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Virulence factors, biofilm-forming ability, and antimicrobial resistance of urinary *Escherichia coli* strains isolated from hospitalized patients

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Background/aim: *Escherichia coli* is the most frequent cause of urinary tract infections. We investigated the possible associations between the origin of strains, antimicrobial resistance, the presence of urovirulence factors, and biofilm-forming ability.

Materials and methods: Antibiotic susceptibility of *E. coli* strains was tested by disk diffusion method. Hemagglutination assays were performed for phenotypic characterization of the cell surface. Multiplex PCR was used for detection of virulence genes and for determination of phylogenetic relationships.

Results: The resistance to ampicillin (55.5%) and tetracycline (39.3%) was significantly more frequent than to other antimicrobial agents. The *fim* gene was present in 92.5% of strains. The *sfa* and *pap* genes were found in 53.8% and 38.7% of strains, respectively. The *pap* gene was significantly less frequently detected in strains from dialysis patients. The *hly* gene was present in 18.5% of strains. The *aer* gene was detected in 52.6% and *cnf* in 12.1%, while *afa* was detected in 4.6% of strains. Most strains belonged to the B2 and D phylogenetic groups. The *aer* gene was significantly associated with strains producing strong biofilms.

Conclusion: The *E. coli* strains causing cystitis in hospitalized patients differed in terms of resistance to antibiotics, virulence genes, and potential for biofilm formation.

Key words: Uropathogenic *Escherichia coli*, antibiotic resistance, virulence genes, biofilm, phylogenetic groups

1. Introduction

Escherichia coli is the major factor of community-acquired urinary tract infection (UTI) (70%–95%) and a large part of nosocomial UTIs (50%). The virulence factors responsible for pathogenesis outside of the gastrointestinal tract belong to various functional groups. They include fimbrial adhesins (e.g., P, S, and type 1 fimbriae), afimbrial adhesin Afa I, toxins (e.g., α-hemolysin, cytotoxic necrotizing factor 1 – CNF1), and iron acquisition systems (e.g., the aerobactin system) (1). The formation of biofilm is another pathogenic mechanism, allowing *E. coli* to persist in the genitourinary tract and hindering the eradication of microorganisms. Different bacterial species colonize urinary catheters and bacterial biofilm is the primary factor for the development of nosocomial UTIs (2). These infections are often difficult to treat and they are a major reservoir of resistant pathogens (3).

In the present study, we compared the antimicrobial resistance of *E. coli* strains causing cystitis in hospitalized patients in different units of the hospital. We also determined the possible relationships between the origin of strains, the presence of urovirulence factors, phylogenetic group, and biofilm formation on urinary catheters.

2. Materials and methods

2.1. Bacterial strains

A total of 173 *E. coli* strains were isolated from urine samples of patients with cystitis. These strains were derived from the urine samples of symptomatic patients in which $\geq 10^5$ colony-forming units of *E. coli* per milliliter were found. Strains were collected from January 2007 through December 2008 from five groups of patients hospitalized in internal, pediatric, neurological, obstetric (pregnant women), and dialysis units of a district hospital. *E. coli* identification was performed according to standard procedures (4). The identification of the strains was confirmed by using the ID 32 E system (bioMérieux).

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2.2. Antimicrobial susceptibility testing

The antimicrobial susceptibility of the strains was tested by disk diffusion method (5). Extended-spectrum β-lactamase (ESBL) production was detected using the double disk synergy test (6).

E. coli ATCC 25922 and *E. coli* ATCC 35218 were used as control strains.

2.3. Phenotypic characterization of the cell surface

The strains were grown on trypticase soy agar (TSA; BBL, Becton Dickinson) at 37 °C for 24 h. Hemagglutination assays were performed using human O group, sheep, calf, guinea pig, adult chicken, and pig erythrocytes as described previously (7). The presence of type 1 fimbriae was defined by mannose-sensitive hemagglutination (MSHA), whereas the presence of P, S, and other adhesins was determined by mannose-resistant hemagglutination (MRHA).

2.4. Detection of virulence genes by PCR

Genomic DNA was isolated from *E. coli* strains by using the Genomic Mini (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's protocol.

