Analysis of biofilm production in *Enterococcus faecium* strains depending on clinical source

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ABSTRACT

Purpose: *Enterococcus faecium* strains have been reported worldwide as etiologic factors of many nosocomial infections, which are difficult to manage because of the constantly increasing resistance of these microorganisms to antibiotics and the ability to form biofilm. The aim of this study was to analyze the ability to produce a biofilm in *E. faecium* strains, depending on the patient's clinical material.
Materials and methods: Sixty-six *E. faecium* strains were investigated. Identification and susceptibility testing were conducted by the VITEK2 system. The ability to form biofilm was assessed by phenotypic methods. The presence of selected virulence genes was established by PCR followed by gel electrophoresis and sequencing.
Results: Among the tested *E. faecium* isolates, 72.7% were biofilm-positive (BIO+) and 27.3% biofilm-negative (BIO-). Strains were collected mostly from rectal swabs (30.4%) and blood (18.3%). BIO+ strains from infections constituted 31.8% (52.4% isolated from blood) and from colonization 40.9% (48.2% from rectal swabs). 91.7% of the Blood Group strains and 68.5% of the Other Group strains produced biofilm. Strains from the Colonization Group produced biofilm in a proportion similar to the Infection Group (about 75%). There were no statistically significant differences in virulence and resistance, except for vancomycin (more resistant BIO+ Other than the BIO+ Blood Group, and more resistant BIO+ Colonization than BIO+ Infection Group) and teicoplanin (more resistant BIO+ Colonization than the BIO+ Infection Group).
Conclusion: The majority of *E. faecium* isolates carries high levels of resistance to many antimicrobials, is well equipped with virulence genes, and possesses the ability to form biofilm.
Key words: *Enterococcus faecium,* biofilm, antibiotic, resistance, virulence

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INTRODUCTION

Enterococcus faecium strains have been reported worldwide as etiologic factors of many nosocomial infections, which are difficult to manage because of the constantly increasing resistance of these microorganisms to antibiotics and their ability to form strong biofilms [1,2]. The largest threat is infections caused by vancomycin-resistant *E. faecium* (VRE), particularly for critically ill or immunocompromised patients [3,4]. Moreover, VRE strains are often simultaneously resistant to β-lactams and aminoglycosides, and are considered multidrug resistant (MDR) [2,4]. Alarmingly, antimicrobial resistance genes from MDR strains can be transferred by transposons or pheromone-mediated conjugative plasmids not only to susceptible enterococcal isolates, but also to other more virulent nosocomial pathogens, like Staphylococcus aureus [5]. Furthermore, *E. faecium* isolates are characterized by a high frequency of genes encoding putative virulence factors, such as collagen adhesin (*acm* gene), enterococcal surface protein (*esp* gene), hyaluronidase (*hyl* gene), gelatinase (*gelE* gene), endocarditis antigen (*efa* gene), and cytolysin (*cyl* operon) [6].

The ability to form biofilm among *E. faecium* strains is considered to be an important virulence property, and these bacteria are often responsible for conditions in which they may be associated with biofilm, such as endocarditis or catheter-associated urinary tract infections [1, 7]. Unfortunately, due to the rapidly increasing number of conflicting literature reports about biofilm formation among enterococci, we still do not know the true impact of biofilm growth on the expression and transfer of resistance and virulence traits, especially among the *E. faecium* species [8-10].

Moreover, very limited data about biofilm formation, virulence, and antibiotic resistance among *E. faecium* strains are available in Poland [11]. This prompted us to determine the prevalence of the biofilm-forming ability among *E. faecium* clinical strains, depending on the patient's clinical material. In the next step, we searched for differences in resistance and virulence determinants between BIO+ and BIO- *E. faecium* isolates. This study also aimed to investigate the differences among *E. faecium* strains isolated from infections and colonization, and to determine differences between strains isolated from blood and other clinical sources.

MATERIALS AND METHODS

Strains

Tests were performed on sixty-six randomly selected *E. faecium* strains, isolated from clinical specimens from patients hospitalized at the University Hospital in Bialystok (Poland) from December 2013 to January 2015. The majority of strains were collected from intensive care units (42.8%) and a hematology clinic (31.8%).

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 guidelines 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) recommend-dations (breakpoint tables for interpretation of minimum inhibitory concentrations, MIC, and zone diameters; version 5.0. 2015; http://www.eucast.org).

Biofilm production

The Congo red agar (CRA) method [12,13] and the tube method [14,15] were used to assess biofilm-forming ability. Each experiment was repeated three times for each strain. Isolates that demonstrated the ability to form biofilm by both methods were considered biofilm positive (BIO+) strains.

