Typing of *Enterococcus* spp. strains in 4 hospitals in the Małopolska region in Poland

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Abstract

Background. In the Małopolska province, the first case of vancomycin resistant enterococci (VRE) occurrence was an outbreak in 2001 caused by strains of the genus *E. faecium* carrying the *vanA* operon.

Objectives. The aim of this study is to determine the antimicrobial resistance and the occurrence of virulence determinants among *Enterococcus* spp. in patients hospitalized in the Małopolska region in 2015.

Material and methods. Antimicrobial susceptibility was determined by disc diffusion and the E test. The presence of aminoglycoside and glycopeptide resistance genes and virulence genes (*asa1*, *gelE*, *cylA*, *esp*, *hyl*) was investigated using multiplex polymerase chain reaction (PCR). Also, the presence of IS*16* was investigated. The activity of gelatinase, cytolysin (hemolysin), and DNase was tested.

Results. All *E. faecalis* were susceptible to ampicillin, vancomycin, teicoplanin, linezolid and tigecycline. All *E. faecium* strains were susceptible to quinupristin-dalfopristin. Resistance to ampicillin and vancomycin was detected among all *E. faecium* isolates from hospitals C and D. 87.32% of *E. faecium* presented high-level aminoglycoside-resistant (HLAR) phenotype, including 78.33% of strains from hospital C and 100% from hospital D. In hospital C (98.3%) and D (96%), resistance to ciprofloxacin, levofloxacin and norfloxacin was observed. Gene *esp* was detected in all *E. faecium* isolates and the majority of *E. faecium* isolates carried *hyl* (97%). In *E. faecalis*, different combinations of virulence genes were detected. All analyzed *E. faecium* strains showed the presence of IS*16* insertion element.

Conclusions. *E. faecalis* isolates were more susceptible to antimicrobials than *E. faecium*, which were largely multidrug-resistant. *E. faecalis* strains have diverse virulence factors. *E. faecium* showed a high percentage of *hyl* and *esp* genes and the presence of IS*16*.

Key words: virulence factors, healthcare-associated infections, *Enterococcus*, vancomycin resistant enterococci (VRE), antimicrobial susceptibility

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Introduction

Enterococci are now recognized as a significant cause of healthcare-associated infections worldwide.[1–3] The emergence of multidrug resistance (MDR), including high-level aminoglycoside-resistant (HLAR) enterococci and vancomycin-resistant enterococci (VRE), causes great difficulties in clinical antimicrobial therapy.[4] VRE infections have been associated with higher mortality, longer hospital length of stay, and higher costs compared with vancomycin-susceptible isolates.[5,6] The first vancomycin-resistant *E. faecium* (VRE*fm*) strains carrying the *vanA* operon and *vanB* operon were reported in Poland in 1996 and 1999, respectively.[7] In the Małopolska province, the first case of VRE occurrence was an outbreak caused by strains of the genus *E. faecium* carrying the *vanA* operon in 2001.[8,9]

The aim of this study is to determine the virulence factors and antibiotic resistance patterns of enterococcal isolates from 4 hospitals with different specialties from the Małopolska region.

Material and methods

Hospital settings

Pieces of information were obtained from hospitals from which enterococcal strains were isolated, regarding ward locations and body sites from which VRE and other enterococci were recovered. Material for the study was taken according to the following criteria: from patients with symptomatic infection as well as from patients admitted from other hospitals, Social Welfare Homes, Health Care Centers; patients who had previously been treated in another hospital and had been given broad-spectrum antibiotics; patients who had information about VRE colonization in their discharge card; the ones who were repeatedly hospitalized for 12 months.

Characteristic of *Enterococcus* strains

In this hospital-based study, a total of 154 *Enterococcus* strains isolated from 4 hospitals during the period from January 2015 through December 2015 were collected. Six of the samples were collected from hospital A, 13 samples were from B, 60 were from C, and 75 samples were acquired from hospital D. The enterococcal isolates were obtained from different specimens and were classified as colonization or infection. The study comprised consecutive, non-repetitive enterococci isolates.

All strains had already been identified with the species level using conventional biochemical tests and with the VITEK-2 COMPACT fully automated microbiological system in hospital laboratories. The identification was confirmed by species-specific multiplex polymerase chain reaction (PCR) as described by Jackson (*E. faecalis* – 360 bp, *E. faecium* – 215 bp and *E. avium* – 368 bp) using the DNA samples of each *Enterococcus* strain isolated by Genomic Mini (A&A Biotechnology, Gdynia, Poland).[10]

Antibiotic susceptibility testing

Susceptibility to antimicrobials was evaluated by the disc-diffusion method (Oxoid, Basingstoke, England) according to manufacturer's procedure. The minimum inhibitory concentrations (MICs) of ampicillin, ciprofloxacin, tigecycline and linezolid were evaluated by the E tests method (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's procedure.

