RESEARCH ARTICLE

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Antimicrobial susceptibility and biofilm-forming ability of *Enterococcus faecalis* strains isolated from the urogenital tract of outpatients

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ABSTRACT

Enterococci are increasingly associated with infections of the urogenital tract. The two species *Enterococcus faecalis* and *Enterococcus faecium* are the third most commonly isolated pathogens in catheter-associated urinary tract infections (CAUTI). Both species are capable of producing biofilms, with *E. faecalis* being more frequently isolated. This study explores the antimicrobial resistance and biofilm formation ability of 72 *Enterococcus faecalis* strains, collected a one-year period from the urogenital tract of outpatients. The results showed that urinary tract infections (UTI), caused by enterococci are more frequent among children up to 10 years of age, while genital tract infections (GTI) are most often observed in women in reproductive age. Antimicrobial resistance was low, with higher levels for UTI compared to GTI strains. The results demonstrate 100% susceptibility to penicillins, which are the most effective agents for the treatment of infections caused by *Enterococcus faecalis*. The resistance to fluoroquinolones was less than 19 %, with clearly defined cross-resistance. Biofilm formation was established for 26% of the tested strains after 24 h of cultivation on tryptic soy broth, with OD_{630} values for the biofilms in range 0.050-0.200. This categorizes the strains as low-grade biofilm-forming strains. The susceptibility profile of strains tested in the present study and their ability to form stable biofilm confirms the constant need of obligate determination of antibiotic resistance before prescriptions by physicians.

Key words: *Enterococcus faecalis*, antimicrobial susceptibility, virulence factors, biofilm formation

Introduction

The human urogenital tract includes the urinary and reproductive systems. The two systems, although functionally different, are anatomically directly related. Urogenital infections are among the most prevalent in outpatients worldwide. On average, over 3 billion women have at least one such infection a year. The cost of their treatment exceeds $6 billion annually worldwide (Foxman, 2002).

UTI are among the most common in humans and affect up to 150 million people (Flores-Mireles et al., 2015). This group of infections is the most prevalent in hospital but it is often observed at outpatients also (Salvatore et al., 2011). Although both genders can be affected, women have been shown to be more susceptible, with a 50% chance to be affected by UTI in their life span. Approximately 25% of women who have had bacterial cystitis continue to have recurrent UTIs for up to 6 months, with high risk for secondary infections (Foxman, 2014). It has been shown that one in three women suffered from such infection for the first time by the age of 24 (Foxman, 2002). Most commonly, UTIs occur in sexually active women. Another reason why women suffer more from this type of infection is the presence of a shorter urethra, which shortens the distance the infectious agent has to travel to establish an infectious process in the bladder (Barber et al., 2016).

Members of the genus *Enterococcus* are the most commonly isolated Gram-positive species causing UTI in both females and males, accounting for about 53% of all strains (Moretti et al., 2009). The species *E. faecalis* and *E. faecium* are the third most common pathogens in CAUTIs with rates ranging between 15 and 30% (Kline & Lewis, 2016). *Enterococcus faecalis* is isolated more often than *Enterococcus faecium* (5:1) in outpatients, suffering from UTI, internal abscesses or blood infections (Goel et al., 2016).

The enterococci produce a number of virulence factors that contribute to the virulence of the species and facilitate the infection process, such as proteases and biofilm-formation. Proteases are thought to regulate bacterial lysis and extracellular DNA release, thereby promoting biofilm formation (Nešuta et al., 2017). It is a key virulent factor in a number of microorganisms. The formation of biofilms on medical devices (such as catheters or implants), as well as skin, teeth and urinary tract infections, complicates treatment, and often infections become chronic (Ong et al., 2008). The strains are more resistant to antibiotics in the bacterial biofilm than in its planktonic form. The standard antibiotic therapy eliminates mainly planktonic cells (Davey & O'Toole, 2000). Antimicrobial molecules must diffuse through the matrix of the biofilm and inactivate the cells. The extracellular polymeric compounds in the matrix serve as a barrier. Altered growth rates of microorganisms in the biofilm are also reported to be a factor that increases their resistance. Biofilm-bound cells grow significantly slower than planktonic, resulting in slower antimicrobial uptake. This point out the significance of biofilm formation and the expression of different virulence factors of *Enterococcus faecalis* for the pathogenesis. Recently, the study of biofilm formation and his role have been in great interest for many scientists (Kafil & Mobarez, 2015; Fallah et al., 2017).

