ISSN 2449-8955

European Journal of Biological Research

Research Article

Comparison of biofilm-producing *Enterococcus faecalis*, *Enterococcus faecium*, and unusual *Enterococcus* strains

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Received: 14 July 2017; **Revised submission:** 25 September 2017; **Accepted:** 02 October 2017

DOI: http://dx.doi.org/10.5281/zenodo.1000837

ABSTRACT

The present study focused on determining the prevalence of biofilm-forming ability in *Enterococcus faecalis*, *E. faecium*, and unusual *Enterococcus* clinical isolates, and comparison of resistance and the prevalence of selected virulence factors among biofilm-positive strains. The ability to form biofilm was detected in 13.3% of *E. faecalis*, 90% of *E. faecium*, and 57.1% of unusual *Enterococcus* strains (p=0.026). All *E. faecalis* strains were susceptible to β-lactams, while 37.5% of unusual and all *E. faecium* isolates were resistant to these antibiotics. Resistance to gentamicin was detected in 75% of *E. faecalis*, 55.5% of *E. faecium*, and 25% of other strains; resistance to streptomycin in 25%, 83.3%, and 50%, respectively. Analysis of the virulence revealed that the enterococcal surface protein (*esp*) gene was found in all *E. faecium*, 75.0% of *E. faecalis*, and 37.5% of other strains; collagen adhesin gene (*ace*) in 100%, 25.0%, and 37.5%; and hyaluronidase gene (*hyl*) in 83.3%, 0%, and 37.5%, respectively. Analysis of the resistance and virulence patterns showed that *E. faecium* isolates had the greatest variety of virulence and resistance determinants, while the lowest variety was exhibited by unusual strains. These findings indicate that unusual biofilm-producing *Enterococcus* strains have lower resistance and virulence potency than *E. faecalis* and *E. faecium*.

Keywords: *Enterococcus faecalis*; *Enterococcus faecium*; Biofilm; Resistance; Virulence.

1. INTRODUCTION

Today, *Enterococcus* spp. are the fourth most common etiological factor in nosocomial infections in Europe [1]. Although these cocci are members of the microbiota of the human gastrointestinal tract, they often infect the bloodstream, surgical sites, and urinary tract, due to their multiresistance to many antimicrobials [2, 3]. *Enterococcus* spp. have an intrinsic resistance to cephalosporins, lincosamides, and low levels of aminoglycosides, and they can easily acquire resistance, most prominently to glycopeptides and aminoglycosides (high-level resistance), by means of mutations or as a result of transfer and incorporation of genes located on mobile genetic elements, such as plasmids and transposons [1, 4]. Moreover, these bacteria have the ability to form strong biofilm structures, and to produce several virulence factors, such as enterococcal surface protein (Esp), aggregation substance

(As), collagen adhesion (Ace), hyaluronidase (Hyl), and gelatinase (GelE) [5-7]. Esp is the factor that mediates the colonization, and, together with GelE, has been suggested to be involved in biofilm formation [5-7]. Ace and EfaA are principal virulence traits associated with infective endocarditis, whereas Hyl causes tissues damages [5-7]. The majority of nosocomial enterococcal infections are caused by *E. faecalis* and *E. faecium*. However, today there is an increasing prevalence of infections caused by other rarely isolated species, for example: *E. avium, E. gallinarum, E. durans*, and *E. casseliflavus* [8-10].

Biofilm is an assemblage of microbial cells enclosed in a self-produced polysaccharide matrix and attached to a biotic or abiotic surfaces, providing an optimal microenvironment for growth, and facilitates transmission of mobile determinants between microorganisms [11, 12]. Evidence suggests that bacteria in biofilms are more resistant to antimicrobials and hosts factors than other microorganisms and are extremely difficult to eradicate [13]. Likewise, among *Enterococcus*, it is suggested that an ability to produce biofilm is a very important virulence factor which has a major impact on the course of nosocomial infections [5, 7]. Unfortunately, our knowledge about the mechanisms and determinants involved in the process of biofilm formation among enterococci is still insufficient [14]. The ability to create biofilm has been suggested to occur less frequently among *E. faecium* strains compared to *E. faecalis* strains, but, astonishingly, data about biofilm-forming ability among unusual enterococcal species are very limited and unclear [14, 15]. Furthermore, there are only a few reports about the differences in resistance and virulence of various biofilm-producing *Enterococcus* species [13, 16, 17]. This prompted us to determine the prevalence of biofilm-forming ability among *E. faecalis*, *E. faecium*, and unusual *Enterococcus* spp. clinical isolates. Then, we focused on the comparison of the antibiotic resistance, the ability to hemolyze, and the presence of selected virulence genes among these three groups of biofilm-producing *Enterococcus* spp. strains. Moreover, the next goals of this study were to determine their exact resistance profiles, and to indicate the antibiotic with the highest activity against these strains.