The presence of HLAR phenotype, HLGR (high-level gentamicin resistance) and HLSR (high-level streptomycin resistance) phenotypes were identified by the disk susceptibility tests with streptomycin and gentamicin. VRE phenotype was detected by teicoplanin and vancomycin. The HLAR and VRE phenotypes were confirmed by the E tests method. All tests carried out using both methods were done on freshly prepared Mueller Hinton II Agar (Biocorp, Warszawa, Poland). The interpretation was performed in accordance with the recommendations of the European Committe on Antimicrobial Susceptibility Testing (EUCAST) guidelines.[11]

Assay of gelatinase activity

The production of gelatinase in *Enterococcus* strains was detected with the method described by Strzelecki.[12] Gelatinase activity was observed as a transparent halo around the enterococcal colonies.

Detection of antimicrobial resistance genes

To detect genes responsible for HLAR phenotype in the genomes of all the strains resistant to gentamicin and/or streptomycin, multiplex PCR was applied according to the method of Vakulenko.[13] The results of VRE testing by the phenotype method were confirmed by investigating *vanA* (732 bp) and *vanB* (635 bp) operons using the multiplex PCR as described by Biendo.[14]

Table 1. Characteristics of the studied hospitals

Hospital	Body sites			Units		
	blood	rectal swab	others*	ICU	surgery	others**
A	5	0	1	2	0	4
B	0	0	13	2	7	4
C	1	49	10	25	15	20
D	2	55	18	57	7	11
Total	8	104	42	86	29	39

* pleural effusion, cerebrospinal fluid, urine, bronchial aspirates, surgical wound; ** Clinical Department of Interventional Cardiology, Department of Pulmonary Diseases, Department of Internal Medicine, Department of Orthopedic Trauma, Department of Neurology, Stroke Unit, Department of Urology; ICU – intensive care unit.

Detection of virulence factor genes

To detect the presence of genes encoding selected virulence factors (*asa1*, *gelE*, *cylA*, *esp*, *hyl* – 375bp, 213 bp, 688 bp, 510 bp, 276 bp, respectively) in the enterococcal DNA, multiplex PCR was applied according to the methods of Vankerckhoven.[15] The presence of quorum-sensing genes (*fsrA*, *fsrB*, *fsrC*) in the genomes of all the strains positive for the *gelE* gene was tested by multiplex PCR and was applied pursuant to Qin.[16] The product sizes for *fsrA*, *fsrB* and *fsrC* were 484 bp, 574 bp and 580 bp, respectively.

Assay of cytolysin (hemolysin) activity

Cytolysin activity in *Enterococcus* strains was detected by the method described by Kowalska-Krochmal.[17] The presence of a clear zone surrounding the studied strain colonies indicated the production of cytolysin.

Assay of DNase activity

DNase activity was detected on BD DNase Test Agar (Becton Dickinson, Oxford, England) described by Kowalska-Krochmal.[17] DNase positive organisms will be surrounded by clear zones of depolymerized DNA. Colonies of DNase negative organisms will not show any clearing around the colonies.

Detection of a specific mobile insertion element IS*16* by PCR

PCR was performed with primers encoding gene fragment of the insertion sequence IS*16*.[18] The product sizes were 547 bp, which is specific for IS*16* element.

Results

Hospital settings

Generally, over half of the strains were isolated from patients hospitalized in ICU (55.84%), of which the highest proportion of enterococci was isolated from patients in hospital D and accounted for, respectively, the highest percentage (76%) of strains in that hospital and among other hospitals.

In hospital A, no strain originated from a patient operated on, while in hospital B, it was the surgical unit that was the source of most strains (53.84%). In general, most enterococci were isolated from perianal swabs, which resulted from a high rate of isolation in hospitals C and D. In hospitals A and B, no enterococci were isolated from rectal swabs. Only in hospital A, the biggest proportion of enterococci was isolated from the blood. At the remaining facilities, enterococci were significantly more often isolated from materials other that blood, as there is no procedure for screening for VRE on admission (Table 1).

