RESEARCH ARTICLE

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Antibiotic and serum resistance of *Enterobacteriaceae* strains isolated from outpatients with infections of urogenital tract

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ABSTRACT

The present study is focused on the antibiotic and serum resistance of *Enterobacteriaceae* strains associated with community infections of the urogenital tract. During a one-year period from April 2016 to March 2017 total of 318 *Enterobacteriaceae* strains were collected from urine samples, vaginal and urethral secrets and ejaculates of outpatients with urinary and genital tract infections at IMDL "Chronolab" - Plovdiv, Bulgaria. The most common etiological agent was *Escherichia coli* (64.8%), followed by *Klebsiella spp*. (17%) and *Proteus mirabilis* (10.37%). The antibiotic susceptibility tests showed high resistance against ampicillin (49%), mecillinam (71%), doxycycline (41%) and high susceptibility to cephalosporins (cefuroxime 84.6%; cefoxitin 83.7%; cefotaxime 91.5%; cefepime 87.7%) and fluoroquinolones (ciprofloxacin 85%, norfloxacin 79%, levofloxacin 83%;). Significant resistance was established to nitrofurantoin (24%). Amongst the tested strains, 8.5% produced extended spectrum beta-lactamases (ESBLs). We tested serum susceptibility of strains and found that 84% of strains were resistant to the bactericidal activity of the normal human serum. Our data suggest that most likely the resistance to complement is one of the mandatory virulence factors for the majority of the *Enterobacteriaceae* strains associated with urogenital infections. The susceptibility profile of the tested *Enterobacteriaceae* strains confirms the need for constant data update on the antibiotic resistance and patterns of virulence of etiological agents of the urogenital infections.

Key words: *Enterobacteriaceae*, UTI, antibiotic resistance, serum resistance

Introduction

The antibiotic resistance of medically significant microorganisms acquires catastrophic proportions globally in the 21st century. A number of reviews describe a frightening picture of a growing number of pathogenic microorganisms resistant to three or more classes of antibiotics (Gould, 2009; Davies & Davies, 2010; Spellberg et al., 2013; Spizek & Havlicek, 2015). The increasing numbers and variety of emerging broad-spectrum beta-lactamases among Gram-negative bacteria are causing no less serious concern (Rawat & Nair, 2010; Lukac et al., 2015; Ruppé et al., 2015).

Urinary tract infections (UTI) are one of the most frequent human infectious diseases affecting millions of people worldwide. It is estimated that 40-50% of the women and 5% of the men will develop a UTI in their lifetime (Ulett et al., 2013). The disturbance of the balance in normal microbiota after an antibiotic intake, catheterization, pregnancy, low level of estrogen, immunosuppression can cause undesirable infection (Grabe et al., 2008). Between one-quarter and one-half of all women, experience at least one urinary tract infection (UTI) in their lifetime. Recurrent infections are observed in 25-30% of women with initial infection. Bacterial vaginosis (BV) is the most common cause of vaginitis worldwide. It has been associated with a risk of preterm birth and is clearly a risk factor for the acquisition and perhaps transmission of sexually transmitted diseases (Eschenbach, 1993). BV is influenced by multiple factors. Lower estrogen levels influence the number and diversity of vaginal lactobacilli and raise the risk for urogenital infections in women (Mendling, 2016).

One of the host's first line defense against invading microorganisms in the urogenital tract includes the bactericidal effect of the serum, which is mediated primarily by complement proteins. A number of studies have demonstrated the involvement of many factors in providing bacterial resistance to complement (Lambris et al., 2008). These factors form different patterns of resistance in the strains tested. Some of the most investigated mechanisms include the presence of protective lipopolysaccharides and an

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extracellular polysaccharide capsule. Lipopolysaccharides (LPS) have been implicated as a major factor in the ability of Gram-negative bacteria to resist the serum bactericidal activity. Merino et al., (1992) suggested some possibilities for the complement resistance of *Klebsiella pneumoniae*. They report that the capsular polysaccharide may shield the LPS and it makes the cell surface not suitable for complement-fixing. On the other hand, the capsule and lipopolysaccharides may be exposed together to the surface, but this results in complement-fixing far from the cell surface without the possibility for bacterial lysis. (Merino et al., 1992; Doorduijn et al., 2016).