The aim of this study was to evaluate antimicrobial susceptibility, hydrolytic activities and biofilm formation of *Enterococcus feacalis* strains associated with outpatients infections of urogenital tract.

Materials and Methods

Bacterial strains collection and identification

The strains were collected from urine, vaginal and cervical samples from 53 female and 18 male outpatients at SMDL „Chronolab", Plovdiv, Bulgaria for a one-year period. Culture purity was evaluated by spread plate technique on blood agar (7% blood). All strains were stored at -80°C in skim milk (Merck, Germany). Taxonomic identification to species level was done by semi-automatic system MICRONAUT (Germany). *Enterococcus faecalis* ATCC 29212 was used as a control strain for the identification.

Antibiotic susceptibility

The antimicrobial susceptibility testing was performed according to EUCAST version 6.1, 2016, by the disk diffusion method. In brief Mueller-Hinton agar plates were inoculated by swabbing with 0.5 McF colony suspension. Antibiotic disks were applied within 15 min after inoculation. Six antimicrobial agents were tested: penicillin 10 units (P), ampicillin 10µg (AMP); gentamicin 10µg (GEN); ciprofloxacin 5µg (CIP), levofloxacin 5µg (LE) and norfloxacin 10µg (NX). After disk application plates were inverted and incubated at 35±1°C for 18±2 h. The zones' edges were read at the point of complete inhibition and diameters were interpreted into susceptibility categories according to the current breakpoint tables.

Production of hydrolytic enzymes

In the current study, we analyzed the exoenzyme activities of caseinase and gelatinase of the strains. Briefly, the gelatinase assay was carried out by adding an inoculum from a pure culture into tubes containing 12% gelatin in 0.8% nutrient broth (HiMedia, India). Tubes were incubated for 24-72h at 37°C and then placed in the refrigerator for approximately 30 min. The liquefaction of gelatin was considered as a positive result (Andrea et al., 2007). Production of caseinase was determined on Todd-Hewitt agar (HiMedia, India) containing 10 g of casein (Sigma, Germany) per liter. Single colonies were streaked onto plates, grown overnight at 37°C, and examined for zones of clearance around the colonies, indicating hydrolysis (Eaton & Gasson, 2001). Caseinase activity (Pz) was expressed as a ratio between the diameter of the colony and the diameter of the precipitation zone. The Pz value was divided into four categories: 1.0 – no enzyme activity; 0.990 to 0.700 – weak producers; 0.699 to 0.400 – good producers and lower than 0.399 – excellent producers.

Biofilm formation

The test was done according to Mohamed et al. (2004) with some modifications. Ten microliters overnight bacterial cultures were inoculated in ninety microliters tryptic soy broth (NCIPD, Bulgaria) supplemented with 0.25% glucose in 96-well culture plates (Costar) and incubated for 24 h at 37°C. Plankton growth of cultures was obtained after the transfer of the samples in new plates and measured at 630 nm. Wells of the first plate were washed three times with sterile saline and stained with 0.1% crystal violet (CV) for 15 min, and finally rinsed again with sterile saline. CV was solubilized with 95% ethanol and optical density was determined at 630 nm on MULTISKAN FC microplate reader (Thermo Fisher Scientific, Shanghai, China).