The presence of the *pap* (encoding P fimbriae), *sfa* (S fimbriae), *afa* (Afa I), *hly* (α-hemolysin), *aer* (aerobactin), and *cnf* (CNF1) genes was investigated by multiplex PCR. The specific primers are shown in Table 1.

2.5. Phylogenetic analysis

The phylogenetic group (A, B1, B2, and D) was determined by triplex PCR performed with a combination of three DNA markers (*chuA*, *yjaA*, and the DNA fragment *TSPE4.C2*) (11).

2.6. Biofilm formation assay

Biofilm formation by *E. coli* strains on a silicone 20-Fr Foley catheter (Romed, Herenweg, the Netherlands) was analyzed using a procedure previously described (12).

2.7. Statistical analyses

Chi-square statistics in Statistics 9.0 (Analytical Software, Tallahassee, FL, USA) was used for analysis. Differences at $P \leq 0.05$ were considered statistically significant.

3. Results

3.1. Antimicrobial resistance

More than half of *E. coli* (55.5%) exhibited phenotypic resistance to ampicillin and the resistance to this antibiotic was significantly more frequent than to other antimicrobial agents. Numerous strains were resistant to tetracycline (39.3%) and the percentage of strains resistant to this antibiotic was highly significantly higher than to the remaining antimicrobial agents (Figure). The resistance to ampicillin occurred significantly less in the strains from pregnant women (10%) than internal medicine patients (60.9%) ($P = 0.008$) and also significantly less in the strains from children (57.4%) ($P = 0.016$) and neurological patients (57.7%) ($P = 0.028$) (Table 2). The resistance to piperacillin was significantly less detected among the strains from children (7.4%) than internal medicine (32.8%) ($P = 0.002$) and dialysis patients (31.6%) ($P = 0.025$) (Table 2). The resistance to cephalothin was detected significantly more frequently in the strains from internal medicine (20.3%) and dialysis patients (42.1%) than in strains isolated from children (5.5%) ($P = 0.039$

Table 1. Primers used in the study.

Target genes	Primer sequences (5' → 3')	Amplicon length (bp)	Reference
pap3	GCAACAGCAACGCTGGTTGCATCAT	336	(8)
pap4	AGAGAGAGAACTGGGTGCATCTTAC		
hly1	AACAAGGATAAGCACTGTTCTGGCT	1177	(8)
hly2	ACCATATAAGCGGTCATTCCCGTCA		
aer1	TACCGGATTGTCATATGCAGACCGT	602	(8)
aer2	AATATCTTCCTCCAGTCCGGAGAAG		
cnf1	AAGATGGAGTTTCCTATGCAGGAG	498	(8)
cnf2	CATTCAGAGTCCTGCCCTCATTATT		
sfa1	CTCCGGAGAACTGGGTGCATCTTAC	410	(9)
sfa2	CGGAGGAGTAATTACAAACCTGGCA		
afa1	GCTGGGCAGCAAACTGATAACTCTC	750	(9)
afa2	CATCAAGCTGTTTGTTCGTCCGCCG		
fim1	GAGAAGAGGTTTGATTTAACTTATTG	559	(10)
fim2	AGAGCCGCTGTAGAACTGAGG		