Recent reviews (Miajlovic & Smith, 2014) have shown that the major etiological agent in urinary infections *E. coli* often combines in the serum resistance mechanisms the production of protective extracellular polysaccharide capsules and the expression of factors that inhibit or interfere with the complement cascade. Phan et al., (2013) confirmed the role of 46 genes in serum resistome. The majority of them encode membrane proteins or factors involved in lipopolysaccharide (LPS) biosynthesis (Phan et. al., 2013). According to Lüthje & Brauner (2014), pathogenic strains have evolved mechanisms to resist attacks from phagocytes and complement system, which allows them to cause more invasive infections than sensitive strains. Complement resistance is not conventionally tested in clinical practice. One of the reasons is that serum resistance tests are labor-intensive and time-consuming (Taylor, 1984; Verduin et al., 1995). The culture-and-spot assay proposed by Verduin et al. (1995) is an easy and inexpensive procedure that allows highlighting one of the features involved in the success of the pathogens in the urogenital tract.

Materials and Methods

Bacterial strains

The strains included in the current study (N=318) were collected for the period April 2016 – March 2017 from samples of urine, vaginal and urethral secrets and ejaculates of outpatients with different urogenital infections at IMDL "Chronolab" Plovdiv, Bulgaria. They are distributed as follows: 227 strains from urine samples – 182 from women and 45 from men with symptomatic and asymptomatic urinary tract infections (UTI), 91 strains from vaginal, cervical secrets, and ejaculates. The identification was based on conventional methods according to the BBL Enterotube II test system (BD) for identification of species of the *Enterobacteriaceae* family.

Antibiotic susceptibility testing

Antimicrobial susceptibility testing was performed by the disk diffusion method following the EUCAST Guidelines (Matuschek et al., 2014). Sixteen antimicrobials from seven antibiotic classes (HiMedia, India) were chosen for the *Enterobacteriaceae* strains: ampicillin 10µg (AMP), mecillinam 10µg (MEC), amoxicillin-clavulanic acid 30µg (AMC), ampicillin-sulbactam 20µg (A/S), cefuroxime 30µg (CXM), cefotaxime 30µg (CTX), cefoxitin 30µg (CX), cefepime 30µg (CPM), amikacin 30µg (AK), gentamicin 10µg (GEN), doxycycline 30µg (DO), ciprofloxacin 5µg (CIP), norfloxacin 10µg (NX), levofloxacin 5µg (LE), nitrofurantoin 300µg (NIT), trimethoprim/sulfamethoxazole 1.25/23.75µg (COT). Strains were defined as susceptible or resistant according to EUCAST version 7.1, (2017). *Escherichia coli* ATCC 25922 was used as a control in each test. The double disk synergy test was performed to detect extended-spectrum β-lactamase (ESBL)-producing strains.

Serum resistance

Serum resistance was tested by the culture-and-spot test as described by Verduin et al. (1995). Veronal-buffered saline (5 mM barbital, 145 mM NaCl, pH 7.4), containing 0.15 mM Ca^{2+} and 0.5 mM Mg^{2+} (VBS^{2+}) was used for serum resistance assay. The normal human serum (NHS) was pooled from five healthy volunteers at IMDL „Chronolab". The serum was diluted to 50% with VBS^{2+} prior to analysis. The strains were cultured in Mueller-Hinton broth (HiMedia, India) for 24 h at 37°C. The cells were pelleted by centrifugation at 5 000 rpm for 10 min and resuspended in VBS^{2+} to 1-2.10^8 cfu/ml (OD_{600} 0.5 McF). Samples of 100 µl from the suspensions were spread-plated on Mueller-Hinton agar. When the agar has completely absorbed the sample, 50 µl of 50% NHS in VBS^{2+} was added dropwise in duplicate. As a negative control, 50 µl of 50% human inactivated serum (HIS) (inactivated at 56°C for 30 min) in VBS^{2+} was added dropwise too on every plate. Immediately after the serum was added, the plates were cultured at 37°C. Colonies were counted in the serum droplet zones after 24 hours. The tested strains were distributed in three groups: 1. sensitive – with 0 to 50 colonies in NHS droplet zones; 2. intermediate sensitivity - intermediate growth in NHS droplet zones; 3. resistant - normal growth in NHS droplet zones.

