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Comparison of antibiotic resistance and virulence between biofilm-producing and non-producing clinical isolates of *Enterococcus faecium**

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An increase in the antibiotic resistance among *Enterococcus faecium* strains has been observed worldwide. Moreover, this bacteria has the ability to produce several virulence factors and to form biofilm that plays an important role in human infections. This study was designed to compare the antibiotic resistance and the prevalence of genes encoding surface protein (*esp*), aggregation substance (*as*), surface adhesin (*efaA*), collagen adhesin (*ace*), gelatinase (*gelE*), and hialuronidase (*hyl*) between biofilm-producing and non-producing *E. faecium* strains. Therefore, ninety *E. faecium* clinical isolates were tested for biofilm-forming ability, and then were assigned to two groups: biofilm-positive (BIO$^+$, n=70) and biofilm-negative (BIO$^-$, n=20). Comparison of these groups showed that BIO$^+$ isolates were resistant to β-lactams, whereas 10% of BIO$^-$ strains were susceptible to ampicillin (statistically significant difference, $p=0.007$) and 5% to imipenem. Linezolid and tigecycline were the only antibiotics active against all tested isolates. Analysis of the virulence factors revealed that *ace*, *efaA,* and *gelE* genes occurred more frequently in BIO$^-$ strains (*ace* in 50% BIO$^+$ *vs*. 75% BIO$^-$; *efaA* 44.3% *vs*. 85%; *gelE* 2.9% *vs*. 15%, respectively), while *hyl* gene appeared more frequently in BIO$^+$ isolates (87.1% BIO$^+$ *vs*. 65% BIO$^-$). These differences were significant ($p<0.05$). We concluded that BIO$^+$ strains were more resistant to antibiotics than BIO$^-$ strains, but interestingly, BIO$^-$ isolates were characterized by possession of higher virulence capabilities.

Key words: *Enterococcus*; biofilm; virulence; resistance

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INTRODUCTION

Enterococcus, gut commensals in a wide variety of hosts, are today among the leading causative agents of nosocomial infections due to their multiresistance to many antimicrobials. These bacteria are responsible for bacteremia, endocarditis, urinary tract and wound infections (Amyes, 2007; Bronk & Samet, 2008). For a long time, these infections were mostly caused by *E. faecalis*. In the last two decades, *E. faecium* has become one of the most prevalent nosocomial pathogens, increasing the total number of enterococcal infections and partially replacing *E. faecalis* as a cause of hospital-associated infections (Top *et al.*, 2007; Diani *et al.*, 2014). This change is related to the fact that *E. faecium* has a number of mechanisms of intrinsic resistance to cephalosporins, lincosamides, low levels of aminoglycosides, and many β-lactams (Sieńko *et al.*, 2014). Moreover, this species is also able to acquire resistance by means of mutations or as a result of the transfer of genes located on plasmids, transposons, or due to the incorporation of integrons (Chen *et al.*, 2009; Sieńko *et al.*, 2014). In the standard treatment of enterococcal infections, the use of a cell wall active agent (β-lactam, glycopeptide) with an aminoglycoside results in synergistic bactericidal activity (Rodriguez-Bano *et al.*, 2005). *E. faecium* has high-level resistance to many β-lactams as a consequence of overproduction and modification of penicillin-binding proteins (PBP), particularly PBP5 (Rodriguez-Bano *et al.*, 2005). Although rare, this resistance is mediated by the production of a β-lactamase enzyme (Rodriguez-Bano *et al.*, 2005; Comerlato *et al.*, 2013). Bacteria resistant to glycopeptides produce cell wall precursors with decreased affinity for the drug administered to treat an infection, which prevents the antibiotic from blocking cell wall synthesis (Sacha *et al.*, 2008; Cheng *et al.*, 2014). High-level aminoglycoside resistance (HLAR), caused by production of aminoglycoside-modifying enzymes (AMEs), makes standard therapy with aminoglycosides and ß-lactams or vancomycin ineffective (Vakulenko *et al.*, 2003).