Results and Discussion

Distribution of the Enterococcus faecalis strains amongst tested outpatients by age and gender

Seventy-two strains affiliated to genus *Enterococcus* were isolated from samples of outpatients with UTI and GTI from Plovdiv region, Bulgaria. Based on the results obtained by the semiautomatic identification system MICRONAUT all strains were identified as *Enterococcus faecalis.* Enterococci are opportunistic pathogens and natural inhabitants of the normal human intestinal microbiota (Duprè et al., 2003). Over the last decade, the species has proven to be one of the most widespread pathogens after *Proteus mirabilis*, showing a

disturbing tendency for rapidly increasing antibiotic resistance among clinical strains (Goel et al., 2016; Kahlmeter, 2000).

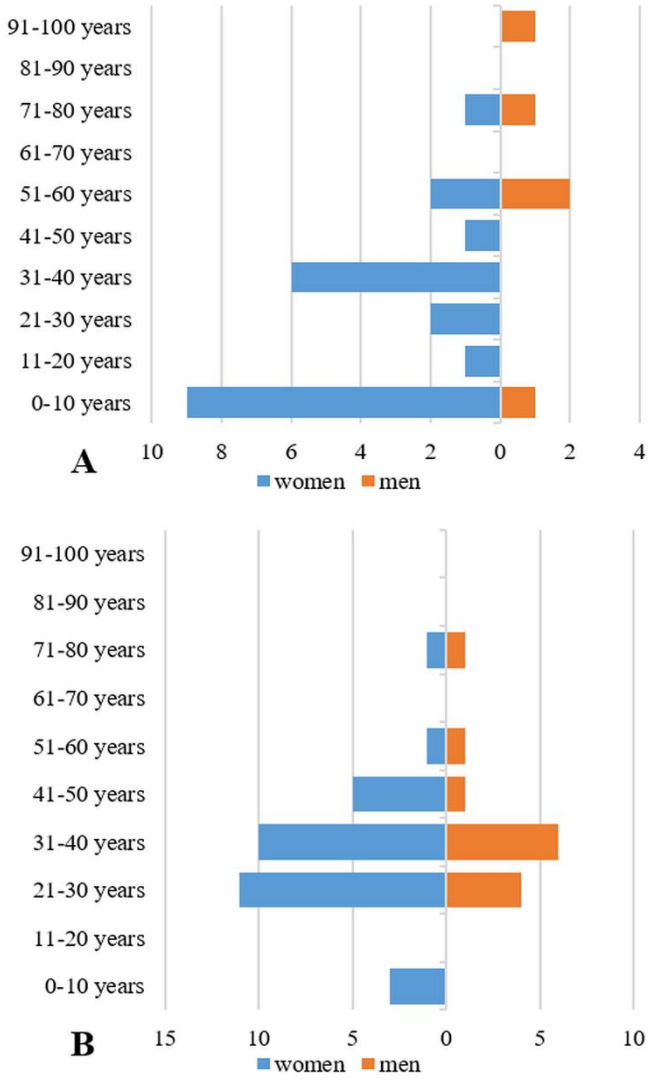

Figure 1. *Age and gender distribution of the Enterococcus faecalis strains isolated from urinary tract (A) and genital tract (B) of outpatients.*

The results showed that enterococci are most frequently isolated from female outpatients. (Figure 1A and Figure 1B). The age characteristics differ significantly between patients with infections of the urinal and genital tract. UTI are more frequent among children up to 10 years of age, while GTI are most often observed in women in reproductive age. The findings are in agreement with Sanchez et al. (2016), who reports that enterococci are the fourth most frequently isolated species from women with UTI. Seven percent of the strains were from pregnant women and in 3% of tested patients the enterococci were detected as coinfecting agents with *Candida albicans* or *Pseudomonas aeruginosa*. The data confirms the statements of other authors that enteroccocal infections in pregnant women vary between 4% and 18% (Bayó et al., 2002; Tansarli et al., 2017). A similar tendency is observed for male outpatients with GTI, while UTI infections were more frequently established in male outpatients over 50 years of age.