Results

Our data on the distribution of the studied samples of outpatients by gender and age showed the characteristic picture of predominantly afflicted women and infants and elders from both genders (Figure 1). The 83% of *Enterobacteriaceae* strains in our study were collected from female samples while the others 17 % were from male samples. Our results show that the kids under 10 years old (17.6% - girls 11% and boys 6.6% respectively) and the adults after 61 years (32.6%) are the groups with the highest risk for infection of urinal tract. Most of the positive urine samples from women are from patients over 61 years of age

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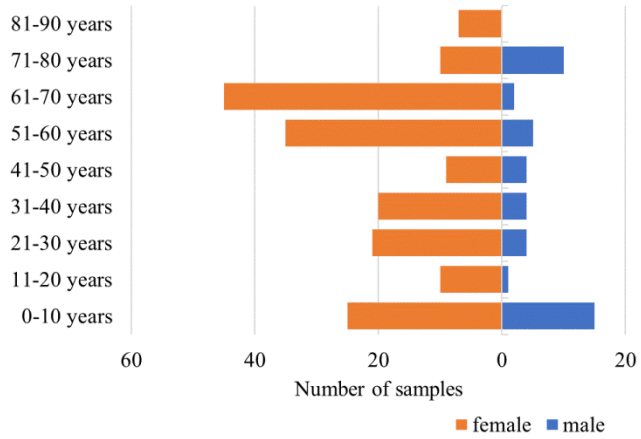

Figure 1. *Demographic distribution according to age and gender of patients with urinary tract infection (UTI).*

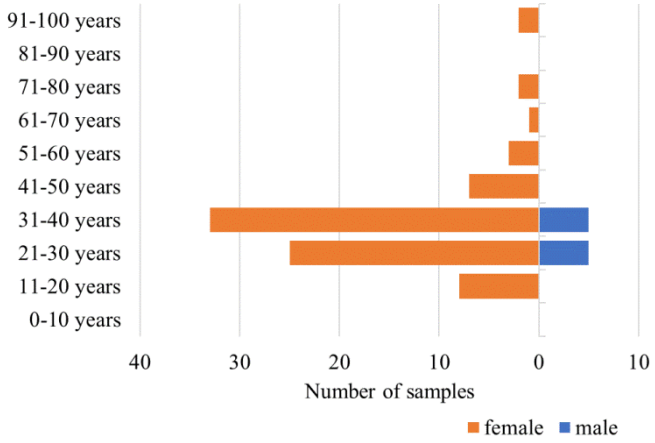

Figure 2. *Demographic distribution according to age and gender of patients with infection of genital system.*

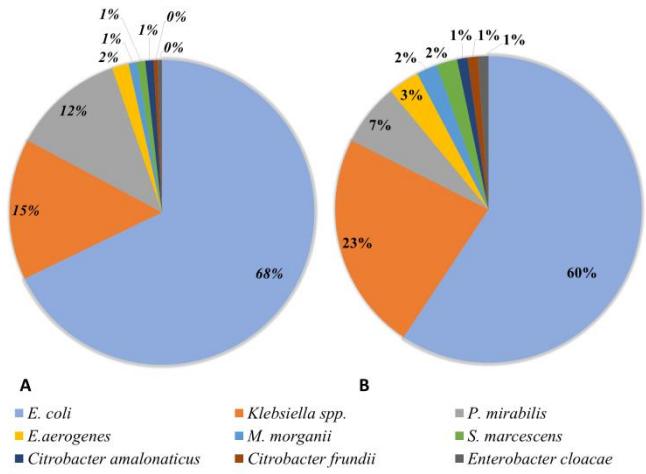

Figure 3. *Taxonomic distribution of the urogenital isolates. A – UTI, B – genital tract.*

(24.7%) followed by samples from girls under 10 years old (13.7%). The isolates from men` urine samples showed age distribution predominantly between children under 10 years (33.3%) and adults of over 70 years (22.2%) (Figure 1).

Our data of the occurrence of bacterial infections of the genital tract showed a strong correlation with sexual active lifetime period for both women and men. Approximately 71.6% of tested female samples were from outpatients between 21 to 40 years old, 9% of them being pregnant. In comparison, all of the male positive samples were from outpatients of the same age groups (Figure 2).