Distribution of *Enterococcus* species in various clinical specimens

The highest prevalence of *E. faecium* was demonstrated in rectal swabs (75%), followed by wounds (12.5%), lower respiratory tract specimens (5.8%), urine (3.7%), blood (2.2%), and others (0.8%). The highest prevalence of *E. faecalis* was detected in wounds (47%), followed by blood and lower respiratory tract specimens (23.5% each), and cerebrospinal fluid (6%). In our study, only one *E. avium* isolate was detected, and it was found in a postoperative wound swab.

Table 2. Prevalence of antimicrobial resistance in *Enterococcus* species isolated from hospitals A, B, C, and D (disk diffusion method)

Antibiotic	Hospital							
	A		B		C		D	
	S	R	S	R	S	R	S	R
Ampicillin	5 (83.3)	1 (16.7)	8 (61.5)	5 (38.5)	0 (0)	60 (100)	0 (0)	75 (100)
Ciprofloxacin	4 (66.7)	2 (33.3)	6 (46.2)	7 (53.8)	1 (1.7)	59 (98.3)	3 (4)	72 (96)
Levofloxacin	4 (66.7)	2 (33.3)	7 (53.8)	6 (46.2)	2 (1.7)	60 (98.3)	3 (4)	72 (96)
Norfloxacin	4 (66.7)	2 (33.3)	6 (46.2)	7 (53.8)	3 (1.7)	61 (98.3)	3 (4)	72 (96)
Gentamicin	3 (50)	3 (50)	6 (46.2)	7 (53.8)	3 (5)	57 (95)	0 (0)	75 (100)
Streptomycin	6 (100)	0 (0)	8 (61.5)	5 (38.5)	8 (13.3)	52 (86.7)	9 (12)	66 (88)
Vancomycin	6 (100)	0 (0)	12 (92.3)	1 (7.7)	0 (0)	60 (100)	0 (0)	75 (100)
Teicoplanin	6 (100)	0 (0)	12 (92.3)	2 (7.7)	15 (25)	45 (75)	70 (93.3)	5 (6.7)
Tigecycline	6 (100)	0 (0)	13 (100)	0 (0)	60 (100)	0 (0)	75 (100)	0 (0)
Linezolid	6 (100)	0 (0)	13 (100)	0 (0)	60 (100)	0 (0)	75 (100)	0 (0)
Quinupristin-dalfopristin	1*(100)	0 (0)	3*(100)	0 (0)	60*(100)	0 (0)	75*(100)	0 (0)

S – susceptible; R – resistant; n (%) – number (percentage) of strains; * quinupristin-dalfopristin was evaluated for *E. faecium* only.

Comparison of antimicrobial resistance between *E. faecium* and *E. faecalis* and antimicrobial resistance in *Enterococcus* species isolated from various hospitals

The E test method confirmed the resistance to antimicrobials shown by the disc diffusion method (vancomycin, teicoplanin, ampicillin, gentamicin, ciprofloxacin, linezolid, and tigecycline) (Tables 2, 3). A strain resistant to linezolid and tigecycline was not reported among the tested enterococcal strains. All *E. faecalis* were also susceptible to ampicillin, vancomycin and teicoplanin. HLAR, HLGR and HLSR phenotypes were present in 33.3%, 20% and 6.6% of *E. faecalis* strains, respectively. All enterococci from *E. faecium* species were also susceptible to quinupristin-dalfopristin. Among all *E. faecium* isolates from hospitals C and D, resistance to ampicillin and vancomycin was detected. A high percentage of *E. faecium* (87.32%) presented HLAR phenotype, including 78.33% of strains from hospital C and 100% from D. Furthermore, in hospital D, HLSR (1.67%) and HLGR (10%) were detected in *E. faecium*. Also, very high rates of resistance to ciprofloxacin and 2 other fluoroquinolones (levofloxacin and norfloxacin) were observed in hospitals C (98.3%) and D (96%). In our study, *E. faecalis* isolates were more susceptible to antimicrobials than *E. faecium*, which were largely multidrug-resistant. Detailed results are shown in Tables 2 and 3. In hospitals A and B, MDR was not reported among the tested *Enterococcus* strains. In hospitals C and D, 95% and 96%, respectively, were classified as MDR strains.

Prevalence of resistance genes

In the study group there were 136 gentamicin resistance strains containing the *aac(6')-Ie-aph(2")-Ia* gene, which encodes the bifunctional enzyme AAC(6')-APH(2"), and 6 strains encoding the *aph(2")-Id* gene that also mediates resistance to gentamicin. All 123 streptomycin resistance strains contained the *aph(3')-IIIa* gene. 115 *vanB* genes and only 21 *vanA* genes were detected in enterococci. PCR analysis confirmed the phenotypic analysis.

