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Distribution of virulence determinants and biofilm-forming among clinical urinary isolates

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ABSTRACT

Urinary tract infections are serious health and economic burden for society. Selected bacterial species as *Escherichia coli, Klebsiella spp., Proteus spp.* are often isolated from patience's urine samples. We investigated twenty strains *Escherichia coli*, isolated from urine of 14 patients with different urinary infections, 4 patients with asymptomatic bacteriuria and 2 pregnant women. Distribution of virulence determinants as adhesins, motility, hemolysins, serum resistance and biofilm-forming were investigated phenotypically and with multiplex PCR in correlation. We found that strains poses different combinations of virulence capabilities and the structure statistically most often found is the type 1 pili.

Key words: virulence determinants, uropathogens, *Escherichia coli*, biofilms

Introduction

Bacterial infections of urinary tract (UTI) are the most frequently diagnosed infection among humans. Annually the number of UTI infection reaches 130-175 million people worldwide (Anderson et al., 2004). Most likely, the actual number is higher because in many cases infection is without symptoms. Women are significantly more likely to experience UTI than men. Almost half of all women will experience 1 UTI during their lifetime (Foxman, 2002). UTIs may be symptomatic (e.g. pyelonephritis and cystitis) and asymptomatic.

The most common etiological agent *Escherichia coli* are responsible for about 70-95% of the cases. *Staphylococcus saprophyticus, Proteus mirabilis, Klebsiella spp.* and other species of family *Enterobacteriaceae* may also be a reason for UTI but they are isolated less frequently. On the other side they are often associated with complicated nosocomial infections in immunocompromised patients, elderly people and patients with catheter (Wright & Hultgren, 2006). In some cases, a single group of uropathogenic *Escherichia coli* (UPEC) emerge in community also via contaminated food, water or other supplies (Todorov et al., 2012) Additionally, isolated from sexually active patients UPEC strains show similarities with fecal isolates from their partners after analysis, suggesting the possibility UTI can be sexually transmitted (Wiles et al., 2008).

For colonization of urinary tract the bacterial cells should have some virulence factors (VFs) – motility, adhesion, toxins, formation of biofilm, which is a direct reflection of their complex relationship with macroorganism and environmental conditions. Motility is a property, associated with the presence of certain structures called flagella on the cell surface. Other structures - fimbriae provide the attachment to certain suitable places in the host and the most common are type 1 and type P. Type 1 pili are heteropolymeric fimbriae produced by nearly all members of the *Enterobacteriaceae* family. Type 1 pili bear the mannose-binding FimH adhesin, which is serologically conserved throughout the *Enterobacteriaceae* (Jones et al., 1995). P fimbriae bind globoseries glycosphingolipids. Receptors for P fimbriae are present on erythrocytes from humans, pigs, pigeons, fowl, goats, and dogs but not on those from cows, guinea pigs, or horses. The abundance of receptors for P fimbriae in human renal tissue, the importance of P fimbriae in upper urinary tract colonization in mice, and the association of P fimbriae with acute pyelonephritis in humans suggest that P fimbriae are required for colonization and invasion of the human upper urinary tract (Johnson, 1991).

The ability to form surface associated, structured and cooperative consortia, called biofilms, is one of the most remarkable and ubiquitous characteristics of bacteria

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(Costerton et al., 1987). Immobilized consortium of bacteria provides increased antibiotic resistance and offers fine adaptive response to many factors that would otherwise be adversely affected bacterial activity and viability. The resistance of biofilm-forming microorganisms is extremely actual with relation to growing amount of isolated biofilm-forming strains with a multiple antibiotic resistance. Globally, there are very negative consequences such as increased morbidity and mortality and difficult control of socially significant diseases.

We isolated and studied some virulence characteristics of 20 strains *Escherichia coli* from urine of patients with cystitis (10), pyelonephritis (4), asymptomatic bacteriuria (4) and urine of two pregnant women. Virulence determinants and characteristics which presumable participate in initial stage and establishment of infections: motility, adhesion, biofilm-forming, hemolysins, were investigated phonotypical and with multiplex colony PCR to obtain data about their correlative distribution.

