

## **VIRULENCE FACTORS AND ANTIBIOTIC RESISTANCE IN *ENTEROCOCCUS FAECALIS* ISOLATED FROM URINE SAMPLES**

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**Abstract:** Haemolysin, enterococcal surface protein (Esp), aggregation substance and gelatinase are some markers that have been proposed as possible enterococci virulence factors. The aim of this study was to detect the presence of haemolysin, gelatinase and enterococcal surface protein in enterococci isolated from urine and to determine their susceptibility to antimicrobial agents. A total of 50 strains of *Enterococcus faecalis* isolated from urine samples was examined. UTI agar (Oxoid) was used for the isolation and identification of the strains as *Enterococcus spp.* The differentiation of the species was done by the Vitek automated system (GPI-card). Haemolysin production was detected phenotypically on Columbia CNA agar as a zone of  $\beta$  haemolysis around the streak. Production of gelatinase was determined as a clear halo around the colonies on trypticase soy agar supplemented with 1.5% skim milk. Esp was proved by detection of the *esp* gene using PCR after DNA extraction. Antibiotic sensitivity to ampicillin, ceftriaxone, vancomycin, nitrofurantoin and ciprofloxacin was examined by the agar diffusion method. In 16 *Enterococcus faecalis* strains (32%) all the virulence factors were present. Two factors were found in 19 (38%) strains and only one in 11 strains. There were only 4 strains without any virulence factor. Esp was the most frequently determined factor (in 38 isolates). All the strains were susceptible to vancomycin and nitrofurantoin; 12 isolates were resistant to ampicillin, 17 to ceftriaxone and 14 to ciprofloxacin. No relationship was found between virulence factors and resistance to an antibiotic.

**Key words:** *Enterococcus faecalis*, virulence factors, antibiotic resistance.

### Introduction

Enterococci are found in the intestine of nearly all animals, from cockroaches to humans. Enterococci normally colonize the human gastrointestinal tract. They are found in  $10^5$ – $10^7$  organisms per gram of human faeces [1].

Infections caused by the genus *Enterococcus* include urinary tract infections, bacteraemia, intra-abdominal infections, and endocarditis [1, 2]. Enterococci rank third among microorganisms isolated in nosocomial urinary tract infection [3] and are a cause of chronic and recurrent urinary tract infections especially associated with morphological and functional abnormalities and instrumentation in that tract [1, 3].

Of 14 or more enterococcal species [1], only *E. faecalis* and *E. faecium* commonly colonize and infect humans in detectable numbers. *E. faecalis* is isolated from approximately 80% of human enterococcal infections, and *E. faecium* from most of the rest. Infections with other enterococcal species are rare. Since both organisms are frequently isolated from the commensal flora, this bias suggests that *E. faecalis* strains show a greater degree of virulence.

Enterococci must first be able to colonize the mucosal surfaces and then cause the infection. After the colonization, bacteria produce pathological changes in the host through direct toxic activity, or indirectly by inducing an inflammatory response. Enterococci are not highly toxigenic, nor highly invasive, but they cause human infections. Some markers have been proposed as possible virulence factors in enterococci. Haemolysin, gelatinase (caseinase), enterococcal surface protein (esp) and aggregation substance are the most frequently mentioned virulence determinants [2, 4, 5].

Haemolysin is a cytolytic protein that lyses human, horse and rabbit erythrocytes and also has a bactericide activity against many Gram-positive bacteria. Strains enriched with this protein have been found among enterococci associated with an increased severity of infection [1, 4, 6, 7].

Gelatinase is an extracellular protease capable of hydrolyzing gelatin, collagen, casein, haemoglobin and other peptides. Gelatinase-producing strains of enterococci have been shown to be virulent in animal models inducing caries formation in germfree rats. Epidemiological studies suggest associations between protease production and infection. The sequence analysis of gelE has shown amino acid similarity to the elastase of *Pseudomonas aeruginosa*.

Esp is a cell wall-associated protein significantly more common in clinical isolates than among strains from normal flora [8, 9]. This protein, like other surfaced proteins, may facilitate adherence to the host epithelium. Some studies have suggested that esp plays an important role in the adherence of enterococci to the uro-epithelium [9].

Enterococci are intrinsically resistant to many antibiotics. Unlike acquired resistance and virulence traits, which are usually transposon or plasmid encoded, intrinsic resistance is based on chromosomal genes, which are non-transferable. Penicillin, ampicillin, piperacillin, imipenem, and vancomycin are among the few antibiotics that show inhibitory, but not bactericidal, activity against *E. faecalis*. *E. faecium* is less susceptible to  $\beta$ -lactam antibiotics than *E. faecalis* due to the lower affinities of their penicillin-binding proteins for antibiotics [2].