The identification of the collected strains showed that *Escherichia coli* is the most common etiological agent from the tested outpatients` urogenital infections, followed by *Klebsiella spp*. and *Proteus mirabilis*. The other etiological agents like *Enterobacter spp*., *Citrobacter spp*., *Serratia spp*. and *Morganela morganii* caused less than 5% of the bacterial urogenital infections (Figure 3).

The results of the antibiotic susceptibility tests of the collected *Enterobacteriaceae* strains showed increased resistance to some of the tested antimicrobials (Figure 4 and Figure 5). The highest resistance was established for penicillins like ampicillin – 49% and mecillinam – 71.4% (for UTI strains). We`ve also registered a high resistance to tetracyclines – 43.6%, trimethoprim/sulfamethoxazole – 22.5%, and fluoroquinolones: ciprofloxacin – 15%; norfloxacin – 21%; levofloxacin – 17%. We have established significant resistance of up to 24% for tested strains to nitrofurantoin, which is one of the first-line drugs for lower UTI. We found that 27 strains (8.5%) produced ESBLs – 22 isolates from urine and 5 strains from genital samples.

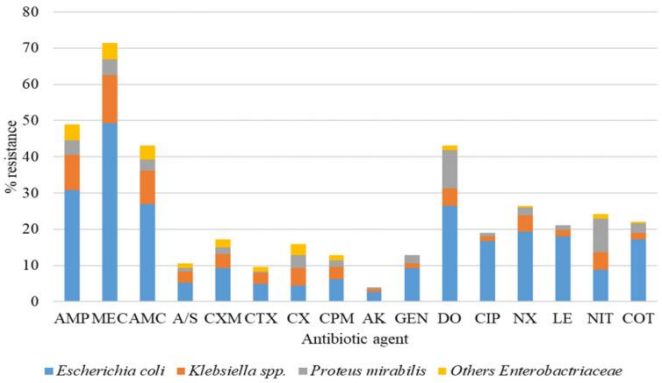

Figure 4. *Antimicrobial resistance of strains isolated from outpatients with UTI. AMP – ampicillin, MEC – mecillinam, AMC – amoxicillin-clavulanic acid, A/S – ampicillin-sulbactam, CXM – cefuroxime, CTX – cefotaxime, CX – cefoxitin, CPM – cefepime, AK – amikacin, GEN – gentamicin; DO – doxycycline, CIP – ciprofloxacin, NX – norfloxacin, LE – levofloxacin, NIT – nitrofurantoin, COT – trimethoprim/sulfomethoxazole.*

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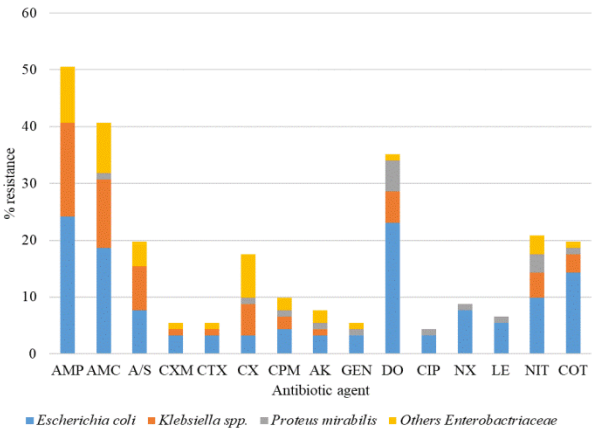

Figure 5. *Antimicrobial resistance of strains isolated from outpatients with genital tract infection. AMP – ampicillin, AMC – amoxicillin-clavulanic acid, A/S – ampicillin-sulbactam, CXM – cefuroxime, CTX – cefotaxime, CX – cefoxitin, CPM – cefepime, AK – amikacin, GEN – gentamicin; DO – doxycycline, CIP – ciprofloxacin, NX – norfloxacin, LE – levofloxacin, NIT – nitrofurantoin, COT – trimethoprim/sulfomethoxazole.*