Detection of virulence factor genes

Esp genes were detected in all *E. faecium* isolates and the majority of *E. faecium* isolates carried *hyl* (97%), in contrast to *E. faecalis* strains, in which different combinations of *asa1*, *esp*, *gelE*, and *cylA* genes were detected (Fig. 1).

Phenotypic analysis of virulence factors

In hospitals A and B, where *E. faecalis* constituted the majority of the isolated strains, cytolysin as well as gelatinase were produced. 100% of the gene responsible for

Table 3. Comparison of in vitro activity of 7 antimicrobials against enterococci isolated from hospitals A, B, C, and D

Antimicrobials	Hospital															
	A				B				C				D			
	MIC range	MIC_{50}	MIC_{90}	% resistant strains	MIC range	MIC_{50}	MIC_{90}	% resistant strains	MIC range	MIC_{50}	MIC_{90}	% resistant strains	MIC range	MIC_{50}	MIC_{90}	% resistant strains
Vancomycin	0.5–2.0	0.75	1.5	0	0.5–>256	2	3	7.7	>256	256	256	100	>256	256	256	100
Teicoplanin	0.5	0.5	0.5	0	0.38–>256	0.5	0.95	7.7	0.25–>256	0.75	256	75	0.38–>256	0.5	0.75	93.3
Ampicillin	2.0–>256	2	129	16.7	0.26–>256	2	256	38.5	>256	>256	>256	100	>256	256	256	100
Gentamicin	32–192	128	192	50	8.0–>256	16	256	53.8	192–>256	256	256	95	192–>256	256	256	100
Ciprofloxacin	0.5–32	2	22	33.3	0.5–256	6	32	53.8	2–256	256	>256	98.3	2–256	256	>256	96
Linezolid	0.75–4.0	2	3	0	1.0–4.0	2	2	0	0.75–2.0	1.25	2	0	0.75–2.0	2	2	0
Tigecycline	0.125–0.5	0.25	0.5	0	0.125–0.75	0.38	0.7	0	0.016–0.5	0.25	0.5	0	0.032–0.5	0.125	0.5	0

MIC – minimum inhibitory concentration; $MIC_{50/90}$ – MICs at which 50% and 90% of the isolates were inhibited, respectively; MIC values are given in mg/L.

producing cytolysin underwent expression. The activity of gelatinase was detected only for 5 strains that had all 3 regulator genes (*fsrA*, *fsrB* and *fsrC*) from 9 enterococci containing the gene encoding gelatinase. No DNase activity was observed in any of the tested strains from all the hospitals (Table 4).

Detection of a specific mobile insertion element IS*16*

Molecular analysis of all analyzed *E. faecium* strains showed the presence of a gene fragment specific for IS*16* insertion element.

Discussion

In our study, the majority of patients were hospitalized in ICU, which is similar to other reports and seems to be an important risk factor for enterococci infections.[19,20]

Table 4. Distribution of cytolysin (hemolysin), gelatinase and DNase activity in the genus *Enterococcus* isolates from hospitals A, B, C, and D

Hospital	Virulence determinants		
	cytolysin	gelatinase	DNase
A	1	2	0
B	2	3	0
C	0	0	0
D	0	0	0
Total	3	5	0

Antimicrobial resistance

Our study confirms that *E. faecalis* is still a rare reservoir of acquired vancomycin resistance, and this trend is well-known in our country and in Europe.[2,21] The majority of *E. faecalis* strains were susceptible to ampicillin and other antimicrobials, and it seems that *E. faecalis* species is not a therapeutic problem in hospitals in Małopolska.

The major determinant of vancomycin resistance among Polish VRE*fm* is still the VanA gene cluster, which is also most prevalent in some European counties.[2,19,22] However, in our study, *E. faecium* VanA phenotype strains were in the minority, while VanB phenotype isolates were in the majority. This seems to be the characteristic feature for VRE*fm* from the Małopolska region, because in other provinces VanA phenotype is predominant.[8,23,24] In Małopolska, in recent years, the presence of both VanA and VanB phenotypes has been described.[25–27] All VREfm strains isolated in this study were MDR and this is a problem for the therapeutic management of patients. The most important problem with the multidrug resistance of the studied strains is that they are able to easily acquire (by plasmids and/or transposon transfer) new resistance traits between enterococci.