Additionally, *E. faecium* strains have the ability to produce several virulence factors and have the ability to form biofilm that plays an important role in human infections (Di Rosa *et al.*, 2006; Sava *et al.*, 2010). The most prominent of the virulence determinants are aggregation substance (as), collagen adhesin (ace), surface adhesins (efaA), hialuronidase (hyl), enterococcal surface protein (esp), and gelatinase (gelE) (Fisher & Philips, 2009; Özden Tuncer *et al.*, 2013). As, encoded by a plasmid *as* gene, causes binding to the host epithelium and mediates bacterial aggregation during conjugation (Fisher & Philips, 2009). Ace (*ace* gene), which binds to collagen types I and IV, and efaA (*efaA*) have been identified as the principal virulence factors associated with infective endocarditis (Fisher & Philips, 2009). Hyl (*hyl*) degrades hyaluronic acid and is associated with tissue damage (Wu

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Abbreviations: BIO$^+$, biofilm-positive; BIO$^-$, biofilm-negative; PCR, polymerase chain reaction; ace, collagen adhesin; efaA, cell wall adhesin; hyl, hialuronidase; esp, enterococcal surface protein; as, aggregation substance; gelE, gelatinase; PBP, penicillin-binding proteins

et al., 2007). Esp (*esp*), which mediates the colonization, and GelE (*gelE*), a zinc metalloprotease, have been suggested to be involved in the process of biofilm formation (Di Rosa *et al.*, 2006; Heikens *et al.*, 2007; Fisher & Philips, 2009; Diani *et al.*, 2014).

Biofilm is an assemblage of bacterial cells attached to a biotic or abiotic surface and enclosed in a self-produced polysaccharide matrix (Mohamed & Huang, 2007). Its structure provides an optimal microenvironment for growth and facilitates transmission of mobile genetic elements between bacteria. Data suggest that microorganisms in biofilms are more highly resistant to antibiotics than others, extremely difficult to eradicate, and are a source of many chronic infections (Heikens *et al.*, 2007, Paganelli *et al.*, 2013). Among *Enterococcus*, a multistep process of biofilm formation has been reported to occur less frequently among *E. faecium* strains compared to *E. faecalis* species (Baldassari *et al.*, 2001; Almohamad *et al.*, 2014). It has been suggested that several virulence determinants are implicated in biofilm formation among *Enterococcus* (Di Rosa *et al.*, 2006; Paganelli *et al.*, 2013). Nevertheless, there are many conflicting literature reports about their contribution in biofilm production (Di Rosa *et al.*, 2006; Mohamed & Huang, 2007; Heikens *et al.*, 2007; Fisher & Philips, 2009). Moreover, in the case of *E. faecium,* data about biofilm-forming ability are still very limited (Almohamad *et al.*, 2014). This prompted us to determine the prevalence of biofilm-forming ability among *E. faecium* clinical isolates. We focused on the search for differences in virulence between biofilm-producing (BIO^+) and non-producing (BIO-) *E. faecium* strains. Moreover, due to the alarming increase in resistance among *Enterococcus* in Poland (Dworniczek *et al.*, 2014; Antimicrobial resistance surveillance in Europe 2013. Stockholm: ECDC; 2014), the next goals of our study were to compare the susceptibility of tested strains to antibiotics between BIO^+ and BIO^- isolates, to determine their resistance profiles, and to indicate the antibiotic with the highest activity.

MATERIALS AND METHODS

Tests were performed on ninety *E. faecium* strains, randomly selected from the collection of the Department of Microbiological Diagnostics and Infectious Immunology (Medical University of Białystok, Poland). Strains were isolated from clinical specimens of patients hospitalized at the University Hospital in Białystok (Poland) from December 2013 to January 2015. Isolates were recovered from various clinical materials, mostly rectal swabs, faeces, blood, urine, and pus. Most of collected isolates were gathered from the intensive care unit and a hematology clinic.

Identification and susceptibility testing. The identification and susceptibility testing of study isolates were conducted on the automated VITEK 2 system (bioMérieux, France) according to the manufacturer's instructions using VITEK 2 GP and AST-P516 cards, respectively. Susceptibility to ampicillin, imipenem, gentamicin, streptomycin, vancomycin, teicoplanin, linezolid, and tigecycline was interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (breakpoint tables for interpretation of minimum inhibitory concentrations (MIC) and zone diameters; version 5.0, 2015; http://www.eucast.org.). *E. faecalis* ATCC 29212 was used as a reference strain. Later, identification to the species level was confirmed by polymerase chain reaction *(*PCR) with primers targeted to specific sequences in the *ddl* (d-Ala-d-Ala ligase) chromosomal genes (Table 1).