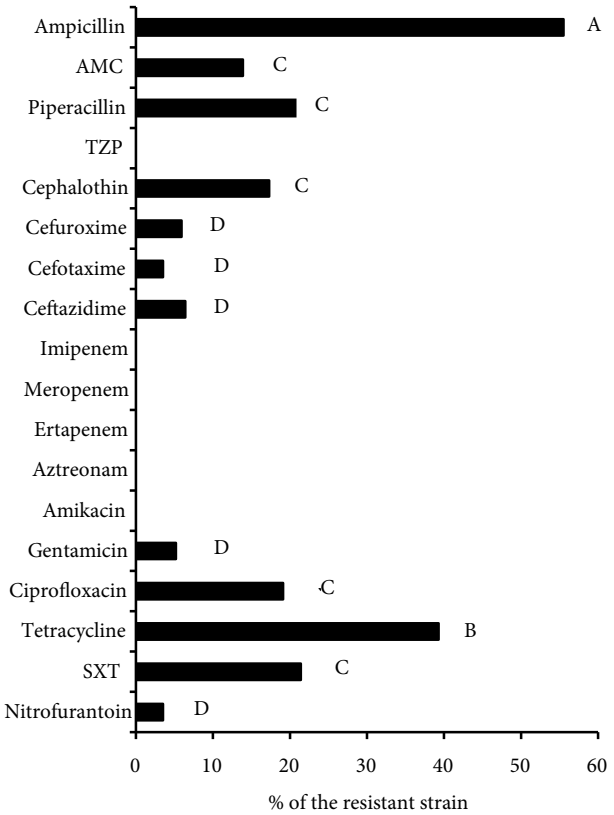

Figure. Antimicrobial resistance of *E. coli* strains causing urinary tract infections in hospitalized patients. The designation of columns with different letters indicates that the percentage of the resistant strains differs significantly at $P \leq 0.05$. AMC – Amoxicillin plus clavulanic acid, TZP – piperacillin plus tazobactam, SXT – trimethoprim plus sulfamethoxazole.

and P = 0.001, respectively). The strains showing resistance to ciprofloxacin were highly significantly more detected in neurological (30.8%) and internal medicine patients (25%) than in children (3.7%) (P = 0.002 and P = 0.003, respectively) (Table 2). ESBLs were expressed by 6 (3.5%) strains recovered from patients hospitalized in internal (n = 4) and neurological (n = 2) units.

3.2. Phenotypic characterization of fimbriae

The percentage of strains with type 1 fimbrial expression was the highest among *E. coli* isolated from dialysis patients (86.1%) but the presence of type 1 fimbriae was not significantly associated with any group of strains (Table 3). MRHA was observed in 82 (47.4%) *E. coli* strains. The highest percentage of strains with MRHA was isolated from children (59.3%). MRHA was significantly more common in the group of strains from children compared to the strains isolated from dialysis patients (31.6%) (P = 0.049) (Table 3).

3.3. Distribution of virulence genes

The *fim* gene encoding type 1 fimbriae was detected in 160 (92.5%) strains. The presence of *sfa* and *pap* genes was found in 93 (53.8%) and 67 (38.7%) strains, respectively. The presence of the *pap* gene was statistically more frequently detected in *E. coli* strains isolated from internal medicine patients (39.1%), children (40.7%), patients of the neurological ward (42.3%), and pregnant women (70%) than in strains from dialysis patients (10.5%) (P = 0.0401, P = 0.017, P = 0.022, and P = 0.002, respectively). Statistical analysis showed that the presence of the *sfa* gene was significantly more frequent in strains isolated from pregnant women (90%) compared to the strains isolated from patients hospitalized in the internal ward (46.8%) (P = 0.028), from dialysis patients (52.6%) (P = 0.05), and from patients hospitalized in the neurological ward (38.5%) (P = 0.007). The *sfa* gene was also significantly more frequently detected in strains isolated from children (63%) than neurological patients (P = 0.04).

The *afa* gene was detected in only 4.6% of strains. The *hly* gene encoding α-haemolysin was found in 32 (18.5%) strains and the presence of this gene was significantly more frequent in strains isolated from dialysis patients (36.8%) than in strains from internal medicine patients (1.6%) (P = 0.0002). The *hly* gene was also detected in the strains from children (33.3%) and patients from the neurological ward (23.1%) significantly more than in the strains from internal medicine patients (P = 0.0002 and P = 0.003, respectively). The *aer* gene was detected in 91 (52.6%) *E. coli* strains and this gene was significantly more often detected in the strains isolated from patients hospitalized in the neurological ward (73.1%) and children (64.8%) compared to internal medicine patients (34.3%) (P = 0.0009 and P = 0.001, respectively) (Table 3).

3.4. Phylogenetic groups

Among the 173 *E. coli* strains from patients with UTIs, 66 strains (38.1%) belonged to phylogenetic group B2, 61 (35.3%) to group D, 32 (18.5%) to group A, and 14 (8.1%) to group B1. More than half of the strains from children (55.5%) belonged to group B2, significantly more than the strains from internal medicine patients (P = 0.0007). The percentage of strains isolated from patients of the obstetric ward that belonged to phylogenetic group D was 60% and the frequency of these strains in group D was significantly higher than the strains from dialysis patients (10.5%) (P = 0.009). The frequency of strains from dialysis patients in phylogenetic group D was also significantly lower than strains from internal medicine patients (43.8%) (P = 0.018) (Table 3).

