic determinants of linezolid resistance such as the *optrA* and *cfr*, raises concerns about the possible decrease in the effectiveness of linezolid in the future. Moreover, the linezolid-resistant *E. faecium* showed high prevalence of vancomycin resistance (90.2%) in the study of Gawryszewska *et al.* [15]; similar observations were reported by Krawczyk *et al.* [9]. It should also be mentioned that over 70% of LRE investigated by Gawryszewska *et al.* [15] were isolated from patients of the ICUs and

haematology wards. In these, the highest use of antimicrobials, including linezolid, is reported. Previous administration of linezolid and treatment with glycopeptides and possibly with carbapenems (imipenems) are considered factors that may favour colonization and subsequent development of infections with LRE [9].

Our study has several limitations, including a limited number of the analyzed isolates and the lack of genetic investigation regarding the clonality of the isolates. Nevertheless, we consider our results a further contribution to the analysis of the epidemiology of the enterococcal HAIs and trends in their antimicrobial resistance. The monitoring of the prevalence and antimicrobial resistance of *Enterococcus* species is of the uppermost significance to control and prevent infections caused by this group of bacteria.

CONCLUSIONS

Multidrug-resistance in the hospital-associated enterococci is a disturbing phenomenon, particularly in *E. faecium*. Understanding of emergent mechanisms of resistance in enterococci can provide insights into the best treatment approaches for these opportunistic pathogens and help to guide physicians in making rational therapeutic decisions. Surveillance of the epidemiologic situation regarding enterococci is crucial to control and prevent infections caused by this group of bacteria.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

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REFERNCES

1. Sadowy E. Mobile genetic elements beyond the VanB-resistance dissemination among hospital-associated enterococci and other Gram-positive bacteria. *Plasmid.* 2021;114:102558.
2. Miller WR, Murray BE, Rice LB, Arias CA. Resistance in vancomycin-resistant enterococci. *Infect Dis Clin North Am.* 2020;34:751-71.
3. Kamińska W, Grochowska M, Chmielarczyk A, Olszewska A, Skolimowska G, Dzierżanowska-Fangrat K. Genetic typing of *Enterococcus faecium* VRE strains isolated in three hospitals in Warsaw and Siedlce in 2015-2016. *Przegl Epidemiol.* 2019;73:49-60.
4. Miller WR, Murray BE, Rice LB, Arias CA. Vancomycin-resistant enterococci: Therapeutic challenges in the 21st Century. *Infect Dis Clin North Am.* 2016;30:415-39.
5. Bender JK, Cattoir V, Hegstad K, Sadowy E, Coque TM, Westh H, Hammerum AM, et al. Update on prevalence and mechanisms of resistance to linezolid, tigecycline and daptomycin in enterococci in Europe: Towards a common nomenclature. *Drug Resist Updat.* 2018;40:25-39.
6. Fiore E, Van Tyne D, Gilmore MS. Pathogenicity of Enterococci. *Microbiol Spectr.* 2019;7:10.1128/microbiolspec.GPP3-0053-2018.
7. García-Solache M, Rice LB. The enterococcus: a model of adaptability to its environment. *Clin Microbiol Rev.* 2019;32: e00058-18.
8. Santajit S, Indrawattana N. Mechanisms of antimicrobial resistance in ESKAPE pathogens. *Biomed Res Int.* 2016;2016:2475067.
9. Krawczyk B, Wysocka M, Kotłowski R, Bronk M, Michalik M, Samet A. Linezolid-resistant *Enterococcus faecium* strains isolated from one hospital in Poland – commensals or hospital-adapted pathogens? *PLoS One.* 2020;15:e0233504.
10. Sadowy E. Linezolid resistance genes and genetic elements enhancing their dissemination in enterococci and streptococci. *Plasmid.* 2018;99:89-98.
11. Talaga-Ćwiertnia K, Bulanda M. Analysis of the world epidemiological situation among vancomycin-resistant *Enterococcus faecium* infections and the current situation in Poland. *Przegl Epidemiol.* 2018;72:3-15.
12. Chmielarczyk A, Pomorska-Wesołowska M, Romaniszyn D, Wójkowska-Mach J. Healthcare-associated laboratory-confirmed bloodstream infections-species diversity and resistance mechanisms, a four-year retrospective laboratory-based study in the south of Poland. *Int J Environ Res Public Health.* 2021;18:2785.
13. Talaga K, Odrowąż-Konduracka D, Paradowska B, Jagiencarz-Starzec B, Wolak Z, Bulanda M, Szczypta A. Typing of *Enterococcus* spp. strains in 4 hospitals in the Małopolska region in Poland. *Adv Clin Exp Med.* 2018;27:111-7.
14. Gawryszewska I, Żabicka D, Bojarska K, Malinowska K, Hryniewicz W, Sadowy E. Invasive enterococcal infections in Poland: the current epidemiological situation. *Eur J Clin Microbiol Infect Dis.* 2016;35:847-56.
15. Gawryszewska I, Żabicka D, Hryniewicz W, Sadowy E. Linezolid-resistant enterococci in Polish hospitals: species, clonality and determinants of linezolid resistance. *Eur J Clin Microbiol Infect Dis.* 2017;36:1279-86.
16. Dutka-Malen S, Evers S, Courvalin P. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *J Clin Microbiol.* 1995;33:24-7.
17. Protocol for PCR amplification of *E. faecium* and *E. faecalis* recommended by the EURL-AR. 3rd Version – January 2014. DTU Food National Food Institute. [https://www.eurl-ar.eu/CustomerData/Files/Folders/21-protocols/281_protocol-for-enterococcus-final-vs3.pdf]
18. Vakulenko SB, Donabedian SM, Voskresenskiy AM, Zervos MJ, Lerner SA, Chow JW. Multiplex PCR for detection of aminoglycoside resistance genes in enterococci. *Antimicrob Agents Chemother.* 2003;47:1423-6.
19. Kobayashi N, Alam M, Nishimoto Y, Urasawa S, Uehara N, Watanabe N. Distribution of aminoglycoside resistance genes in recent clinical isolates of *Enterococcus faecalis, Enterococcus faecium* and *Enterococcus avium*. *Epidemiol Infect.* 2001;126:197-204.
20. Asgin N, Otlu B. Antibiotic resistance and molecular epidemiology of vancomycin-resistant enterococci in a Tertiary Care Hospital in Turkey. *Infect Drug Resist.* 2020;13:191-8.
21. Haghi F, Lohrasbi V, Zeighami H. High incidence of virulence determinants, aminoglycoside and vancomycin resistance in enterococci isolated from hospitalized patients in Northwest Iran. *BMC Infect Dis.* 2019;19:744.
22. Jia W, Li G, Wang W. Prevalence and antimicrobial resistance of *Enterococcus* species: a hospital-based study in China. *Int J Environ Res Public Health.* 2014;11:3424-42.
23. *The Center for Disease, Dynamics Economics & Policy. Resistance Map: Antibiotic resistance.* 2021. [https://resistancemap.cddep.org/AntibioticResistance.php] Date accessed: July 7, 2021.
24. Shete V, Grover N, Kumar M. Analysis of aminoglycoside modifying enzyme genes responsible for high-level aminoglycoside resistance among enterococcal isolates. *J Pathog.* 2017;2017:3256952.
25. Gorrie C, Higgs C, Carter G, Stinear TP, Howden B. Genomics of vancomycin-resistant *Enterococcus faecium*. *Microb Genom.* 2019;5:e000283.