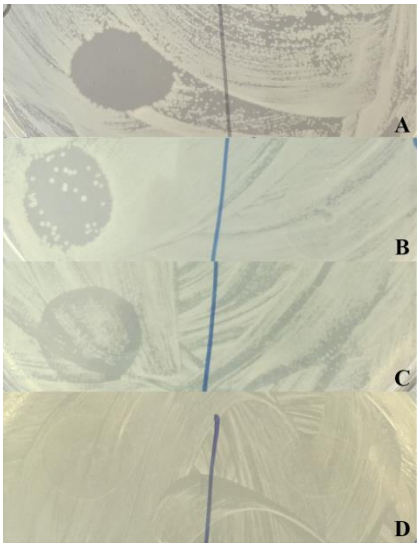

Figure 6. *Culture-and-spot test for serum resistance. Strains were confirmed as complement sensitive [A, B], intermediate sensitive [C], resistant [D] according to growth in NHS droplet zones (left) and control HIS droplet zones (right).*

We have used the pattern of growth in presence of 50% NHS as a measurement for the strains` resistance to complement lysis with a convenient and easy-to-do procedure (Figure 6).

Our results showed a significantly high frequency of serum-resistance strains (84%) – 80.8% of the urine isolates and 92.1% of the genital tract isolates respectively (Figure 7). Intermediate-resistance was observed only for 13 of all strains (4.1%).

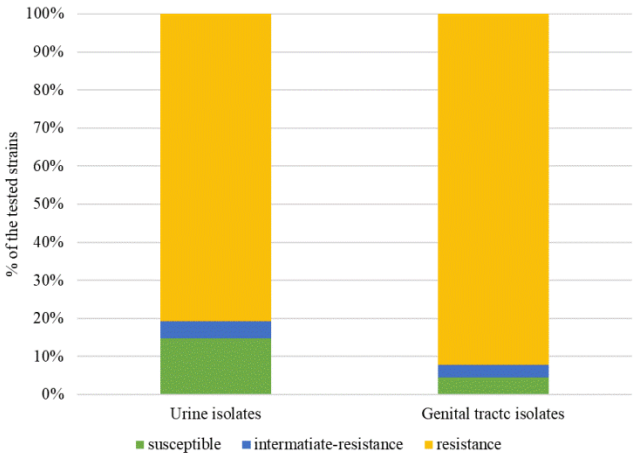

Figure 7. *Distribution of serum resistance among tested Enterobacteriaceae strains associated with urogenital infections.*

Discussion

Gender and age are one of the potential risks for urogenital infection (Eschenbach et al., 1988; Foxman, 2002). For the tested period, 83% of the affected outpatients were women – 70% with UTI and 30% with infections of the genital tract. The overwhelming part of tested *Enterobacteriaceae* strains was isolated from female samples – 81% from urine and 89 % of genital samples (Figures 1 and 2). The genital tract of women is much more susceptible to infections compared to men. It is normal for women who are sexually active to experience occasional UTI, unlike men where the urethra is much longer and the distance between the anus and urethral meatus is greater than in woman. In elder men, the increased prostate, which leads to incomplete bladder emptying, occur to be the main factor for UTI (Harper & Fowlis, 2007). Bacterial vaginosis (BV) is mainly associated at sexual active women with a new sex partner, multiple male or/and female partners, lack of condom use and replacement of hydrogen peroxide-producing *Lactobacillus* spp. Women who were not sexually active or were with decreasing sexual activity are less affected (Fethers et al., 2009).

Our findings were in accordance with previous studies suggesting that the boys are more susceptible in the first year of life, and mostly in the first 3 months, thereafter the incidence of UTI is subsequently higher in girls (Larcombe, 2004; Grabe et al., 2008). The use of diapers and personal hygiene often explains the development of UTI (Fahimzad et al., 2010). Neonatal epidemiologic studies have shown that

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the prevalence of UTI in term infants is nearly 1% with a male to female ratio between 2:1 and 6:1. After the age of 3 months, the prevalence of UTI in febrile children falls to around 8% in females and 2% in male children (Jones & Kausman, 2018). UTI among young man is less than 5-8 episodes per 10,000 men per year (Stamm & Norrby, 2001). Older age, recurrent UTI, immunosuppression, recent hospitalization, and antibiotic therapy are the main risk factors associated with community-acquired UTI (Chervet et al., 2018).