Enterococci often acquire antibiotic resistance through an exchange of resistance-encoding genes carried on conjugative transposons, pheromone-responsive plasmids and other plasmids [2, 10]. Among several phenotypes of vancomycin-resistant enterococci, VanA (resistance to vancomycin and teicoplanin) and VanB (resistance to vancomycin alone) are most common [1]. Inducible genes encoding these phenotypes can be transferred from enterococci to other bacteria [1], for example to *Staphylococcus aureus*.

Some studies have examined the relationship between antibiotic resistance and virulence factors, but there should be more investigation to reach further conclusions [2, 11].

The aims of this study were:

- to detect the presence of haemolysin, gelatinase and enterococcal surface protein in *E. faecalis* isolated from urine;
- to determine the susceptibility of strains to antimicrobial agents and whether there is a relationship between the presence of virulence factors and resistance to antimicrobial agents.

### *Materials and Method*

A total of 50 strains of *E. faecalis* isolated from urine samples was examined. Chromogen UTI agar (Oxoid) and routine microbiological tests were used for isolation and identification of strains such as *Enterococcus spp.* Differentiation of *E. faecalis* was done by the Vitek automated system (GPI-card). Only strains detected in more than 100,000 viable bacteria per ml urine were chosen for examination.

The presence of haemolysin was detected as phenotypical on Columbia CNA agar. Inoculated plates were incubated for 24 hours at 37°C. Haemolysin production was determined as a zone of  $\beta$ -haemolysis around the streak of colonies. Production of gelatinase was determined as a clear halo around the colonies on trypticase soy agar supplemented with 1.5% skim milk.

Esp was proved by detection of the *esp* gene using PCR. The extraction of total DNA from enterococci was performed by Shankar's protocol [9].

PCR for the *esp* gene is described bellow.

<b>Primers (Sigma)</b>	<b>length of the product</b>
<i>esp</i>	
5'- TTGCTAATGCTAGTCCACGACC-3'	933 bp
5'- GCGTCAACACTTGCATTGCCGAA-3'	

The PCR reaction mixture contained: 250 ng DNA template, 200  $\mu$ M dNTP, 1.5 mM MgCl<sub>2</sub>, 2 U AmplyTaq DNA polymerase, 20 pmol F (Forward) and R (Reverse) primers and 1 $\times$  concentrate buffer. Amplification was performed in a thermal cycler (initial denaturation at 95<sup>0</sup>C for 2 min was followed by 30 cycles: denaturation at 94<sup>0</sup>C for 45 sec; annealing at 63<sup>0</sup>C, 45 sec; extension at 72<sup>0</sup>C for 1 min).

The reaction products were subjected to electrophoresis in 1.5% agarose gel.

The antibiotic sensitivity of the strains to ampicillin, ceftriaxone, vancomycin, nitrofurantoin and ciprofloxacin was tested. These antibiotics were chosen because they are used in therapy for enterococcal infections, but also there may be different susceptibilities among enterococcal strains. Sensitivity was examined by the agar diffusion method on Muller-Hinton agar. Results were interpreted using NCCLS recommendations.

### Results

The examined strains of *E. faecalis* showed varying presences of virulence factors (Table 1).

Table 1 – Табела 1

*Presence of virulence factors in examined strains*  
*Присусиство на фактори на вируленција кај испитаниите соеви*

Virulence factor	Esp <sup>1,2,*</sup>	Gelatinase <sup>1,3,*</sup>	Haemolysin <sup>2,3,*</sup>	Total
Combination of factors	+	+	+	16
	+	+	-	10
	+	-	-	8
	+	-	+	4
	-	+	+	5
	-	+	-	3
	-	-	-	4
Total	38	34	25	50

Cochran Q test: <sup>1</sup>Q = 0.8 (p > 0.05); <sup>2</sup>Q = 7.35 (p < 0.05); <sup>3</sup>Q = 4.76 (p < 0.05); \*Q = 12.1 (p < 0.005)

In 16 strains (32%) all three virulence factors were present. Two factors were found in 19 (38%) strains and only one in 11 strains. There were only 4 strains without any virulence factor. Esp was the most commonly determined factor (in 38 isolates). Statistically significant differences (Cochran Q test) were found in the presence of all virulent determinants, between Esp and gelatinase and between gelatinase and haemolysin (Table 1).

All strains were susceptible to vancomycin and nitrofurantoin; 12 isolates were resistant to ampicillin, 17 to ceftriaxone and 14 to ciprofloxacin (Figure 1).