2. MATERIAL AND METHODS

2.1. Strains

Tests were performed on sixty-four enterococcal isolates: thirty *E. faecalis*, twenty *E. faecium*, and fourteen others (five *E. avium*, three *E. casseliflavus*, three *E. gallinarum*, three *E. durans*), isolated from clinical specimens from patients hospitalized at the University Hospital in Bialystok (Poland) from December 2013 to January 2015. Isolates were recovered from various clinical materials, mostly blood, peritoneal fluid, bronchoalveolar lavage (BAL), feces, urine, and pus. Most of the collected isolates were gathered from the intensive care unit and a hematology clinic.

2.2. Identification and susceptibility testing

The identification and susceptibility testing were conducted on the automated VITEK 2 system (bioMérieux, France) according to the manufacturer's instruction 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).

2.3. Biofilm and hemolysin production

The tube method [18, 19] and Congo red agar (CRA) method [20, 21] were used to assess the ability of tested isolates to biofilm formation. Each experiment was repeated three times for each strain. Strains that demonstrated the ability to produce biofilm by both methods were considered as biofilm positive (BIO+) isolates. Hemolysin production was determined on Columbia blood agar supplemented with 5% sheep blood (OXOID, United Kingdom) [22].

2.4. DNA extraction

In the next step, genomic DNA was extracted

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

2.5. PCR detection of virulence genes

Then, PCR assays were performed to detect the following virulence genes: *gelE*, *ace*, *hyl*, *esp*, *as*, and *cyl*. The primers 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).

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). The positions of obtained products were estimated with the molecular weight marker Perfect^{TM} 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 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.

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

Virulence gene	Primers	Product size (bp)	Annealing temperature (°C)	Reference
gelE	5' AAT TGC TTT ACA CGG AAC GG 3' 5' GAG CCA TGG TTT CTG GTT GT 3'	548	52	[23]
ace	5' GGC CAG AAA CGT AAC CGA TA 3' 5' CGC TGG GGA AAT CTT GTA AA 3'	353		
hyl	5' ACA GAA GAG CTG CAG GAA ATG 3' 5' GAC TGA CGT CCA AGT TTC CAA 3'	276	55	[24]
esp	5' AGA TTT CAT CTT TGA TTC TTG G 3' 5' AAT TGA TTC TTT AGC ATC TGG 3'	510		
as	5' CACGCTATTACGAACTATGA 3' 5' TAAGAAAGAACATCACCACGA 3'	375		
cyl	5' TGG ATG ATA GTG ATA GGA AGT 3' 5' TCT TTC ATC ATC TGA TAG TA 3'	517		

2.6. Statistical analysis

STATA 13.1 (StataCorp LP, USA) was used for statistical analysis. Differences among *E. faecalis*, *E. faecium*, and unusual enterococcal strains were assessed by the Chi-square test and Fisher's exact test. Results with $p<0.05$ were considered significant.

3. RESULTS AND DISCUSSION

The present study focused on determining the prevalence of biofilm-forming ability among three enterococcal groups: *E. faecalis*, *E. faecium*, and other clinical isolates, and on comparison of the antibiotic resistance and the prevalence of selected virulence traits between BIO+ strains from these groups. Interestingly, we found that the ability to form biofilm occurred in 4/30 (13.3%) *E. faecalis*, 18/20 (90%) *E. faecium*, and 8/14 (57.1%) rarely isolated strains: 5 *E. avium*, 1 *E. durans*, 1 *E. casseliflavus*, and 1 *E. gallinarum* (statistically significant difference, $p=0.026$). Studies by other authors showed different results; in Greece, the ability to

produce biofilm was found in 60.9% of *E. faecalis* isolates [25], in Italy - in 80% of *E. faecalis* strains [26]. In the case of *E. faecium* isolates, in India, Italy, Turkey, and Spain, this ability occurred much less frequently (0%, 28.8%, 48%, and 75%, respectively) [5, 8, 27, 28]. Lleo et al. [17] described the biofilm-forming ability among four out of twelve unusual enterococcal strains (33.3%), which our study supports. However, in contrast to our findings, Dworniczek et al. [29] indicated the lack of these features in rare species. These varied results indicate that the level of the ability to produce biofilm among different *Enterococcus* species varies with geographic location.

