Virulence factors and antibiotic resistance in *Enterococcus faecalis* and *Enterococcus faecium* strains isolated from patients hospitalized in the University Hospitals in Bialystok, Poland.

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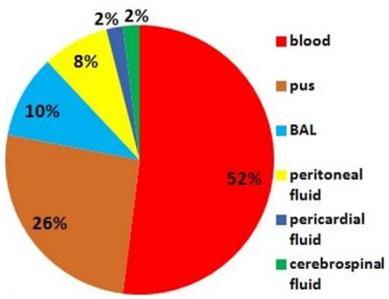
Introduction and purpose:

Enterococcus spp. has in recent years become one of the most common etiological factors in nosocomial infections, because of their virulence and resistance to a variety of antimicrobials. This bacteria causes urinary tract infections, wound infections, sepsis, endocarditis and intraabdominal infections. *Enterococcus* exhibit intrinsic resistance to a variety of antibiotics (ß-lactams, low concentration of aminoglycosides, lincosamides, tripethoprim/sulfamethoxazole and fluorochinolones) and acquire resistance determinants by horizontal genetic exchange, such as *van* and *AMEs* (aminoglicosides modifying enzymes) genes. Moreover, *Enterococcus* have the ability to produce several virulence factors and the ability to form strong biofilm. *Enterococcus faecalis* and *Enterococcus faecium* are the most prevalent species, accounting for more than 90% of clinical isolates.

The aim of this study was to compare the antibiotic resistance, the prevalence of genes encoding selected virulence factors, hemolytic activity and biofilm forming ability between *Enterococcus faecalis* and *Enterococcus faecium* strains isolated from invasive nosocomial infections.

Methods:

Fifty *Enterococcus* strains (30 *E. faecalis* and 20 *E. faecium*) from invasive infections were analyzed. Strains were isolated from February 2013 to December 2014 from patients hospitalized in different wards of Medical University of Białystok Clinical Hospital and the Medical University of Bialystok Children's Clinical Hospital in Poland (Fig. 1).



Hospital department	E. faecalis		E. faecium	
Intensive Care Unit	7	23,4%	10	50%
Surgery	10	33,3%	5	25%
Gynecology	5	16,8%	-	-
Cardiology	2	6,7%	2	10%
Children's Clinical Hospital	1	3,3%	1	5%
Orthopedics	1	3,3%	-	-
Haematology	1	3,3%	-	-
Hospital in tapy	1	3,3%	-	-
Endocrinology	1	3,3%	-	-
Rheumatology	1	3,3%	-	-
Gastrology	-	-	1	5%
Neurosurgery	-	-	1	5%

Fig. 1. Distribution of *E. faecalis* and *E. faecium* isolates among clinical materials and different hospital wards.

Each isolate was identified by automated VITEK2 system (bioMerieux, USA) and confirmed by PCR amplification with primers targeted to the *ddl* chromosomal gene. Susceptibility to 8 antibiotics (ampicillin, imipenem, gentamicin, streptomycin, vancomycin, teicoplanin, linezolid and tigecycline) was determined using the E-test method (Fig. 2, a.) and interpreted according to the newest EUCAST guidelines. The biofilm forming ability was determined using two methods – the tube method and Congo red agar method (Fig. 2, b - c). Each experiment was repeated three times for each strain. Hemolysin production was evaluated on Columbia blood agar plates (Fig. 2, d). Genes encoding selected virulence factors (*esp* - enterococcal surface protein, *as* - aggregation substance, *ace* - collagen adhesin) were investigated by PCR using specific primers (Tab. 1) followed by gel electrophoresis and DNA sequencing. Differences in the prevalence of tested features between *E. faecalis* and *E. faecium* strains were assessed by Chi-square and Fisher tests (significance level $p < 0.05$). STATA 13.1 (StataCorp, USA) was used.

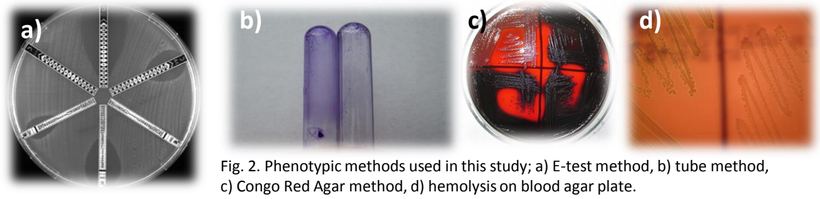


Fig. 2. Phenotypic methods used in this study; a) E-test method, b) tube method, c) Congo Red Agar method, d) hemolysis on blood agar plate.

virulence gene	primers	product size (bp)	annealing temperature (C)
esp	AGA TTT CAT CTT TGA TTC TTG G	510	55
	AAT TGA TTC TTT AGC ATC TGG		
as	GCACGCTATTACGAACTATGA	375	
	TAAGAAAGAACATCACCACGA		
ace	GGC CAG AAA CGT AAC CGA TA	616	52
	CGC TGG GGA AAT CTT GTA AA		

Tab. 1. Primers used in this study.
Source: 5. Ozden Tuncer B, Ay Z, Tuncer Y (2013) Occurrence of enterocin genes, virulence factors, and antibiotic resistance in 3 bacteriocin-producer *Enterococcus faecium* strains isolated from Turkish tulum cheese. Turk J Biol 37: 443-449

Results:

Figure 3 presents an exact comparison of antibiotic susceptibility between *E. faecalis* and *E. faecium* isolates.