Hemolysin production

Hemolysin production was established on Columbia blood agar with 5% sheep blood (OXOID, United Kingdom), as previously described [16].

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 instructions.

PCR detection of virulence genes

PCR assays were performed to detect the following virulence genes: *gelE*, *acm*, *hyl*, *esp*, *efaA*, and *cyl*. The primers used in this survey 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 denaturation at 94^{O}C for 5 min, followed by 30 cycles of denaturation at 94^{O}C for 1 min, annealing at an appropriate temperature for 1 min, and elongation at 72^{O}C for 1 min using a DNA thermocycler (SensoQuest GmbH, Germany).

Table 1. PCR primers, annealing temperatures, and product sizes for the detection of 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	[17]
acm	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	[18]
esp	AGA TTT CAT CTT TGA TTC TTG G AAT TGA TTC TTT AGC ATC TGG	510		
efaA	CACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA	375		
cyl	TGG ATG ATA GTG ATA GGA AGT TCT TTC ATC ATC TGA TAG TA	517		

PCR products were separated electrophoretically on the Sub-Cell GT apparatus (Bio-Rad, USA) at 5 V/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). To confirm the presence of the above-mentioned virulence genes, DNA sequencing was carried out on selected PCR products by the 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 between various groups of *E. faecium* strains were assessed using the Chi-square and Fisher's exact tests. Results with $p<0.05$ were considered significant.

RESULTS

Sixty-six *E. faecalis* strains were divided into various groups based on their source of isolation: Infection Group, strains isolated from blood (18.2%), urine (13.7%), pus (3%), and bronchoalveolar lavage (BAL) (3%); Colonization Group, isolates from rectal swabs (30.3%), feces (12.1%), pharyngeal swabs (7.6%), and groin swabs (3%); Blood Group, isolates only from blood (18.2%); and Other Group, isolates from all other clinical materials (71.8%). Moreover, after determining the biofilm-forming ability of all tested *E. faecium* strains, we created BIO+ (72.7%) and BIO- (27.3%) groups. We also divided the previous groups into BIO+ subgroups: BIO+ Infection/BIO+ Colonization, and BIO+ Blood/BIO+ Other.

The exact characteristics of differences in virulence and antibiotic resistance between the tested *E. faecium* groups are presented in Table 2. A significant difference ($p=0.001$) was reported only in the case of the phenotypic ability to hemolyze (97.9% BIO+ and 72.2% BIO-). The most frequent virulence genes among the tested isolates were *acm* (>95.5%) and *efa* (>81.8%). There were no statistically significant differences in the prevalence of all tested virulence genes ($p>0.05$).

All tested *E. faecium* groups showed high resistance to ampicillin (>96.3% resistant isolates) and imipenem (>94.4% resistant isolates). Resistance to gentamicin was detected in more than 41.7% of the tested isolates, whereas more than 81.5% were resistant to streptomycin. Differences between the various groups of *E. faecium* were not statistically significant ($p>0.05$), except for glycopeptides (Table 2). In the case of vancomycin, 71.1% of *E. faecium* from the Colonization group and 17.9% of *E. faecium* from the Infection group ($p<0.001$), 70.4% of the BIO+ Colonization group and 19% of the BIO+ Infection Group ($p<0.001$), 55.6% of other, 16.7% of blood isolates ($p=0.015$), 56.8% of BIO+ other, and 18.2% of BIO+ blood isolates ($p=0.026$) were resistant. Resistance to teicoplanin was detected in 63.2% of strains from the Colonization group and 14.3% of strains from the Infection group ($p<0.001$), in 59.3% of the BIO+ Colonization group and 14.3% of the BIO+ Infection group ($p<0.001$), and in 48.1% of other and 16.7% of blood isolates ($p=0.046$). Linezolid and tigecycline had the highest activity against all studied isolates (100% susceptibility).