In the next step of our research, only BIO+ strains (30/64) were chosen for further investigation. A comparison of antibiotic resistance among *E. faecalis*, *E. faecium* and other isolates showed that all *E. faecalis* strains were susceptible to tested ß-lactams, while 37.5% of other strains and all *E. faecium* isolates were resistant to these antibiotics. These results strongly overlap with results recently published by us [30] and other researchers [9, 16, 31]. Resistance to gentamicin was detected in 75% of *E. faecalis*, 55.5% of *E. faecium*, and 25% of other strains; resistance to streptomycin in 25%, 83.3%, and 50%, respectively. Findings from our previous work showed that more *E. faecium* isolates were resistant to aminoglycosides: 76% to gentamicin, and 91.4% to streptomycin [30]. Interestingly, a study by Tan et al. [9] demonstrated that all unusual enterococcal isolates from blood were susceptible to gentamicin and around 80% of them were susceptible to ß-lactams. Therefore, the authors concluded that combination therapy (penicillin with aminoglycosides) could be easily used for the treatment of serious infections caused by rare species of *Enterococcus*, such as bacteremia and sepsis. This finding is not confirmed in our survey. We revealed that resistance to glycopeptides occurred only in the case of four (22.2%) *E. faecium* isolates; two strains from the rare group, *E. gallinarum* and *E. casseliflavus*, showed intrinsic resistance to vancomycin. Similar results were obtained by other authors [9, 32]. We concluded that tigecycline and linezolid had the highest activity against all studied isolates (100% susceptibility), including those resistant to glycopeptides and aminoglycosides. Many studies confirmed that these antibiotics are a valuable therapeutic option in serious enterococcal infections [33-35]. Unfortunately, resistance to these drugs has been recently reported [34, 36, 37], indicating that resistance to newer antibiotics is also increasing, and development of new targeted enterococcal drugs is needed.

Our comparative analysis of the prevalence of virulence genes among *E. faecalis*, *E. faecium*, and other strains revealed that the *esp* gene was found in all *E. faecium*, 75% of *E. faecalis*, and 37.5% of other strains. Similar proportions were seen by other researchers [6, 11, 25, 38, 39, 40]. These findings indicate that this gene has a connection with biofilm-forming ability, especially in *E. faecium* strains. However, many authors found that there is no association between the presence of the *esp* gene and biofilm production [5, 14, 29, 41]. These conflicting results suggest that *esp* requires interactions with other virulence traits to result in biofilm enhancement.

Considering the presence of other virulence factors in our studied BIO+ groups, we found that the *ace* gene occurred in all *E. faecium*, 25% of *E. faecalis*, and 37.5% of unusual isolates; *hyl* in 83.3%, 0%, and 37.5%, respectively. The *gelE* gene was detected only in *E. faecalis* strains. According to the literature, the presence of *gelE* and *as* genes among *E. faecalis* is very common, whereas they are extremely rarely present in *E. faecium* and rare enterococcal isolates; consequently, they are not necessary in the process of biofilm formation among these species [5, 25, 28, 42, 43]. These assumptions are confirmed by our survey. However, some researchers imply that there is a strong relationship between *gelE* and the ability to form biofilm [12, 15]. Other virulence genes, *cyl* and *as*, were also found only in *E. faecalis* isolates, which is in accordance with other studies [22, 40, 44].

The exact resistance and virulence patterns among all tested BIO+ strains are shown in Table 2. No predominant profile among each group was identified, not only due to small sample size, but also because of high interindividual variability of examined traits among tested *Enterococcus* spp. groups. However, we have found that *E. faecium* isolates showed the greatest variety of virulence and resistance determinants, while the lowest variety was exhibited by the unusual strains group. Moreover, all *E. faecium* strains carried resistance to

three or more antibiotics and had the ability to hemolyse. Different results were seen in recent research by Tsikrikonis et al. [25], who detected only 1.9% of hemolysin-producing *E. faecium* clinical isolates. We also found that one *E. faecalis* isolate and three *E. avium* isolates were susceptible to all tested antibiotics.