In our research, we isolated only 1 strain which belonged to the *E. avium* species and included the HLAR phenotype but was susceptible to other antimicrobials. Therefore, it seems that other enterococcal species are not a threat to the therapeutic situation in the Małopolska region nowadays. In our study, only a few *E. faecalis* strains and a large group of *E. faecium* strains, including all VRE*fm* isolates, were resistant to ampicillin. It is a very disturbing and

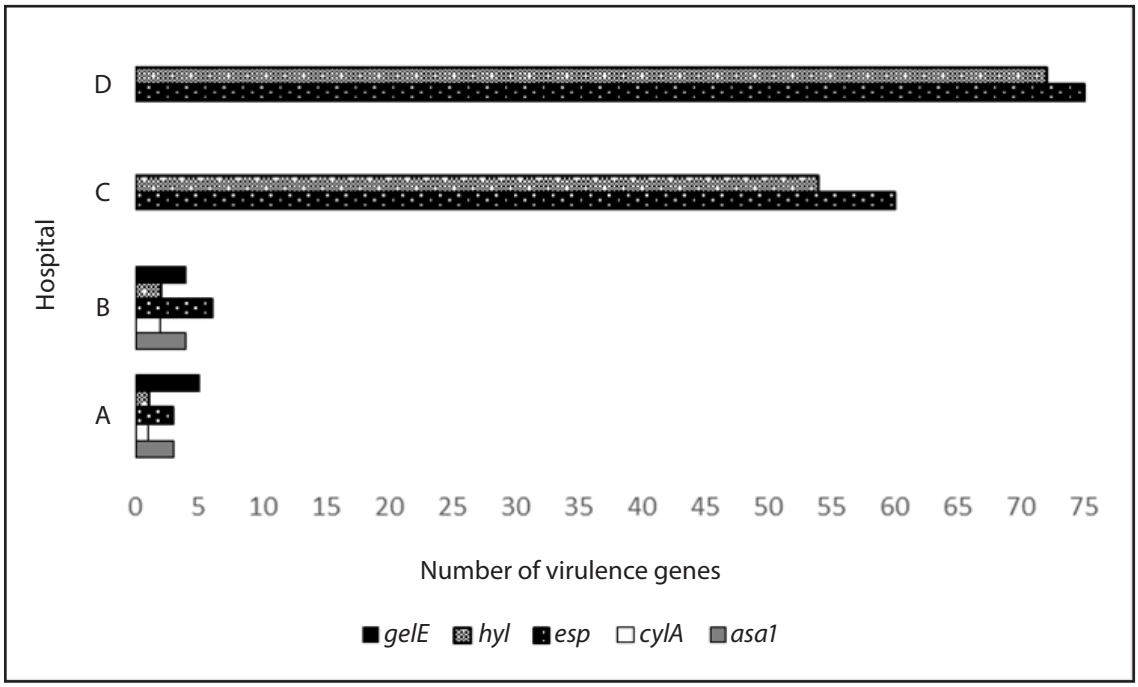

Fig. 1. Occurrence of virulence determinants *asa1*, *gelE*, *cylA*, *esp* and *hyl* in enterococci isolates from hospitals A, B, C, and D