Biofilm production. The tube method (Christensen *et al.*, 1982; Oliveira & Cunha, 2010) and Congo red agar (CRA) method (Freeman *et al.*, 1989; Cabrera-Contreras *et al.*, 2013) were used to assess biofilm-forming ability. Each experiment was repeated three times for each strain. In the first test, trypticase soy broth (Emapol, Poland) with sucrose (WARCHEM, Poland) was inoculated with a loopful of the bacteria from an overnight culture and incubated for 24 h at 37ºC. The tubes were then decanted and washed with phosphate-buffered saline (PBS) (pH 7.2) (BTL, Poland) to remove non-adherent cells. The tubes were then dried and stained with 0.1% crystal violet (Graso, Poland). After 30 minutes, the excess stain was washed off with distilled water and the tubes were left to dry in an inverted position and observed for biofilm formation. A visible film lining the bottom and the sides of the tube was considered to be indicative of biofilm production. In the second method, for preparing CRA medium, 50 g/l sucrose and 0.8 g/l Congo red

Table 1. PCR primers, annealing temperatures, and product sizes for detection of *ddl* gene and virulence genes.

Virulence gene	Primers	Product size (bp)	Annealing temperature (°C)	Reference
gelE	AAT TGC TTT ACA CGG AAC GG GAG CCA TGG TTT CTG GTT GT	548	52	Camargo *et al.*, 2006
ace	GGC CAG AAA CGT AAC CGA TA CGC TGG GGA AAT CTT GTA AA	353		
hyl	ACA GAA GAG CTG CAG GAA ATG GAC TGA CGT CCA AGT TTC CAA	276	55	Zou *et al.*, 2011
esp	AGA TTT CAT CTT TGA TTC TTG G AAT TGA TTC TTT AGC ATC TGG	510		
as	CACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA	375		
efaA	AAC AGA TCC GCA TGA ATA CAT TTC ATC ATC TGA TAG TA	735	50	Özden Tuncer *et al.*, 2013
ddl	GGC AGA GCA TGA AGT GTC CA CTT CTG GGT TTT CTG CTT TTG TA	557	56	Dutka-Mahlen *et al.*, 1995

stain (WARCHEM) were added to brain heart infusion agar (Sigma-Aldrich, USA) and autoclaved at 121°C for 15 m. Strains were inoculated onto CRA and incubated at 37°C for 24 h. Reading was done after 24 h and 48 h. A positive result was indicated by black colonies with black crystalline morphology; non-biofilm producers produced pink-colored colonies. Isolates that demonstrated the ability to produce biofilm by both methods were identified as BIO$^+$ strains, others as BIO$^-$ isolates.

β-lactamase and hemolysin production. Strains were tested for β-lactamase production by a chromogenic cephalosporinase method (Pitkälä *et al.*, 2007) using nitrocefin discs (OXOID, United Kingdom) as per manufacturer's instruction. *Staphylococcus aureus* ATCC 29213 was used as a positive control. Hemolysin production was evaluated on Columbia blood agar supplemented with 5% sheep blood (OXOID, United Kingdom), as previously described (Vergis *et al.*, 2002).

DNA extraction. Genomic DNA was extracted from overnight *E. faecium* cultures using a Genomic Mini Kit (A&A Biotechnology, Poland) according to the manufacturer's protocol.

PCR detection of virulence genes. PCR assays were performed to detect the following virulence genes: *gelE*, *ace*, *hyl*, *esp*, *as*, and *efaA*. The primers used in this assay were selected from the literature and their sequences are listed in Table 1. PCR amplification was performed in 25 μl mixtures using 2 μl of DNA solution, 1 μl of each primer, 8.5 μl of nuclease-free water, and 12.5 μl of PCR master mix (DNA Gdańsk, Poland). Samples were subjected to an initial cycle of denaturation at 94°C for 5 min, followed by thirty cycles of denaturation at 94°C for 1 min, annealing at an appropriate temperature for 1 min, and elongation at 72°C for 1 min using a programmable DNA thermocycler (SensoQuest GmbH, Germany).