Table 2. Characteristics of resistance and virulence patterns among BIO+ *E. faecalis*, BIO+ *E. faecium*, and other BIO+ *Enterococcus* strains. AMP, ampicillin; IMP, imipenem; CN, gentamicin; S, streptomycin; VA, vancomycin; TEI, teicoplanin; *esp*, enterococcal surface protein; *as*, aggregation substance; *gel*, gelatinase; *hyl*, hyaluronidase, *ace*, collagen adhesin; *c*, cytolysin; α, β, types of hemolysis.

No. of inactive antibiotics	Resistance pattern	No. of virulence genes	Virulence pattern	No. (%) of strains
BIO+ *E. faecalis* (n = 4)				
4	AMP IMP CN S	3	*as* *gel* *c* α	1 (25)
1	CN	4	*esp* *as* *gel* *c*	2 (50)
0		5	*esp* *as* *gel* *ace* *c*	1 (25)
BIO+ *E. faecium* (n = 18)				
5	AMP IMP S VA TEI	3	*esp* *ace* *hyl* α	2 (11.1)
	AMP IMP CN VA TEI		*esp* *ace* *hyl* α	1 (5.5)
4	AMP IMP		*esp* *ace* *hyl* α	1 (5.5)
	AMP IMP CN S		*esp* *ace* *hyl* α	5 (27.8)
	AMP IMP CN S	2	*esp* ace β	2 (11.1)
3	AMP IMP S	3	*esp* *ace* *hyl* α	5 (27.8)
	AMP IMP CN		*esp* *ace* *hyl* α	2 (11.1)
Unusual BIO+ *Enterococcus* (n = 8)				
4	AMP IMP CN VA	2	*esp* *hyl* α	1 (12.5)
	AMP IMP CN S	1	*esp* β	1 (12.5)
3	AMP IMP S	2	*esp* *hyl*	1 (12.5)
1	S		*ace* *hyl* β	1 (12.5)
	VA	0		1 (12.5)
0		1	*ace* β	2 (25)
		0	β	1 (12.5)

In conclusion, we observed that the proportion of isolates producing biofilm was the highest among *E. faecium* isolates, at the middle level among the unusual *Enterococcus* spp. group, and the lowest in *E. faecalis* isolates. Interestingly, our data demonstrated that unusual biofilm-forming *Enterococcus* strains have lower resistance to antibiotics and are characterized by possession of lower virulence capabilities than BIO+ *E. faecalis* and BIO+ *E. faecium* clinical isolates. Moreover, *E. faecium* strains showed the highest resistance and virulence levels. It is well known that *E. faecium* isolates resistant to ß-lactams, aminoglycosides, and glycopeptides are considered as multidrug resistant (MDR) bacteria, and they represent a particular threat to immunocompromised patients [9]. The problem with these strains becomes even more serious when they are also able to produce biofilm, and persist in hospital environments for a very long time. However, the high percentage of biofilm-

forming ability among unusual *Enterococcus* species, observed in this study, indicates that these isolates could also stay in the medical environment and, consequently, slowly acquire resistance and virulence traits. Therefore, the infections caused by these strains should not be underestimated, and determination of their susceptibility should always be performed. The changing epidemiology and increasing resistance to antibiotics among *Enterococcus* species stress the need to search in new directions for the treatment and new methods for preventing the spread of enterococcal nosocomial infections.

ACKNOWLEDGEMENTS

We thank Steven J. Snodgrass for editorial assistance. The results of this work were presented in part at the Biofilms 7 Conference 2016, Porto, Portugal (26-28.06.2016).

AUTHOR'S CONTRIBUTION

AS: Conception and design, Development of methodology, Acquisition of data, Analysis and interpretation of data, Writing, review and/or revision of the manuscript; DO: Acquisition of data, Analysis and interpretation of data, Administrative, technical, or material support; PM: Analysis and interpretation of data, Administrative, technical, or material support; PS and PW: Writing, review and/or revision of the manuscript; ET: Writing, review and/or revision of the manuscript, Study supervision. The final manuscript has been read and approved by all authors.

SOURCE OF FUNDING

This work was supported by funds from Medical University of Bialystok (Poland), and from the Leading National Research Centre (137/KNOW/ 2015) in Bialystok.

TRANSPARENCY DECLARATION

The authors have no conflict of interest to declare.

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