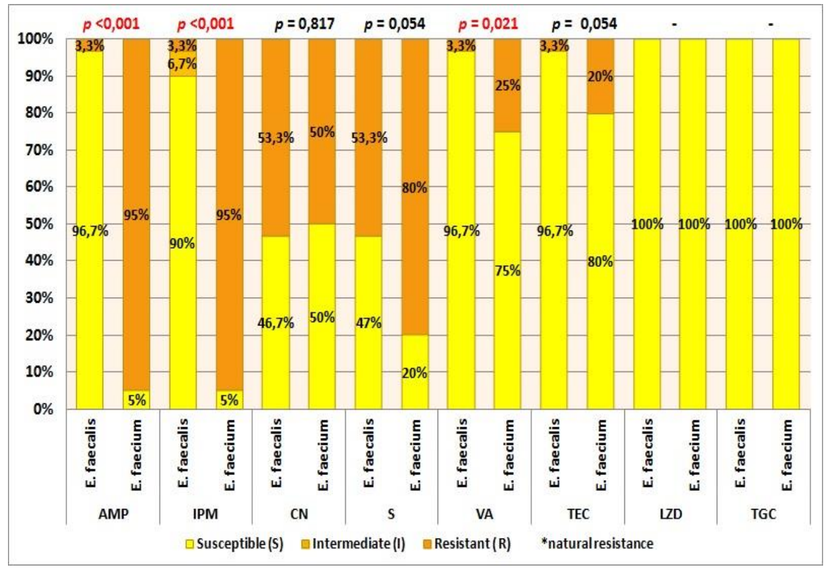


Fig. 3. Antibiotic resistance among tested strains; AMP – ampicillin, IPM – imipenem, CN – gentamicin, S – streptomycin, VA – vancomycin, TEC – teicoplanin, LZD – linezolid, TGC – tigecycline

The most effective antibiotics against both groups were linezolid and tigecycline (100% susceptible strains). Resistance to gentamicin was detected in 46.7% *E. faecalis* and 50% *E. faecium* strains, resistance to streptomycin in 47% and 20% strains, resistance to teicoplanin in 3.3% and 20% strains, respectively; these differences were not statistically significant. The statistically significant differences ($p<0.05$) were found in the case of ampicillin, imipenem (3.3% *E. faecalis* and 95% *E. faecium* resistant strains) and vancomycin (3.3% vs 25% resistant strains).

The ability to produce biofilm was detected in 90% *E. faecium* and 13.3% *E. faecalis*; hemolytic activity in 95% *E. faecium* and 13.3% *E. faecalis* strains (statistically significant differences, $p<0.001$, Tab. 2).

All strains carried one or more of the virulence genes (Fig. 4). *Esp* gene was found in 66.7% *E. faecalis* and 95% *E. faecium* strains, *ace* in 50% and 100% strains, respectively. Ninety seven percent of *E. faecalis* strains had *as* gene; that gene were not found in any of the *E. faecium* isolates. All differences were statistically significant (Tab. 2).

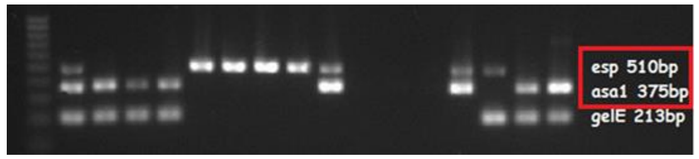


Fig. 4. Example of amplified products of virulence genes generated by Multiplex PCR

		E. faecalis	E. faecalis %	E. faecium	E. faecium %	p-value
biofilm	+	4	13,3%	18	90%	<0,001
	-	26	86,7%	2	10%	
hemolysis	ß	3	10%			<0,001
	α	1	3,3%	19	95%	
	-	26	86,7%	1	5%	
Esp	+	20	66,7%	19	95%	0,018
	-	10	33,3%	1	5%	
As	+	29	96,7%			<0,001
	-	1	3,3%	20	100%	
Ace	+	15	50%	20	100%	<0,001
	-	15	50%			

Tab. 2. Statistical analysis of differences in the prevalence of virulence factors between *E. faecalis* and *E. faecium* strains.

Conclusion:

E. faecium strains causing invasive infections were significantly often *esp* and *ace* positive, had higher antibiotic resistance, were more hemolytic and produce biofilm more often than *E. faecalis* strains. This findings indicate that *E. faecium* has greater ability to adhesion to artifical surfaces, persistance and spread in hospital environment and may cause more serious nosocomial infections.