PCR products were separated electrophoretically on Sub-Cell GT apparatus (Bio-Rad, USA) at 5V/cm for 100 min on a 1.5% agarose gel (Sigma-Aldrich, USA) containing 0.5% ethidium bromide (MP Biomedicals, USA) in Tris-borate-EDTA (ethylenediaminetetraacetic acid) buffer. Then, amplicons were visualized and photographed using the ChemiDoc XRS imaging system and Quantity One 1-D analysis software (Bio-Rad). The positions of obtained products were estimated with the molecular weight marker, Perfect™ 100–1000 bp DNA ladder (EURx, Poland). To confirm the presence of the above-mentioned virulence genes, DNA sequencing was carried out on selected PCR products by GENOMED S.A. company in Poland. The sequences were aligned and compared with reference sequences achieved using GenBank with the Basic Local Alignment Search Tool (BLAST) algorithm.

Statistical analysis. STATA 13.1 (StataCorp LP, USA) was used for statistical analysis. Differences in the prevalence of antibiotic resistance and virulence determinants between BIO$^+$ and BIO$^-$ strains were assessed by the Chi-square test and Fisher's exact test. Results with $p < 0.05$ were considered significant.

RESULTS

We observed an ability to produce biofilm in seventy of ninety *E. faecium* strains (77.8%). As mentioned earlier, these isolates were classified as BIO$^+$, and the remaining twenty strains (22.2%) as BIO$^-$. An exact comparison of antibiotic resistance between BIO$^+$ and BIO$^-$ *E. faecium* isolates is presented in Fig. 1. All of the BIO$^+$ isolates showed phenotypic resistance to tested β-lactams, whereas 10% of BIO$^-$ strains were susceptible to ampicillin (statistically significant difference, $p = 0.007$), and 5% were susceptible to imipenem. None of the investigated isolates had the ability to produce the β-lactamase enzyme. Both groups showed high-level resistance to aminoglycosides: resistance to gentamicin was detected in 75.7% of BIO$^+$ and 60% of BIO$^-$ strains, to streptomycin in 91.4% of BIO$^+$ and 85% of BIO$^-$ strains, respectively (insignificant differences, $p > 0.05$). Interestingly, more than half of the tested strains in both groups was resistant to vancomycin and teicoplanin. Linezolid and tigecycline had the highest activity against all studied isolates (100% susceptibility).

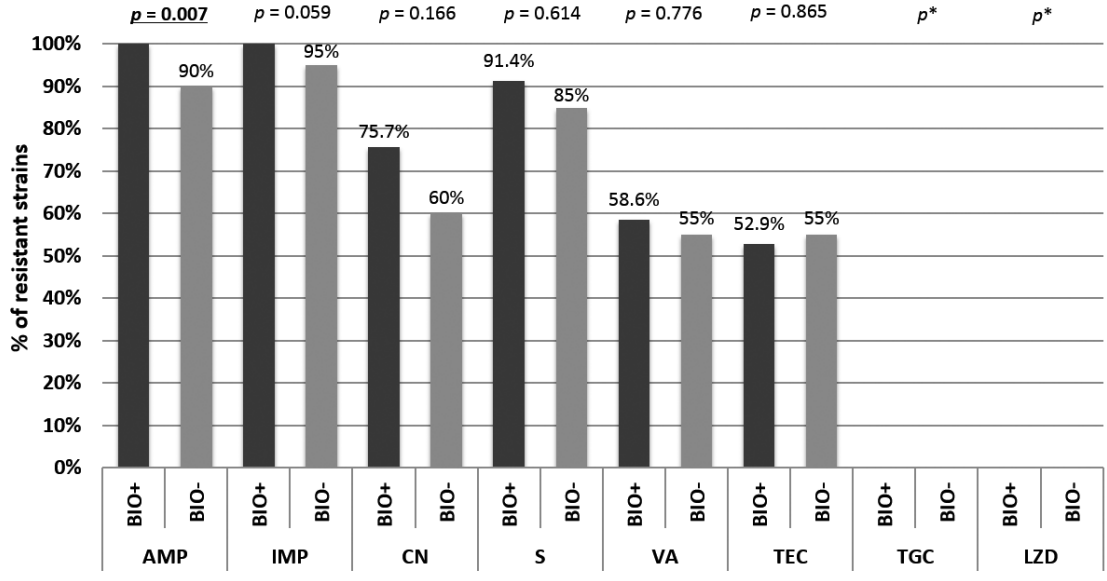

Figure 1. Comparison of resistance to antibiotics among BIO+ (n = 70) and BIO– (n = 20) *E. faecium* strains.
AMP, ampicillin; IMP, imipenem; CN, gentamicin; S, streptomycin; VA, vancomycin; TEC, teicoplanin; TGC, tigecycline; LZD, linezolid; *lack of differences.