Antimicrobial susceptibility

Antimicrobial susceptibility was determined according to the EUCAST guideline, including three different classes of antimicrobials as follows: penicillins (penicillin and ampicillin), aminoglycosides (gentamicin) and fluoroquinolones (ciprofloxacin, norfloxacin and levofloxacin). The results demonstrate 100% susceptibility to penicillins. They have been proven to be the most effective agents for the treatment of infections caused by *Enterococcus faecalis*, while other members of the genus as *E. faecium* demonstrate higher resistance (Kristich et al., 2014). For this reason, susceptibility to penicillin is used for taxonomic differentiation of the two species. The resistance to gentamicin is nearly 30% (Figure 2). The values are close to the previously described by Dadfarma et al. (2013), obtained by screening for highly-resistant to gentamicin enterococci. To ensure an effective treatment in practice, it is usually applied a combination of penicillin with gentamicin or amikacin, but if resistance to either drug is reported, there will be no synergy between the two agents (Patterson & Zervos, 1990).

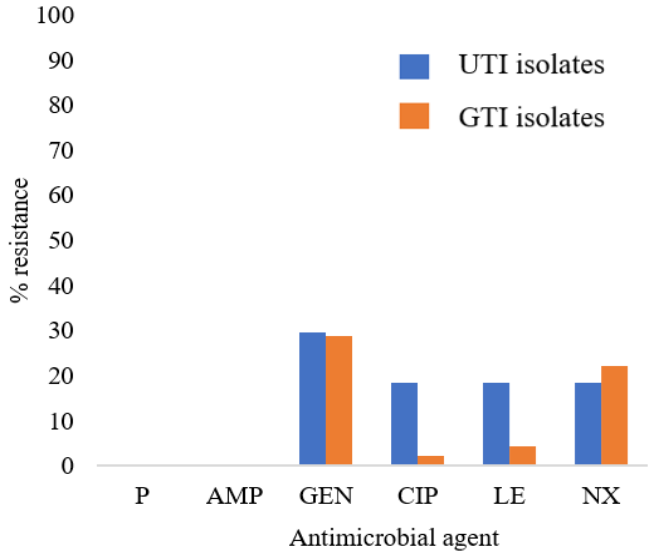

Figure 2. *Antimicrobial susceptibility of Enterococcus faecalis strains, isolated from urogenital tract of outpatients to penicillin (P); ampicillin (AMP); gentamicin (GEN); ciprofloxacin (CIP); levofloxacin (LE); norfloxacin (NX).*

We have found a relatively low resistance to fluoroquinolones – less than 19%, with clearly defined cross-resistance of *E. faecalis* strains associated with UTI. It means that if a strain is resistant to one of the fluoroquinolones, it is usually resistant to all and vice versa. Sixty percent of the strains with cross-resistance were isolated from male outpatients. The studied *E. faecalis* strains associated with GTI showed the highest resistance values against norfloxacin

(22%), which is a second-generation fluoroquinolone, and comparatively lower values for levofloxacin (third generation) and ciprofloxacin (second generation) – 4% and 2% respectively. The low level of resistance confirms the reliability of ciprofloxacin for the treatment of infections associated with *E. faecalis*, as suggested by IDSA (Infectious Disease Society of America). Similar results were described in neighbor countries (Sibel et al., 2012). However, the resistance to fluoroquinolones in last years is increasing (Yasufuku et al., 2011; Anupurba & Banerjee, 2012), with levels more than 50% in some countries like India and Korea (Lee, 2013; Chakraborty et al., 2015). Fluoroquinolone resistance has been reported predominantly after the administration of fluoroquinolones and/or with a combination of other antibiotics, as well as after recent hospitalization (Rattanaumpawan et al., 2011). Globally, levofloxacin resistance is lower than ciprofloxacin (Schouten et al., 1999; Jia et al., 2014), but we have established an opposite trend, and probably one of the reasons is the more frequent prescription of preparations containing levofloxacin rather than ciprofloxacin, applying selective pressure to pathogenic *E. faecalis*. Resistance to both aminoglycosides and fluoroquinolones was detected in 10% of the strains, isolated again from male outpatients.