The current study demonstrates the dominant position of *Escherichia coli* as a major etiological agent related to the UTI, followed by *Klebsiella* spp. and *Proteus* spp. It shows no significant changes compared to the etiological structure of microbial species associated with urogenital tract infections recorded in recent years. The international study of Schito et al. (2009) also suggests that there is no significant differences in the set of species causing uncomplicated UTI through the years with the dominant involvement of *E.coli* in approximate from 60 to 80% of cases (Schito et al., 2009). Data from ECO-SENS confirm that role for *E. coli* as a major pathogen in women with acute, uncomplicated UTI for all phases of the project (Kahlmeter, 2000; Kahlmeter, 2003; Kahlmeter & Poulsen, 2012).

The accumulation of data on the changes of the antibiotic resistance of Gram-negative bacteria, both locally and globally, is of great benefit in drawing up recent and adequate programs for the monitoring and treatment of the urogenital infections. Andersson and Hughes (2010) had discussed the possibilities of reversing the process of developing resistance on the basis of a reduction in selective pressure and prediction and assessment of the fitness cost for the bacteria. Our data about the antimicrobial resistance profile of tested *Enterobacteriaceae* strains reviled some changes in resistance to first-line drugs for treatments of bacterial UTI and infections of genital tract.

Data from monitoring report in 2011 as part of a Resistance surveillance program for selected 21 European nations including Bulgaria, had reviled tetracycline resistance of 43.5% and 27.3% for *E. coli* and *Klebsiella* spp, respectively (Jones et al., 2014). Our study showed similar levels of resistance to tetracyclines. The presence of tetracycline-resistant pathogens limits the use of these agents in the disease treatment.

Our research confirms the increased resistance to trimethoprim/sulfamethoxazole (22%) of the tested urogenital isolates. In many countries, this agent is a first-line choice for management of UTI but the use of short-course of treatment (3 days) make pathogens unsusceptible to this drug (Brown et al., 2002). The results are in agreement with an international survey on the antimicrobial resistance of pathogens associated with uncomplicated UTI enrolled in nine European countries and Brazil for the four-year period from 2003 to 2006 (Schito et al., 2009). Other studies report an even higher level of resistance. Linhares et al. (2013) conducted a ten-year surveillance study from 2000 to 2009 in Aveiro, Portugal which reviled that trimethoprim/sulfamethoxazole is unsuitable to treat UTI infections as the resistance of 25% was higher than the recommended less than 20% value. Kahlmeter and Poulsen (2012) determined the antimicrobial susceptibility of pathogens causing community-acquired uncomplicated UTI, and report an increased resistance to trimethoprim/sulfamethoxazole during eight-year period between the multinational ECO-SENS I (1999-2000) and ECO-SENS II (2007-2008) study.

Our research showed that 13.2% of the isolated strains were resistant to the tested fluoroquinolones. They are potent, broad-spectrum antibiotics that had been used in medical practice for the treatment of severe or resistant infections since the late 1980s. Redgrave et al. (2014) noticed a correlation between fluoroquinolone resistance in *E. coli* and the consumption of medicine in Greece, France, and Sweden. They reached the conclusion that greater consumption leads to a more frequent occurrence of resistance by the pathogen (Redgrave et al., 2014). Resistance rates to ciprofloxacin exceed 10% in Italy, Spain, and Russia for the period from 2003 to 2006 (Schito et al., 2009). Linhares et al. (2013) report that the resistance of the main bacteria implicated in UTI, *E. coli*, to the fluoroquinolone ciprofloxacin – 13.9% was higher in Portugal than that observed in other European countries. The level of resistance described in the current study demonstrates the mandatory need for a revision of the local approach for use of these antibiotics in the treatment of urogenital infections.

We have detected a high resistance to nitrofurantoin – for 24% of the tested strains, most likely associated with frequent prescription and increased consumption of this antibiotic. It is a significant change compared to our previous studies in 2009 when we registered a very low resistance to nitrofurantoin – 3.6% of *E. coli* strains, associated with uncomplicated UTI (Marhova et al., 2009). Nitrofurantoin is an antibiotic used to treat lower urinary tract infections and is available for oral administration. Nitrofurantoin appears to have clinical efficacy equivalent to that of trimethoprim/sulfamethoxazole, ciprofloxacin, and amoxicillin. When given short term for lower UTI, nitrofurantoin has good clinical and microbiological efficacy (Huttner et al., 2015). It has been used to treat asymptomatic bacteria in pregnancy because it does not cross the placenta (Cunha, 2006).