Table 2. Characteristics of resistance and virulence patterns among BIO+ (n = 70) and BIO– (n = 20) *E. faecium* strains.
AMP, ampicillin; IMP, imipenem; CN, gentamicin; S, streptomycin; VA, vancomycin; TEI, teicoplanin; *ace,* collagen adhesin; *efa,* cell wall adhesin; *hyl,* hialuronidase; *esp,* enterococcal surface protein; *as,* aggregation substance; *gelE,* gelatinase.

Antibiotic resistance							Virulence factors								
Number of inactive antibiotics	Resistance pattern						Number of genes	Genes detected by PCR						Hemolysis	No. of strains
E. faecium BIO+ (n = 70)															
6	AMP	IMP	CN	S	VA	TEI	5	*ace*	*efa*	*hyl*	*esp*			α	6
	AMP	IMP	CN	S	VA	TEI	4	*ace*	*efa*		*esp*			α	2
	AMP	IMP	CN	S	VA	TEI	3			*hyl*	*esp*			α	7
	AMP	IMP	CN	S	VA	TEI	2			*hyl*				α	5
	AMP	IMP	CN	S	VA	TEI	1							α	4
5	AMP	IMP		S	VA	TEI	6	*ace*	*efa*	*hyl*	*esp*		*gel*	α	1
	AMP	IMP		S	VA	TEI	5	*ace*	*efa*	*hyl*	*esp*			α	2
	AMP	IMP	CN		VA	TEI		*ace*	*efa*	*hyl*	*esp*			α	2
	AMP	IMP	CN	S	VA			*ace*	*efa*	*hyl*	*esp*			α	2
	AMP	IMP		S	VA	TEI	4	*ace*		*hyl*	*esp*			α	1
	AMP	IMP		S	VA	TEI	3	*ace*	*efa*					α	2
	AMP	IMP		S	VA	TEI				*hyl*	*esp*			α	1
	AMP	IMP		S	VA	TEI	2			*hyl*				α	4
4	AMP	IMP	CN	S			5	*ace*	*efa*	*hyl*	*esp*			α	4
	AMP	IMP	CN		VA			*ace*	*efa*	*hyl*	*esp*			α	2
	AMP	IMP	CN	S			4				*esp*	*as*	*gel*	α	1
	AMP	IMP	CN	S				*ace*		*hyl*	*esp*			α	3
	AMP	IMP	CN	S			3			*hyl*	*esp*			α	10
	AMP	IMP	CN	S						*hyl*		*as*		α	2
	AMP	IMP	CN	S			2			*hyl*				α	1
3	AMP	IMP		S			5	*ace*	*efa*	*hyl*	*esp*			α	5
	AMP	IMP	CN					*ace*	*efa*	*hyl*	*esp*			α	1
	AMP	IMP		S			4	*ace*	*efa*	*hyl*				α	1
	AMP	IMP	CN					*ace*	*efa*	*hyl*				α	1
E. faecium BIO- (n = 20)															
6	AMP	IMP	CN	S	VA	TEI	5	*ace*	*efa*	*hyl*	*esp*			α	2
	AMP	IMP	CN	S	VA	TEI	4	*ace*	*efa*	*hyl*				α	3
	AMP	IMP	CN	S	VA	TEI		*ace*	*efa*	*hyl*	*esp*				1
	AMP	IMP	CN	S	VA	TEI	2		*efa*				*gel*		1
	AMP	IMP	CN	S	VA	TEI	0								1
5	AMP	IMP		S	VA	TEI	3		*efa*	*hyl*	*esp*				1
	AMP	IMP		S	VA	TEI	1							α	1
4	AMP	IMP	CN	S			5	*ace*	*efa*	*hyl*	*esp*			α	2
	AMP	IMP			VA	TEI		*ace*	*efa*	*hyl*	*esp*			α	1
3	AMP	IMP		S			5	*ace*	*efa*	*hyl*	*esp*			α	1
	AMP	IMP		S			4	*ace*	*efa*	*hyl*	*esp*				2
	AMP	IMP	CN				2				*esp*			α	1
2			CN	S			4	*ace*	*efa*			*as*	*gel*		1
		IMP		S				*ace*	*efa*			*as*	*gel*		1
	AMP	IMP					3	*ace*	*efa*					α	1