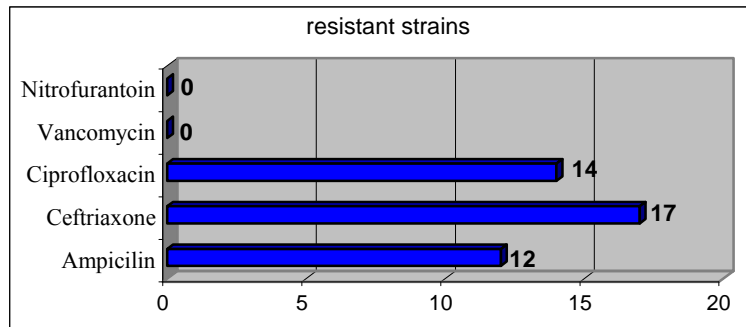


Figure 1 – Strains resistant to antimicrobial agents (N = 50 strains)

Слика 1 – Резистентни соеви кон антибиотици (N = 50 соеви)

The results of the resistance analysis in enterococci with a combination of virulence factors are shown in Table 2.

Table 2 – Табела 2

*Susceptibility to antibiotics of enterococci with combination of virulence factors*  
*Осељливост кон антибиотици на ентерококије со комбинации*  
*на фактори на вируленција*

Virulence factors	Resistant strains to antibiotics			x <sup>2</sup> test
	Ampicillin	Ceftriaxone	Ciprofloxacin	
Esp/Gel/Hem (N = 16)	2	2* <sup>†</sup>	4	NS
Esp/Gel (N = 10)	3	4	4	NS
Esp (N = 8)	3	5 <sup>†</sup>	2	NS
Esp/Hem (N = 4)	2	2	1	NS
Gel/Hem (N = 5)	1	1	2	NS
Gel (N = 3)	0	0	0	NS
None factor (N = 4)	1	3*	1	NS
Total	12	17	14	

NS-not significant; \*<sup>†</sup>p < 0.05

Susceptibility to nitrofurantoin and vancomycin is not shown in the table, because no strains resistant to these antibiotics were found.

Statistical analysis ( $\chi^2$  test) did not show any relationship between the presence of the virulence factors and resistance to all antibiotics ( $p > 0.05$ ). Statistically significant differences were found in resistance to ceftriaxone: between strains with all virulence factors and those with Esp only; and between strains with all virulence factors and those without any virulence determinant (Table 2).

Analysis of separated virulence factors and resistance to different antibiotics is shown in figure 2. No relationship was found between virulence factors and resistance to an antibiotic ( $p > 0.05$ ;  $\chi^2$  test).

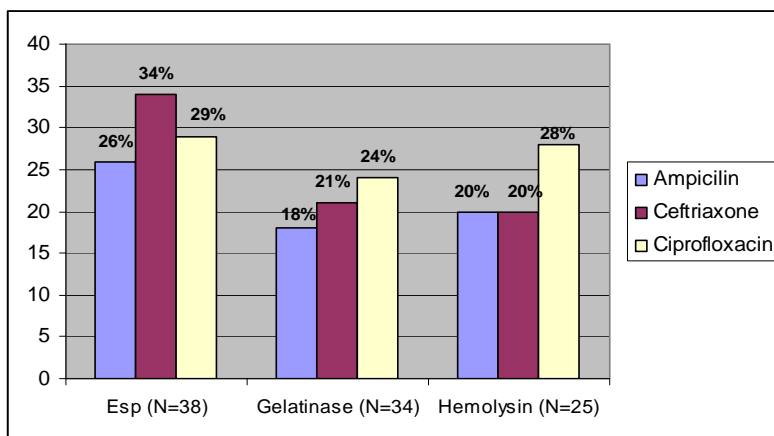


Figure 2 – Resistant strains of enterococci with different virulence factors  
Слика 2 – Резистентни соеви на енџерококи со разни факџори на вируленџија

### Discussion

Enterococci are important causes of urinary tract infections, bacteraemia, intra-abdominal infections and endocarditis.

The virulence of enterococci is due to their adherence and lytic activity, as in other bacteria. The adherence to the uro-epithelium is complex, involving surface adhesions: proteins and carbohydrates [2, 3]. Many factors of virulence in enterococci are known, but the most frequently mentioned are cytolysin/haemolysin, aggregation substance, surfaced proteins and gelatinase.

Enterococcal surface protein, Esp, assists in adherence to the host epithelium, but increased adherence to urinary catheters of Esp positive strains

has been noted [10]. The role of Esp in the persistence of experimentally caused urinary tract infection has been determined, and it is hypothesized that Esp has a function analogous to fimbriae of *Escherichia coli* and *Proteus* in adherence to the uro-epithelium [6, 12].

The presence of Esp in only 3% of stool isolates, according to some investigations, points out its role in infection [2]. In our study Esp was found in 38 *E. faecalis* isolates (76% of 50) from urine. More often Esp was combined with other virulence factors, alone it was present in only 8 strains.