Virulence factors

The primary role of gelatinases is to provide nutrients to the bacterial cell through degradation of the tissues of the host and also to participate in the initial stages of biofilm formation (Gilmore et al., 2002). Twenty-two strains E. *faecalis* produced gelatinase, representing 31% of all strains in the present study. The activity was phenotypically expressed *in vitro* by liquefaction of a culture medium containing the substrate as described by Tsikrikonis et al. (2012). A higher number of strains hydrolyzing gelatin were isolated from the urinary tract (Table 1). Caseinase activity was 2.5 times higher reaching 93% for the UTI and 67% of the GTI strains. The significant gelatinase and caseinase activity differences are due to the detection methods. A positive reaction for gelatin hydrolysis is observed upon complete liquefaction of the culture medium in the tube, while in the hydrolysis of casein, a light zone around the colonies is obtained. In order to liquefy the medium in gelatin tubes, most of the substrate has to be degraded, while even a very low enzyme activity can be detected in casein solid media (Figure 3). This makes casein hydrolysis a more reliable method. These virulence characteristics are most common in clinical strains of *E. faecalis* (Semedo et al., 2003). Kanemitsu et al. (2001) report even higher activity in clinical strains from hospitalized patients – with gelatinase being demonstrated in 45%, with a peak counting about 10 hours from the start of the reaction.

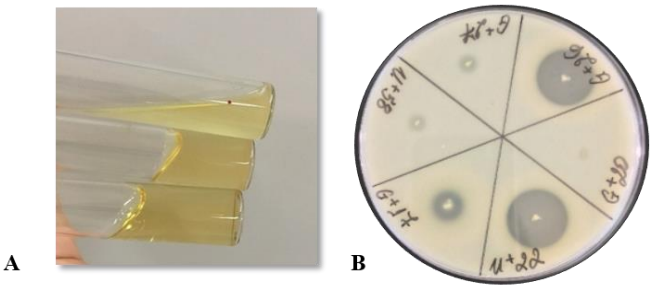

Figure 3. *Gelatin (A) and casein (B) hydrolysis by strains Enterococcus faecalis.*

Biofilm formation

After the initial screening in polystyrene tubes, biofilm formation was observed for eighteen of all 72 tested strains (26%) with varying degrees of intensity. Over 70% of biofilm-forming *E. faecalis* strains were from the GTI, with only two from male outpatients. Higher turbidity of the broth cultures was visualized near the bottom of the tube, where biofilm formation was subsequently observed. Representatives of the *Enterococcus faecalis* are facultatively anaerobic and can survive in an oxygen-free environment. The reduced amount of oxygen decreases the intensity of the metabolic processes in the cells involved in the microbial community, as well as the rate of growth and division. This makes these cells less sensitive to antibiotics that normally affect active cells (Rabin et al., 2015). Enterococci are opportunistic pathogens and natural inhabitants of the normal human intestinal microbiota. A number of studies have shown that *E. faecalis* is involved in biofilm formation significantly more often than *E. faecium* (Baldassarri et al., 2001; Duprè et al., 2003). According to Shridhar & Dhanashree (2019), biofilm-forming *E. faecalis* strains from urogenital infections are relatively less – about 22%, which correlates with our results.

Table 1. *Gelatinase and caseinase enzyme activity of Enterococcus faecalis strains, isolated from urogenital tract of outpatient*

	Urinary tract (n = 27)	**Genital tract (n = 45)**	**Total number (n = 72)**
Gelatinase activity	37%	27%	31%
Caseinase activity	93%	67%	76%
Pz = 1.00	7%	33%	24%
Pz < 0.999-0.700	30%	11%	18%
Pz < 0.699-0.400	19%	40%	32%
Pz < 0.399	44%	16%	26%