Mecillinam is used only for treating urinary tract infections and worldwide is very effective medicine – there was observed more than >90% susceptibility to it in strains studied in Germany, Hungary, Poland and The Netherland (Schito et al., 2009). Our studies showed a disturbingly high

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resistance (71%) to this drug amongst urinary isolates, which renders mecillinam unsuitable for the treatment of UTI. Presumably, the selective pressure of the use and overuse of new antibiotics in the treatment of patients had selected new variants of β-lactamase (Bradford, 2001).

We have established a comparatively low percentage of ESBLs producers amongst the strains we tested – 8%. Even lower ESBLs producers' percentage of 4.2% is described for October 2014 to March 2015 in Paris (Chervet et al., 2018). These data differ from the report of Mazzariol et al. (2017) for a high percentage of isolated ESBLs strains in Bulgaria for 2014: for *E. coli* 40.4% and for *Klebsiella pneumoniae* 74.8%. Jones et al. (2014) reported similar data for high ESBLs rates from surveillance monitoring of antimicrobial resistance for 2011 of 21 European countries including Bulgaria. Bulgaria participated in this study with 100 strains isolated from various infections. Our results revealed significant variability in levels and type of resistance among uropathogens in time and proved the constant need for monitoring and adaptation of the therapeutic approaches.

Multiple drug resistance (MDR) is defined as acquired resistance to at least one agent out of three or more different antimicrobial categories (Magiorakos et al., 2011). In our study, we have established that 81 (35.7%) of *Enterobacteriaceae* isolates from the urine and 30 (33%) of strains from genital samples were with MDR phenotype. The most common antimicrobial resistance pattern in our study was simultaneous resistance to penicillins, tetracyclines, and fluoroquinolones, observed for 60 (54.1%) of the MDR strains, followed by penicillins, tetracyclines and trimethoprim/sulfamethoxazole observed for 43 MDR strains (39%). Kahlmeter and Poulsen (2012) consider that there had to be a pool of such MDR strains *E. coli* in the community. The research of genetics determinant and mobile genetic elements in these strains would contribute to elucidating the components of multidrug resistance.

Cross-resistance is defined as resistance to two or more antimicrobial agents within the same class of antibiotics and is considered to be due to a common genetic mechanism. In the current study, we observed cross-resistance of the tested strains to penicillins – 33.7%, cephalosporins – 10.5%, fluoroquinolones – 13.2% and aminoglycosides – 1.1%.

We have established extremely high serum resistance of over 84% of the tested strains. Our results are in agreement with other authors studying the complement-mediating killing (Raksha et al., 2003). Recent data highlighted the antimicrobial defenses in urinary epithelium and the importance of the innate immune mechanisms that prevent pathogenic strains from gaining success on the invasion of the urinary tract (Zasloff, 2007). According to Merino et al. (1992), the bacterial resistance to complement-mediated killing may be due to either one of two main factors: a complete or nearly complete inability to activate complement or failure of activated complement to exert its effect. It has been previously reported that uropathogenic *E. coli* are capable to utilize complement components secreted by the urothelium and facilitate their own invasion of the urinary tract. These data suggest that complement activation may be disadvantageous in these bacterial infections (Schwab et al., 2017). Bacterial survival in the urinary tract is promoted by expression of cell surface capsules, which enable them to resist the bactericidal actions of complement and phagocytic cells (Lüthje & Brauner, 2014; Abraham & Miao, 2015). This could explain the extremely high serum resistance of the tested *Klebsiella spp*. The study of the complement resistance in the clinical practice would further expand the set of targets for the development of therapeutic and prophylactic strategies to limit infections of the urogenital tract.

The current study reviles the dynamics' in the antimicrobial resistance pattern of *Enterobacteriaceae* strains associated with outpatients' infections of the urogenital tract. The often undue empirical prescription of medications to treat uncomplicated infections is considered one of the reasons for the significantly increasing resistance of the most frequently isolated *Enterobacteriaceae* species causing UTI. There is an urgent need for monitoring the resistance of pathogens associated with the infection of urogenital tract and continual revision of the list of first-line drugs for treatment. Future studies should include an analysis of the genetic determinants associated with the virulence factors and their correlation with the established levels of resistance. This will help to update the trends and strategies of bacterial colonization and invasion of the urogenital tract.

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