Esp was first found among vancomycin-resistant enterococci and it was thought that there is some relationship between them, but under further investigation Esp was detected in 40% of the clinical strains, most often isolated from urine, with a different susceptibility to vancomycin [1]. In the present study no relationship was found between the presence of Esp and resistance to an antibiotic. In fact we did not find any strain resistant to vancomycin.

Gelatinase and haemolysin-producing strains of *E. faecalis* have been shown to be virulent in animal models of enterococcal infections. Gelatinase is a protease produced by enterococci [3] that is capable of hydrolyzing gelatin, collagen, casein and other peptides. Little is known about its occurrence among clinical and stool enterococcal isolates. We found gelatinase in more than half the clinical strains examined (68% of 50), but alone it was present in only 3 strains.

Cytolysin (haemolysin) causes rupture of a variety of target membranes, including bacterial cells, erythrocytes and other mammalian cells, with a haemolytic activity on some types of blood agar [3, 5, 6]. Haemolysin occurs in up to 60% of clinical isolates. In the present study it was found in 25 strains (50 % of all). This toxin was only found combined with two other factors.

The very frequent presence of virulence determinants in combination [7] suggests possible synergistical activity in causes of infection.

In the past two decades the antibiotic resistance of enterococci has increased, which has created therapeutic problems [8].

Vancomycin resistance of enterococci has been reported in many countries [2, 8, 9, 12], not only in hospitals, but also in communities. Zhanel *et al.* [12] examined the susceptibility of 300 isolates of enterococci to nitrofurantoin and did not find any resistant strain, even among those that were resistant to vancomycin. In the present study we did not detect resistance to either of the antimicrobial agents mentioned.

The relationship between virulence and resistance has been examined in some studies [11, 4]. Huycke *et al.*, [10], found that the haemolytic activity of *E. faecalis* was associated with aminoglycoside resistance, 91% of gentamicin-resistant strains were haemolytic. Statistical analysis in our study did not show

any relationship between the presence of a virulent factor and resistance to antibiotics. There should be more extended investigations to reach relevant conclusions about a possible link between virulence and resistance.

### Conclusions

- Uroisolated *E. faecalis* was enriched with virulence factors, 32% of the strains possessed all the examined factors.
- Esp was the most common factor (in 76% of 50).
- All strains were susceptible to vancomycin and nitrofurantoin, resistance to ampicillin was 24%.
- No relationship was noted between virulence determinants and resistance to an antimicrobial agent.

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## Резиме

**ФАКТОРИ НА ВИРУЛЕНЦИЈА И АНТИБИОТСКА РЕЗИСТЕНЦИЈА  
КАЈ *ENTEROCOCCUS FAECALIS* ИЗОЛИРАН ОД УРИНА**

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Хемолизин, ентерококен површински протеин (*Esp*), агрегациона супстанција и желатиназа се некои од маркерите кои се спомнуваат како можни фактори на вируленција кај ентерококите. Целта на оваа студија беше да се детектира присуството на хемолизин, желатиназа и ентерококен површински протеин кај ентерококи изолирани од урина и да се одреди осетливоста кон антимикубни средства. Вкупно беа испитани 50 соеви на *Enterococcus faecalis* изолирани од урина. За изолација и идентификација на соевите како *Enterococcus spp.* беше користен хромоген UTI agar (Oxoid). Диференцијацијата на видовите беше направена со Vitek автоматизиранот систем (GPI-картици). Продукцијата на хемолизин беше фенотипски детектирана на Columbia CNA agar како зона на  $\beta$  хемолиза околу лентата со бактерии. Продукцијата на желатиназа беше одредувана како јасно хало околу колониите на триптиказа соја агар суплементиран со 1,5% обрано млеко. *Esp* беше докажуван преку детекција на *esp* генот со PCR по претходна екстракција на ДНК од бактеријата. Осетливоста кон: ampicillin, ceftriaxone, vancomycin, nitrofurantoin и ciprofloxacin беше испитувана со агар дифузиониот метод. Кај 16 соеви на *Enterococcus faecalis* (32%) беа присутни сите фактори

на вируленција. Два фактори беа најдени кај 19 (38%), а само еден фактор кај 11 соеви. Кај само 4 соеви не беше докажан ниту еден од факторите. Esp беше најчесто присутен фактор (кај 38 изолати). Сите соеви беа осетливи на vancomycin и nitrofurantoin; 12 изолати беа резистентни на ampicilin, 17 на ceftriaxone и 14 на ciprofloxacin. Било каква поврзаност помеѓу факторите на вируленција и резистенцијата кон некој антибиотик не беше најдена.

**Клучни зборови:** *Enterococcus faecalis*, фактори на вируленција, антибиотска резистенција.

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