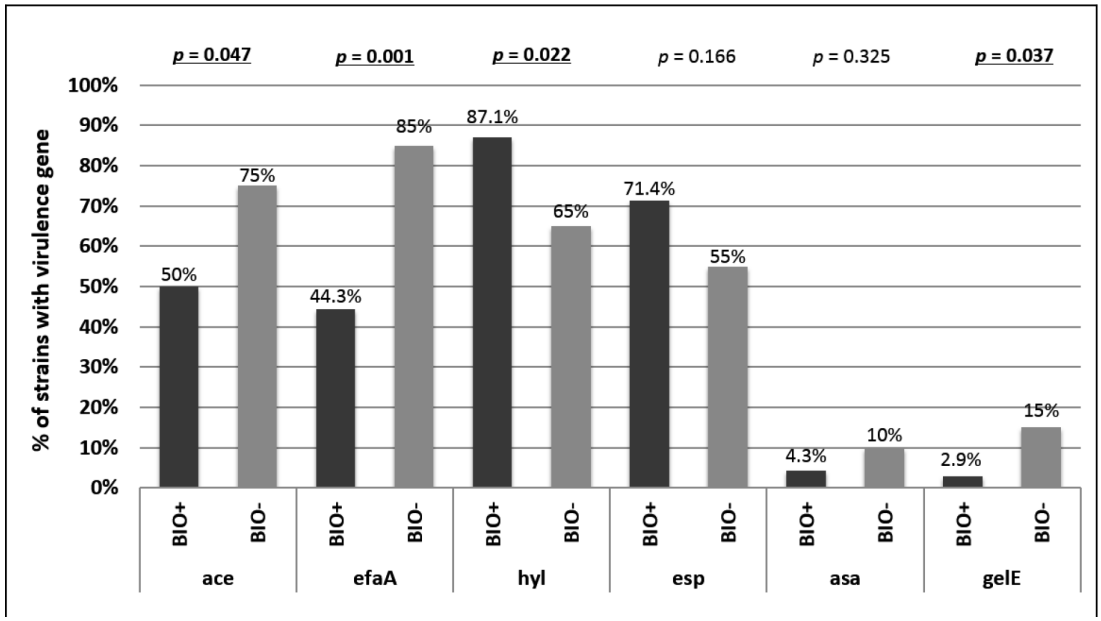

Figure 2. Comparison of the prevalence of virulence genes among BIO+ (n = 70) and BIO– (n = 20) *E. faecium* strains.
ace, collagen adhesin; *efaA*, cell wall adhesin; *hyl*, hialuronidase; *esp*, enterococcal surface protein; *asa*, aggregation substance; *gelE*, gelatinase.

Comparative analysis (Fig. 2) of the prevalence of virulence genes among BIO^+ and BIO^+ strains revealed that *ace*, *efaA,* and *gelE* genes occurred more frequently in BIO^- strains (*ace* in 50% BIO^+ *vs.* 75% BIO-, *efaA* in 44.3% *vs.* 85%, *gelE* in 2.9% *vs.* 15%, respectively), while *hyl* gene appeared more frequently in BIO^+ isolates (87.1% BIO^+ *vs.* 65% BIO^-). These differences were statistically significant ($p < 0.05$). No statistically significant differences were found in the case of *esp* and *as* genes ($p > 0.05$).

Resistance and virulence patterns among all BIO^+ and BIO^- *E. faecium* strains are shown in Table 2. The most frequent antibiotic resistance profile among BIO^+ strains was AMP^R IMP^R CN^R S^R (resistance to ampicillin, imipenem, gentamicin, streptomycin, respectively), which was detected in ten strains. These strains had *hyl* and *esp* virulence genes and the ability to hemolyze. The most frequent resistance and virulence profile of BIO^- isolates, which occurred in three strains, was AMP^R IMP^R CN^R S^R TEC^R VA^R (resistance to ampicillin, imipenem, gentamicin, streptomycin, teicoplanin, and vancomycin, respectively) with *ace, efaA,* and *hyl* genes. All (100%) BIO^+ strains carried resistance to three or more antibiotics and had the ability to α-hemolyze, whilst three (15%) BIO^- strains showed resistance to only two antibiotics, and a smaller number of these strains (n = 12; 60%) exhibited α-hemolysis. BIO^+ isolates showed a greater variety of resistance and virulence determinants than BIO^- strains.