Materials and Methods

Bacterial strains and media

Bacterial isolates were collected from urine samples at medical diagnostic laboratory "Hronolab" (Plovdiv). In the present study were analyzed twenty strains *Escherichia coli*, isolated from urine of patients with different urinary infections: 10 with cystitis, 4 with pyelonephritis, 4 with asymptomatic bacteriuria and 2 pregnant women. Strains were collected in case of significant bacteriuria ($>10^5$CFU/ml) from microbiological tests for analysis of urine. Standard biochemical identification was done according to BBL Enterotube II test system (BD) for identification of species in *Enterobacteriaceae*. *Escherichia coli* ATCC 25922 was used as control in all tests. Strains were maintained on LB agar at 4°C for month. Material from each strain was stored in vials with trypticase soy broth (TSB) and 20 % glycerin at - 20°C.

Motility assay

Motility of different types was tested: swimming motility was tested on PPLO broth (Difco) with 0.3% agarose; swarming motility was analyzed on PPLO broth with 0.5% glucose and 0.5% agar. For twitching motility strains were deep inoculated into LB broth with 1% agar. Petri dishes were cultivated 24 hours and analyzed for typical zones of motility.

Agglutination of yeast cells

Mannose – sensitive and mannose – resistant agglutination of yeast cells was used for detection of expression of type 1 pili and other adhesins (Eshdat et al., 1981). Agglutination assay was conducted on 96 well polystyrene plates and in parallel with slide technique. Yeast cell suspension in PBS (10^9 cell/ml) was mixed in a 1:1 ratio with bacterial cells, washed and resuspended in PBS (10^9 cell/ml) with equal density in presence or absence of 1% mannose (Müller et al., 2009). Probes were examined at every 5 min for half an hour. Cases with positive agglutination in presence of mannose were referred as mannose-resistant yeast agglutination (MRYA). Absence of agglutination in presence of mannose was referred as mannose – sensitive yeast agglutination (MSYA).

Hemolysin assay

Isolates were inoculated with strike technique and cultured onto 5% blood agar plates (BB – NCIPD Ltd.) 24-48 h at 37°C for detection of hemolysins.

Assay of morphotype (CR agar assay)

Strains were tested for their morphotype on Congo Red agar (g/l: 10.0 peptone, 5.0 yeast extract, 17.0 agar, 40 µg/ml Congo Red, 20 µg/ml Coomassie BB G250). The morphotype of each strain was determined by the morphology of the colonies after incubation for 24 hours at 37°C. Morphotype *saw* (white) - deficiency of expression of cellulose and curli fimbriae; *pdar* (pink) - presence of cellulose and the absence of fimbriae; *bdar* (brown) – expresses curli fimbriae; *rdar* (red, dry and rough) morphotype - expresses curli fimbriae and cellulose (Bokranz et al., 2005).

Crystal violet (CV) assay for biofilm formation

Biofilm – forming capabilities were tested in microtiter assay with crystal violet technique as described elsewhere (Soto et al., 2006). Strains were cultured in tryptic soy broth to stationary phase and then subcultured (1:100) in M63 minimal medium in 96 wells U-shape microtiter polystyrene plates (Nunc). Plates were incubated at 37°C for 24 h. After assessment of planktonic growth with measurement of OD at 630 nm (BioTek Ex800), plankton was removed, wells of microtiter plates were washed three times with sterile 0.89% NaCl and biofilm was stained with 0.1 % crystal violet for 10 min. After incubation dye was removed, wells were washed three times with sterile 0.89% NaCl and biofilm-connected

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dye was resolved with 75% ethanol. Absorbance was measured at 630 nm. Test was performed in six wells for a strain in microplate and at least three times for all strains.