Table 2. Characteristics and statistical analysis (Chi square test, significance level α=0.05) of differences in virulence and antibiotic resistance between the tested *E. faecium* groups; BIO+, biofilm-positive; BIO-, biofilm-negative; n, number of strains; *acm*, collagen adhesin; *gelE*, gelatinase; *esp*, enterococcal surface protein; *hyl*, hyaluronidase; *efa*, endocarditis antigen; *cyl*, cytolysin; AMP, ampicillin; IMP, imipenem; GN, gentamicin; S, streptomycin; VA, vancomycin; TEI, teicoplanin; TG, tigecycline; LZD, linezolid; *lack of differences between groups.

virulence															
strains	**n**	**hemolysis**	***p***	***acm***	***p***	***gelE***	***p***	***esp***	***p***	***hyl***	***p***	***efa***	***p***	***cyl***	***p***
BIO+	48	97.9%	**0.001**	97.9%	0.117	4.2%	0.809	87.5%	0.138	83.3%	*	89.6%	0.154	2.1%	0.463
BIO-	18	72.2%		88.9%		5.6%		72.2%		83.3%		100%		5.6%	
Infection	28	92.9%	0.636	100%	0.128	0%	0.128	85.7%	0.656	85.7%	0.656	89.3%	0.408	0%	0.218
Colonization	38	90.9%		95.5%		4.5%		83.3%		83.3%		92.4%		3%	
BIO+ Infection	21	100%	0.373	100%	0.373	0%	0.203	85.7%	0.741	90.5%	0.241	85.7%	0.439	0%	0.373
BIO+ Colonization	27	96.3%		96.3%		7.4%		88.9%		77.8%		92.6%		3.7%	
Blood	12	100%	0.226	100%	0.403	0%	0.403	91.7%	0.392	83.3%	*	83.3%	0.188	0%	0.498
Other	54	88.9%		94.4%		5.6%		81.5%		83.3%		94.4%		3.7%	
BIO+ blood	11	100%	0.582	100%	0.582	0%	0.430	90.9%	0.697	81.8%	0.878	81.8%	0.337	0%	0.582
BIO+ other	37	97.3%		97.3%		5.4%		86.5%		83.8%		91.9%		2.7%	
antibiotic resistance															
		AMP	***p***	**IMP**	***p***	**GN**	***p***	**S**	***p***	**VA**	***p***	**TEI**	***p***	**TG/LZD**	***p***
BIO+	48	97.9%	0.464	100%	0.273	62.5%	0.917	83.3%	0.575	47.9%	0.880	39.6%	0.446	0%	*
BIO-	18	94.4%		94.4%		61.1%		88.9%		50%		50%		0%	
Infection	28	96.4%	0.826	96.4%	0.240	60.7%	0.840	85.7%	0.866	17.9%	**<0.001**	14.3%	**<0.001**	0%	*
Colonization	38	97.4%		100%		63.2%		84.2%		71.1%		63.2%		0%	
BIO+ Infection	21	100%	0.372	100%	*	61.9%	0.940	85.7%	0.696	19%	**<0.001**	14.3%	**<0.001**	0%	*
BIO+ Colonization	27	96.3%		100%		63%		81.5%		70.4%		59.3%		0%	
Blood	12	100%	0.498	100%	0.635	41.7%	0.129	91.7%	0.466	16.7%	**0.015**	16.7%	**0.046**	0%	*
Other	54	96.3%		98.1%		63%		83.3%		55.6%		48.1%		0%	
BIO+ blood	11	100%	0.582	100%	*	45.5%	0.183	90.9%	0.443	18.2%	**0.026**	18.2%	0.098	0%	*
BIO+ other	37	97.3%		100%		67.6%		81.1%		56.8%		45.9%		0%	

DISCUSSION

Our results revealed that 72.7% of the tested *E. faecium* strains had the ability to form biofilm. Studies by other authors showed different results; in India, Italy, and Turkey, the percentages of BIO+ *E. faecium* strains were much lower (25.2%, 28.8%, and 48%, respectively) [8, 19, 20]. When comparing strains from the Infection Group with strains from the Colonization Group, we found that this ability was on a similar level (75% and 72.7%, respectively). Di Rosa et al. [8] described only 35.7% of biofilm-producing *E. faecium* isolated from infections. In our survey, the highest difference in biofilm formation was observed when comparing the Blood Group with the Other Group (91.1% and 68.5%, respectively), but it was statistically insignificant (p=0.103). Researchers from Greece [7] detected 55.9% of BIO+ *E. faecium* strains in blood isolates, while our study revealed that all strains from the analogous group had this ability. Thus, worryingly, we can consider that the percentages of BIO+ *E. faecium* strains in our hospital are very high, and the appropriate surveillance methods should be implemented.

According to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) interpretation tables for clinical breakpoints, almost all (>95%) *E. faecium* isolates were resistant to tested ß-lactams, and more than 60% showed high levels of resistance to aminoglycosides. Moreover, in this research we observed very high rates of resistance to glycopeptides: 48.5% strains were VRE, and 42.4% were also resistant to teicoplanin. Likewise, the latest research conducted in our hospital revealed similar levels of resistance among *E. faecium* strains [21]. Therefore, we can conclude that the problem with MDR *E. faecium* isolates in our hospital environment is large and the infections caused by these strains should not be underestimated. The only antimicrobial agents that showed 100% activity against these strains were tigecycline and linezolid. These findings are consistent with previous surveys that describe these drugs as valuable therapeutic options in infections caused by MDR *Enterococcus* strains, although their clinical use is limited [21-23].