DISCUSSION

The present study focused on determining the prevalence of biofilm-forming ability among *E. faecium* clinical isolates and on comparison of the antibiotic resistance and the prevalence of genes encoding selected virulence factors between biofilm-producing (BIO^+) and non-producing (BIO^-) strains. In this study we observed very high incidence of the ability to form biofilm among randomly selected *E. faecium* clinical strains (77.8%). A similar proportion was observed in Spain (75%) (Latasa *et al.*, 2006). However, studies by other authors showed different results; in India, Italy, and Turkey, *E. faecium* isolates were able to produce biofilm less frequently or even could not form this structure (0%, 28.8%, and 48%, respectively) (Prakash *et al.*, 2005; Di Rosa *et al.*, 2006; Diani *et al.*, 2014). These results indicate that the level of the ability to form biofilm among *E. faecium*, as well as the factors conducive to its formation, vary with geographic location.

The notion that bacteria in biofilms are more resistant to antibiotics than planktonically grown microorganisms (Lewis, 2001; Heikens *et al.*, 2007, Paganelli *et al.*, 2013) was not fully confirmed in our survey. BIO^+ strains were, admittedly, slightly more resistant than BIO^- isolates, but the statistically significant difference between these groups was found only in the case of ampicillin. We were also very astounded due to the fact that in the literature there are only a few reports about the differences in resistance between biofilm-producing and non-producing isolates belonging to *E. faecium* species (Raad *et al.*, 2005; Akhter *et al.*, 2014), compared to the amount of data about *E. faecalis* strains (Chai *et al.*, 2007; Mohamed *et al.*, 2007; Yayanthi *et al.*, 2008; Lins *et al.*, 2013; Frank *et al.*, 2015).

In our study, the majority of *E. faecium* isolates (> 90%) exhibited resistance to β-lactams. None of the tested strains had β-lactamase activity; therefore, we can assume that this resistance is associated with changes in PBPs. These results are in agreement with other studies (Simonsen *et al.*, 2003; Iris *et al.*, 2014). However, researchers from Spain (Rodriguez-Bano *et al.*, 2005) obtained only 28.6% ampicillin-resistant *E. faecium* strains, whereas in the Netherlands (Schouten *et al.*, 1999) 24% of *E. faecium* strains were susceptible to imipenem. It should be noted that in this study most of the tested strains that were resistant to β-lactams were also resistant to gentamicin or streptomycin. Similar results were observed by Simonsen *et al.* (2003). The high rate of co-resistance between ampicillin and aminoglycosides among *E. faecium*, especially in vancomycin-resistant enterococci (VRE) isolates, is worrisome since it eliminates the synergistic effect between β-lactams and aminoglyco-

sides in the treatment of patients (Simonsen *et al.*, 2003; Sieńko *et al.*, 2014). Unfortunately, *E. faecium* isolates resistant to β-lactams, aminoglycosides, and glycopeptides, considered as multidrug resistant (MDR), are now widespread across Europe (Hryniewicz *et al.*, 2009). According to a recent multicenter report (Antimicrobial resistance surveillance in Europe 2013. Stockholm: ECDC; 2014), resistance to glycopeptides has significantly increased over the last four years, including Poland. The high prevalence of *E. faecium* strains with AMP^R IMP^R CN^R S^R TEC^R VA^R resistance patterns, obtained in this study, confirms that the scale of the problem with MDR *Enterococcus* strains is large, and that changing in epidemiology of these strains remains a major infection control challenge throughout Europe. This study showed that linezolid and tigecycline were the most active antibiotics toward all tested strains. Many authors confirm that they are a valuable therapeutic option in infections caused by *E. faecium*, including VRE (Franiczek *et al.*, 2008; Freitas *et al.*, 2011; Praharaj *et al.*, 2013; Sieńko *et al.*, 2014). However, cases of resistance to these antibiotics have been recently reported (Werner *et al.*, 2008; Baldir *et al.*, 2013). This may indicate that resistance to newer antimicrobials is also developing; therefore, new strategies, including combination therapies, are urgently needed.