Multiplex PCR

Presence of genes for some virulence factors was estimated with multiplex colony PCR. Sample preparation and conditions for the PCR reaction (primers, temperature and number of cycles) are consistent with those published by Johnson & Stell (2000) with modifications. The selected primers are divided into groups according to the expected size of the PCR product. In the reaction are included 3 µl of 24 h broth cultures of the selected strains and DreamTaq green PCR master mix (2x) according of the terms of company – manufacturer (Fermentas). Primers and genes selected in this study were for *fimH* which encodes the mannose-specific adhesin subunit of type 1 fimbriae, for toxins genes included *hlyA* and *cnf1*, for capsule synthesis genes which were selected from aligned *kpsMT* sequences for K1 (group II), K5 (group II), and K5 (group III) capsules; for serum survival genes *traT* and *iss*, latest targeted with primers iss f – gtggcgaaaactagtaaaacagc and iss r – cgcctcggggtggataa for whole gene sequence, taken from GenBank - accession No. AF042279.1 (http://www.ncbi.nlm.nih.gov/nuccore/AF042279.1).

Statistical analysis

The functions of MS Excel 2010 to calculate mean values and standard deviations were used.

Results

After standard procedure according to BBL Enterotube II test system strains in present study were identified as *Escherichia coli.* Phenotypical characteristic as motility, morphotype, adhesion, hemolysins and biofilm-forming were investigated with a view to their contribution in virulence.

The motility is perhaps one of the most impressive features in microbial physiology. Movement in water environments or near the surface using various ways and structures for moving was classified into several different types. The investigation of motility showed that almost all of strains had *swimming* phenotype (15 strains - 75%) and eight strains possessed swarming motility (40%) (Figure 1). Studied 20 strains did not show *twitching* translocation.

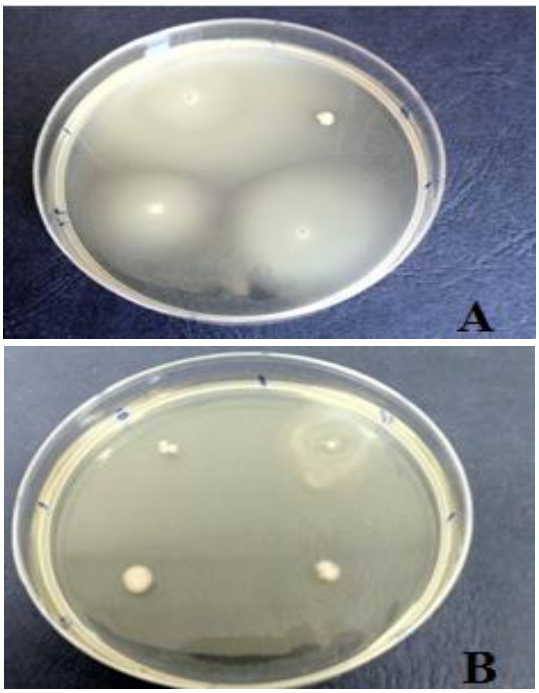

Figure 1. *Swimming (A) and swarming (B) of some of tested strains*

We studied some other phenotypic characteristics of the strains associated with their pathogenic potential. At first the morphotype for all strains was obtained after 24 h incubation on CR agar at 37°C. Three of all four different morphotypes were found. The dominant morphotype was *pdar* (pink colony, expressed cellulose) – 10 strains (50%), *rdar* morphotype (violet colony, expresses curli fimbriae and cellulose) occurs to 8 *E. coli* strains (40%) and only two colonies (10%) were with *saw* morphotype (no expression of fimbriae nor cellulose). The fourth morphotype *bdar* (brown colony, expression of curli) was not found in strains which we studied (Table 1).

Yeast agglutination was performed to detect the presence of type 1 pili on the surface of bacterial cells. The result showed that 15 strains (75%) agglutinated yeast cell in the absence of mannose but not in the present of it – mannose-sensitive yeast agglutination (MSYA) (Table 1). Mannose-resistant yeast agglutination (MRYA) occurs as a result of the expression of P, FIC, Dr and/or S fimbriae and only two strains showed it (10%).