Taking into account the levels of resistance among the tested groups and subgroups, we found no statistically significant differences, except for vancomycin and teicoplanin (Table 2). In the case of our isolates from the Blood Group, we found very high levels of resistance to all tested antibiotics (except linezolid and tigecycline). Previous American research revealed significantly smaller percentages of resistance toward ampicillin (75.6%) and aminoglycosides (about 30%) among *E. faecium* isolated from blood. However, the same study showed higher levels of resistance to vancomycin (22.2%) [24]. Different results were presented by Saeedi et al. [25], who reported resistance to gentamicin in all *E. faecium* blood isolates. Interestingly, we revealed no significant differences in antibiotic resistance between BIO+ and BIO- isolates; therefore, the hypothesis that bacteria in biofilms are more resistant to antibiotics than planktonically grown microorganisms [3,9,26,27] is not confirmed in our study.

Unfortunately, we did not find any statistically significant differences in the prevalence of all tested virulence genes among the tested *E. faecium* groups (p>0.05). The only significant disparity (p=0.001) was reported in the case of the phenotypic ability to hemolyze: more BIO+ (97.9%) than BIO- (72.2%) strains had this feature, indicating that BIO+ isolates are slightly more virulent than the BIO- group.

The results obtained in this study agree with previous statements that there is no relationship between the occurrence of the *esp* gene and biofilm formation among *Enterococcus* strains [3,6,8]. Nevertheless, *esp* seems to be an important virulence trait among *E. faecium* strains. Hallgren *et al.* [28] noticed that it was the only virulence factor found among these species; it occurred in 75% of blood isolates and 70% of rectal isolates. On the other hand, Diani *et al*. [20] found that 46% of blood and 22% of fecal isolates contained this gene. An American survey conducted concurrently revealed that *esp* was present in 33% of BIO+ and 53.8% of BIO- isolates [6].

Interestingly, the *hyl* gene was detected much less frequently, in only 22% of BIO+ and 38.5% of BIO- strains [6]. In our BIO+ Infection Group, 85.7% of strains had the *esp* gene, while Di Rosa *et al.* [8] detected it in only 50% of analogous strains. Unfortunately, in our research we observed much higher rates of these genes among corresponding groups. Astonishingly, Tsikrikonis *et al.* [7] revealed that 83.8% of BIO+ and 26.7% of BIO- *E. faecium* clinical strains had *esp*, and 61.9% of BIO+ and 0% of BIO- fecal isolates carried this gene. The authors concluded that the presence of *esp* has a strong connection with biofilm-forming ability, which is not in concordance with our findings. All of these varied results indicate that *esp* may require certain interactions with other virulence traits to result in biofilm enhancement; more studies are definitely needed in this area.

A noteworthy fact is that the presence of *cyl* and *gelE* genes among *E. faecium* strains is very rare [20,28]. Vankerckhoven et al. [29] did not detect any *cyl* and *gelE* genes with PCR in 271 *E. faecium* isolates. In our study, the majority of *E. faecium* isolates were shown to be cytolysin/hemolysin producers (>89%) on blood agar plates, but only two (3%) strains carried the genes of the *cyl* operon. This may be due to the expression of other hemolysin genes that are not yet known or not so well studied. Interestingly, we

found that these *cyl*-positive strains also had the *gelE* gene. A small percentage of strains with the *gelE* gene have also been reported [30], but without the coexistence of the *cyl* gene.

CONCLUSIONS

In summary, this study demonstrated a lack of significant differences in virulence and resistance among various tested *E. faecium* groups. Nevertheless, we revealed that all *E. faecium* isolates in our hospital carry high levels of resistance to many antimicrobials and are extremely well equipped with virulence genes. Furthermore, the majority of these strains were able to form biofilm structures; therefore, they can persist in a hospital environment for a long time. This creates the need for more effective surveillance and an appropriate antibiotic policy. Only a complete understanding of the exact role of resistance and virulence factors in the development of biofilm can lead to improved strategies for the control of infections caused by MDR *E. faecium* isolates. There is an urgent need for larger multicenter studies to assess reports about levels of resistance and virulence among *E. faecium* strains in Poland.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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