Despite the fact that the biofilm-formation process has an essential impact on the course of enterococcal infections, our knowledge of the mechanisms and factors involved in this process is still insufficient (Almohamad *et al.*, 2014). Therefore, many studies have sought to find the relation between biofilm formation and virulence genes, especially *esp* and *gelE*, among *Enterococcus* (Dupre *et al.*, 2003; Dworniczek *et al.*, 2005; Raad *et al.*, 2005; Di Rosa *et al.*, 2006; Heikens *et al.*, 2007; Fisher & Philips, 2009; Diani *et al.*, 2014). We reported that the prevalence of *esp* gene was higher in BIO^+ isolates (71.4%) than in BIO^- strains (55%), but that this difference was statistically insignificant, and that many *esp*-positive isolates did not form biofilm. These findings suggest that this gene has no connection with biofilm-forming ability. Similar proportions and lack of significant differences were seen by other researchers (Dupre *et al.*, 2003; Dworniczek *et al.*, 2005; Raad *et al.*, 2005; Di Rosa *et al.*, 2006; Almohamad *et al.*, 2014). Nevertheless, many authors found that there is a strong relationship between the *esp* gene and biofilm formation (Heikens *et al.*, 2007; Fisher & Philips, 2009; Top *et al.*, 2013; Diani *et al.*, 2014). Undoubtedly, these varied and conflicting results indicate that Esp may require interaction with other virulence factors to result in biofilm enhancement. Interestingly, researchers from Sweden (Bilström *et al.*, 2008) concluded that *E. faecium* strains that carry the *esp* gene demonstrate higher resistance to β-lactams. Likewise, in our study, we observed the coexistence of resistance to β-lactams and the *esp* gene in the majority of tested strains, and two strains from the BIO^- group that were susceptible to ampicillin did not have this gene. However, more research is definitely needed in this area, particularly studies concerning the expression of virulence genes.

The presence of *gelE* and *as* genes among *E. faecium* strains is very rare, whereas they are widely present in *E. faecalis* isolates (Vankerckhoven *et al.*, 2004; Comerlato *et al.*, 2013). In our study, we observed small percentages of strains with *gelE* and *as* genes (< 15%). Similar results have also been reported by other researchers (Kowalska-Krochmal *et al.*, 2011; Hasani *et al.*, 2012; Comerlato *et al.*, 2013), but two studies (Vankerckhoven *et al.*, 2004, Diani *et al.*, 2014) did not find any of them with PCR in large groups of *E. faecium* isolates. We found that *gelE* and *as* genes occurred more frequently in BIO^- groups than in BIO^+ groups, which suggests that these genes have a greater role in the pathogenicity of *E. faecium* strains unable to form biofilm. This result is not in concordance with those from other studies (Hancock & Perego, 2004; Mohamed *et al.*, 2007; Kafil *et al.*, 2015); however, one study (Di Rosa *et al.*, 2006) confirmed that there is no association between gelatinase and biofilm in *E. faecium* strains.

In the case of other virulence factors, our findings that BIO^- strains had significantly more *ace* and *efaA* genes than BIO^- strains indicate that isolates carrying these genes prefer a planktonic rather than a biofilm lifestyle. We also found that the *hyl* gene occurred more frequently in BIO^+ strains. Different results were observed in one recently published study (Kafil *et al.*, 2015); the authors showed that isolates with the *efaA* gene produced more biofilms than negative ones, while strains with the *hyl* gene had a significantly lower biofilm-forming ability.

In conclusion, our data demonstrated that BIO^+ *E. faecium* strains were slightly more resistant to antibiotics than BIO- strains, but, interestingly, BIO^- isolates were characterized by a higher virulence potency. Nevertheless, these observations are not in agreement with many previously published reports. Our attempts to understand these large numbers of contradictory results have allowed us to conclude that the ability to form biofilm cannot be unambiguously linked to increased virulence and resistance in *E. faecium* strains. This stresses the need to perform more research on regulation and expression of virulence and resistance genes, how to prevent the spread of MDR enterococcal nosocomial infections, and on treatment alternatives. Novel approaches, including the use of metabolomics, proteomics, and genomics, may improve our knowledge of *E. faecium* biofilm, in the light of changing epidemiology and increasing resistance to antibiotics. Novel drugs targeted at specific virulence factors may play a preventative or even therapeutic role in the elimination of MDR *E. faecium* strains.

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