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Table 1. *Motility and morphotype of investigated strains*

Strain	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Motility																				
Swimming	+	+	+	+	-	+	-	+	+	+	+	-	+	+	-	+	+	+	+	-
Swarming	-	-	-	-	-	+	-	-	-	+	-	-	+	+	+	+	+	-	+	-
Twitching	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Morphotype																				
pdar	+	+	-	-	+	+	+	-	+	-	-	+	-	-	-	+	+	+	-	-
rdar	-	-	+	+	-	-	-	+	-	+	+	-	+	+	+	-	-	-	-	-
saw	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
bdar	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Strains causing extra-intestinal infections often secreted α-hemolysin as virulence factor. The protein is extracellular, can induce a lysis of human erythrocytes and have a cytotoxity to several types of human cells. The test on blood agar for α-hemolysin synthesis showed that only 3 of 20 (15%) strains secreting α-hemolysin.

Biofilm-forming capacity was investigated phenotypically. In this study we used CV test to investigate the ability of urinary isolates to form biofilm in M63 medium on polystyrene plates for 24 hours at 37°C.The results are shown in Figure 2.

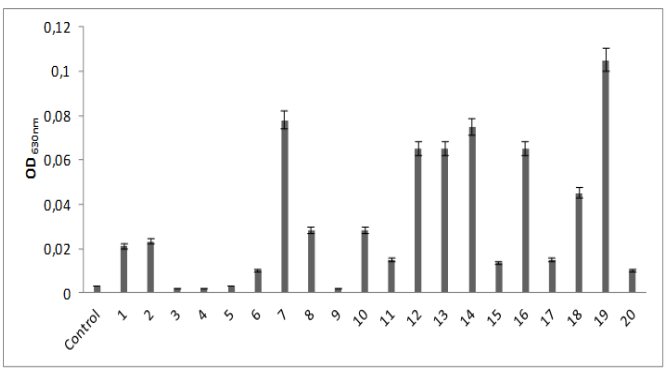

Figure 2. *Biofilm-forming capacity of strains in M63 medium for 24 h at 37°C.*

We conducted multiplex colony polymerase chain reaction (PCR) to investigate presence of genes for presumptive virulence factors for strains included in our study according to published from Johnson & Stell, 2000) data. Selected primers were from different parts of the *pap* operon (*papG II / III*, *papG I*, *papAH*, *papEF*, *papC*), for cytotoxic necrotizing factor-1 gene (*cnf-1*), *traT* and *iss* (serum resistance genes), α-hemolysin gene (*hlyA*), protein capsule genes (*kps MT K5*, *kps MT II*, *kps MT K1*) and *fimH* gene, encoding mannose-sensitive adhesin of type 1 pili. Primers were grouped according to the expected size of the PCR products as follows: System I: forward and reverse primers for *papG II / III*, *cnf 1*, *traT*, *kps MT K5*; System II: forward and reverse primers for *papG I*, *pap AH*, *pap EF*, *kps MT II*, *kps MT K1*; System III: forward and reverse primers for *hlyA*, *iss*, *fim H*, *pap C*. The results of multiplex colony polymerase chain reaction are shown in Table 2.

Discussion

Swimming on the surface occurs when the liquid layer is thick enough as a movement is for each individual cell and is performed by rotating flagella. One of the possible explanations for predominant swimming motility over other two is conditions which offer urinary tract - continuous flow of urine which is difficult surmountable obstacle and in the case opportunities for coordinated movement and adhesion are the key for successful colonization of the uropathogens.

Motility contributes to the fitness of the uropathogen and allows a strain that bears such traits to outcompete strains lacking these capabilities. It is likely that transient expression of flagellum-mediated motility and chemotaxis are necessary at specific stages of infection and at specific sites for most-efficient colonization of the urinary tract. It has been demonstrated that, overall, flagellar genes are down-regulated during infection. This strategy likely succeeds in avoiding the triggering of Toll-like receptor-5-mediated innate immunity (Lane et al., 2005). Our results about motility of investigated strains are expected since most of them were isolated from urine of patients with cystitis and asymptomatic bacteriuria as preliminary diagnosis. It was recently founded that switching from motility to adhesion is based on mutual inhibition of the flagellar and curli control cascades.