dangerous situation, because β-lactam antibiotics are a vital group of drugs employed for the treatment of infections caused by enterococci. Ampicillin resistance also indicates resistance to amoxicillin, piperacillin and preparations combined with β-lactamase (ampicillin/sulbactam, amoxicillin/clavulanic acid, piperacillin/tazobactam).[28] Moreover, it is well known that ampicillin-resistant *E. faecium* are a precursor of multidrug-resistant strains, including VRE, and are widespread in Polish and European hospitals.[2,19] Enterococci are inherently resistant to low concentrations of aminoglycosides, which is associated with reduced permeability of these antibiotics through the cell wall.[29] In agreement with other studies from Poland, also ours, the spread of the *aac(6')-Ie-aph(2")-Ia* gene, which encodes the bifunctional enzyme AAC(6')-APH(2") and eliminates the synergistic effect between β-lactams or glycopeptide antibiotics and aminoglycosides, was responsible for high-level resistance to gentamicin among the majority of enterococci.[22,28] Thus, it precludes its therapeutic application in severe infections caused by enterococci, including bacteremia and endocarditis.[28,29] If the strains are resistant to high concentrations of gentamicin with simultaneous sensitivity to streptomycin (HLGR), streptomycin can be used in combination with β-lactams and glycopeptides. If the strains display HLSR phenotype, which means that they are resistant to high concentrations of streptomycin with simultaneous sensitivity to gentamicin, streptomycin therapy can be applied combined with β-lactams and glycopeptides, since the therapeutic effect is present. Detection of resistance to high concentrations of gentamicin and streptomycin at the same time (HLAR) means that there is no synergy of aminoglycosides with β-lactams and glycopeptides, which was found in a high degree in the strains examined by us.[28,29] For years, a tendency that has appeared among enterococci, also in Poland, regards their growing resistance to fluoroquinolones. In hospital A, this regularity is poorly marked (only 33.3% of resistant strains). In hospital B, it increases to approx. 50% of resistant strains, but it is best marked by a high resistance to ciprofloxacin, levofloxacin and norfloxacin in the enterococci investigated in hospitals C and D. This also proves that a very high percentage of resistant strains is common among the species *E. faecium*, which is also described in other studies originating in Poland.[19,28] Quinupristin-dalfopristin (Q/D) (with the exception of the *E. faecalis* species), linezolid and tigecycline have bacteriostatic activity against VRE, so they are recommended for the treatment of various infections caused by strains simultaneously resistant to several groups of antibiotics, such as ampicillin, glycopeptides, and a high concentration of aminoglycosides. Our data suggests that these antimicrobials may be effective therapeutic options for the treatment of serious infections caused by the studied enterococci strains. Unfortunately, Q/D treatment has failures and adverse effects, whereas acquired resistance to linezolid has been observed in enterococci, but this phenomenon is still rare and associated with the duration of previous linezolid therapy.[3,6]

Virulence factors

Enterococci displaying virulence factors are more likely to cause an infection than the strains devoid of them. There are factors that facilitate colonization and those that facilitate the infection of previously colonized tissue. The majority of VREfm strains from hospitals C and D both contained *esp* and hyl_{Efm} genes. Hyaluronidase (encoded by the *hyl* gene) contributes to the destruction of connective tissue and thus makes it easier for bacteria to spread in an infected organism.[15] The enterococcal surface protein Esp (encoded by *esp*) is associated with increased virulence, colonization and persistence in the urinary tract and biofilm formation.[15,18] In hospitals A and B, the situation was more diverse, and *gelE*, *asa1*, *esp*, hyl_{Efm}, and *cylA* genes in different combinations were detected in the enterococcal isolates. Gelatinase (encoded by *gelE*) has the ability to hydrolyze collagen, gelatin and small peptides.[15,18] Aggregation substance (encoded by *asa1*) facilitates the adhesion of enterococci to host cells, supports aggregation and facilitates the survival of bacteria in macrophages. Cytolysin has bacteriocin activity and the ability to lyse certain eukaryotic erythrocytes and gram-positive bacterial cells.[15,18] Among the studied enterococci, there were also strains which did not have virulence determinants. Moreover, like other researchers, we did not find DNase activity in either *E. faecalis*, *E. faecium* or *E. avium* isolates.[17] Therefore, it seems that this is not a virulence factor that occurs in enterococci.

The correlation between drug resistance and virulence genes

Nowadays, in European hospitals, the vancomycin-susceptible enterocci are replaced by high-level ampicillin and ciprofloxacin resistance, HLAR and VRE phenotype simultaneously, which are typical of hospital-acquired VREfm. The *E. faecium* strains examined in our study also shared other subpopulation characteristics of hospital-acquired strains, such as the presence of *esp*, *hyl* and IS*16*.[18,30] Phenotypic and molecular characterization of the *E. faecium* isolates tested coming from hospitals C and D corresponds to the characteristics of strains with high epidemic potential occurring in hospitals in Europe (Clonal Complex CC17). The specific mobile insertion element IS16 is highly specific for predicting hospital-associated strain types.[30] In our study, all strains belonging to the *E. faecium* species showed the presence of IS*16*, which is a similar result to other studies from Poland.[19,22,23,30]

Conclusions

The *E. faecalis* strains that appear in Małopolska hospitals are largely sensitive to antibiotics and have a variable amount of virulence factors. The *E. faecalis* species were

isolated less frequently than *E. faecium* from patients hospitalized in Małopolska hospitals. In contrast, the strains of the species *E. faecium* are a more uniform group with resistance to a number of significant therapeutic drug types, such as ampicillin, high concentrations of aminoglycosides, fluoroquinolones and glycopeptides (also more often displaying the VanB phenotype). VRE*fm* strains also have a high proportion of *hyl* and *esp* genes, which is characteristic of hospital strains of enterococci.

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