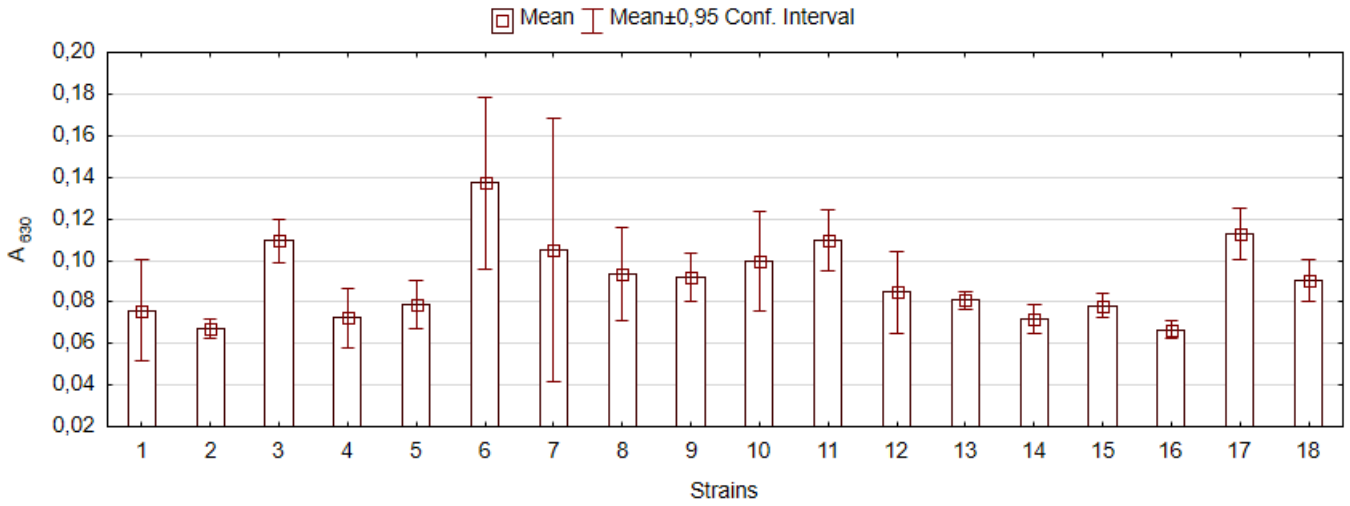

Figure 4. *Biofilm formation after 24 h incubation at 37°C of Enterococcus faecalis strains in 96-wells microtiter plates, represented as mean absorbance values at 630 nm ± 95% confidence intervals.*

The ability of *E. faecalis* strains to form a biofilm was quantitatively tested by cultivation for 24 h at 37°C in sterile 96-wells microtiter plates on tryptic soy broth supplemented with 0.25% glucose (Figure 4). The results were used to classify the strain's ability to form biofilm according to the classification proposed by Seno et al. (2015). Values for OD_{630} were in range 0.05-0.200, categorizing them as low-grade biofilm-forming strains. This group covers the second largest number of *Enterococcus faecalis* strains associated with UTI (Seno et al., 2015). The groups with medium and low-grade biofilm-forming abilities differ in terms of metabolic activity. Proteins associated with secondary metabolites, co-factor synthesis, and tetrahydrofolate synthesis are reduced, while the activity of shikimate kinase, as well as enzymes involved in the transport of sulfate ions, are increased in strict biofilm-forming strains. In the low biofilm-forming isolates, proteins associated with the synthesis of nucleotides and nucleosides are positively affected, whereas those associated with the transport of sugars and sulfate are suppressed after biofilm formation (Suriyanarayanan et al., 2018).

Conclusions

The isolation, identification, and antibiotic susceptibility testing of *Enterococcus* spp. from the samples of outpatients with UTI and GTI in the present study showed complete sensitivity to penicillins and resistance to fluoroquinolones of less than 19 %, with clearly defined cross-resistance. Such data will be helpful to predict patterns of antimicrobial susceptibility and to avoid empirical antibiotic prescription. The positive hydrolase activity demonstrated in a large percentage of *E. faecalis* strains testifies to its importance as a virulent factor in the colonization of the urogenital tract. Further, the present study emphasized the importance of testing the biofilm forming ability of the strains, because neglecting such properties may lead to compromised therapeutic choices.

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