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Table 2. *Results from multiplex colony PCR*

	Strain																			
Gene	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
papG II/III							+	+		+								+		+
cnf 1			+			+		+		+			+	+	+	+				
traT	+	+	+		+	+	+		+	+	+				+	+			+	
kps MT K5			+				+													
papG I																				
pap AH			+				+	+		+					+	+	+			+
pap EF			+			+	+	+		+	+				+					+
kps MT II	+		+					+		+	+				+		+			+
kps MT K1								+	+		+				+		+			+
hly A			+					+		+			+	+						
iss						+														
fim H	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pap C			+			+	+	+		+					+	+		+		+

Additionally was revealed that in a developing biofilm, motile and sedentary subpopulations can coexist (Pesavento et al., 2008).

Fifteen of all strains (75%) agglutinated yeast cells in mannose-sensitive manner which indicate that they expressed type 1 pili on their surface. This is quite expected because type 1 pili are the most common virulence factor in uroisolates.They are needed for the first step of invasion of uroepithelial cells as a way to avoid adversely extracellular environment where antibodies, complement system and other antibacterial molecules are in abundance. Three of isolates did not agglutinate yeast cells neither with nor without mannose. Two of these strains were isolated from urine of patients with asymptomatic bacteriuria. This data suggest that there wasn't expression of type 1 and P pili. According to findings of other authors (Roos et al., 2006) the lack of adhesion could to a large degree account for the inability to cause symptoms in the human host. From the other side this fact leads to another question about how such strains are capable of staying in bladder and urethra. Well documented results in study of ability of ABU strain to exploit human urine as a niche for persistence and survival suggest that these key differences may be exploited for preventative and/or therapeutic approaches (Roos et al., 2006).

A large part of microbial infections are associated with biofilm formation. Furthermore, it is believed that all medical implants are predisposed to bacterial colonization (urinary, venous, abdominal catheters, artificial heart valves, pacemakers, voice prostheses, contact lenses, etc.) (Davey & O'Toole, 2000). Catheter - associated infections are one of the most common nosocomial infections worldwide. Treatment of this type of secondary infections is quite difficult due to the limited diffusion of drugs to cells inside the biofilm. This makes the biofilm formation an important virulence factor for UTI. In our case 80% of urinary isolates have biofilm forming capacity in different degree.

We tested strains for morphotype on CR agar at 37°C in parallel to survey of biofilm forming. The red, dry and rough (rdar) morphotype, a multicellular behavior, includes adhesion to abiotic surfaces (biofilm formation) and expression of curli fimbriae and cellulose as extracellular matrix components (Bokranz et al., 2005). Our data showed that there was no direct correlation between morphotype and biofilm-forming capabilities. To confirm this we need more detailed analysis of both characteristics in larger group bacterial isolates.

The surveys of genotype by multiplex colony polymerase chain reaction (PCR) showed there was no universal

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combination of genes that very likely to makes the strain a successful uropathogen (Table 3). The different virulence factors had various percent presentments. Of all 13 virulence genes only *papG* allele I was not detected in any strain and other had overall prevalence ranging from 5% (*iss*) to 100% (*fimH)*. The tested strains have a specific set of genetic determinants of virulence and the only type 1 pili emerge as a universal tool for successful conservative pathogenesis in the urinary tract. Our survey results match with the findings of other researchers (Moreno et al., 2008).

Table 3. *Distribution of virulence characteristics in tested strains E. coli*

Characteristics	Number of strains, possessing the characteristic / total number of strains / %
Motility	
- *Swimming*	15/20/75%
- *Swarming*	8 /20/40%
- *Twitching*	0/20/0%
MSYA	15/20/75%
MRYA	2/20/10%
α-hemolysin secretion	3/20/15%
Colony morphotype:	
- *pdar*	10/20/50%
- *rdar*	8/20/40%
- *saw*	2/20/10%
Biofilm formation	16/20/80%
Gene	
- *cnf-1*	8/20/40%
- *traT*	12/20/60%
- *hlyA*	5/20/25%
- *iss*	1/20/5%
- *fimH*	20/20/100%
- *papG II/III, papAH, pap EF, papC*	4/20/20%
- *kps MT K5*	2/20/10%
- *kps MT II*	8/20/40%
- *kps MT K1*	6/20/30%

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