3.5. Biofilm formation on urinary catheter

Out of 173 strains, moderate and strong biofilm producers were found to be 45.1% and 37%, respectively. The highest percentage of strong biofilm producers was among the

Table 2. Distribution of antimicrobial resistance according to the origin of *E. coli* strains.

Antimicrobial agents	Number (%) of resistant strains				
	Internal n = 64	Pediatrics n = 54	Neurological n = 26	Obstetric n = 10	Dialysis n = 19
Ampicillin	39[a] (60.9)	31[a] (57.4)	15[a] (57.7)	1[b] (10.0)	10[ab] (52.6)
AMC	8[a] (12.5)	6[a] (11.1)	5[a] (19.2)	1[a] (10.0)	4[a] (21.0)
Piperacillin	21[a] (32.8)	4[b] (7.4)	6[ab] (23.1)	1[ab] (10.0)	6[a] (31.6)
Cephalothin	13[ac] (20.3)	3[b] (5.5)	6[abc] (23.1)	0[ab] (0.0)	8[c] (42.1)
Cefuroxime	6[a] (9.4)	1[a] (1.8)	2[a] (7.7)	0[a] (0.0)	1[a] (5.3)
Cefotaxime	5[a] (7.8)	0[b] (0.0)	1[ab] (3.8)	0[ab] (0.0)	0[ab] (0.0)
Ceftazidime	6[a] (9.4)	1[a] (1.8)	3[a] (11.5)	1[a] (100	0[a] (0.0)
Gentamicin	5[a] (7.8)	2[a] (3.7)	2[a] (7.7)	0[a] (0)	0[a] (0)
Ciprofloxacin	16[a] (25.0)	2[b] (3.7)	8[a] (30.8)	2[ab] (20.0)	5[ab] (26.3)
Tetracycline	31[a] (48.4)	18[a] (33.3)	10[a] (38.5)	1[a] (10.0)	8[a] (42.1)
SXT	13[ab] (20.3)	7[b] (13.0)	10[ac] (38.5)	2[ab] (20.0)	5[ab] (26.3)
Nitrofurantoin	6[a] (9.4)	0[a] (0.0)	0[a] (0.0)	0[a] (0.0)	0[a] (0.0)

AMC – Amoxicillin plus clavulanic acid; SXT – trimethoprim plus sulfamethoxazole.
The designation with different letters indicates that number of resistant strains differs significantly among patients from different hospital wards at $P \leq 0.05$.

Table 3. Distribution of MSHA and MRHA fimbriae, virulence genes, and phylogenetic groups according to the origin of *E. coli* strains.

	Number (%) of strains with trait				
Trait	Internal n = 64	Pediatrics n = 54	Neurological n = 26	Obstetric n = 10	Dialysis n = 19
Fimbriae					
MSHA	55[a] (85.9)	47[a] (87.0)	21[a] (80.8)	8[a] (80.0)	18[a] (94.7)
MRHA	28[ab] (43.7)	32[b] (59.3)	11[ab] (42.3)	5[ab] (50.0)	6[a] (31.6)
Genes					
pap	25[a] (39.1)	22[a] (40.7)	11[a] (42.3)	7[a] (70.0)	2[b] (10.5)
sfa	30[ab] (46.8)	34[bcd] (63.0)	10[a] (38.5)	9[c] (90.0)	10[ad] (52.6)
fim	58[a] (90.6)	51[a] (94.4)	25[a] (96.2)	8[a] (80.0)	18[a] (94.7)
afa	2[a] (3.1)	1[a] (1.8)	4[a] (15.4)	0[a] (0.0)	1[a] (5.3)
hly	1[a] (1.6)	18[b] (33.3)	6[b] (23.1)	0[ab] (0.0)	7[b] (36.8)
cnf	11[a] (17.2)	4[a] (7.4)	1[a] (3.8)	2[a] (20.0)	3[a] (15.8)
aer	22[a] (34.4)	35[bcd] (64.8)	19[cd] (73.1)	5[ad] (50.0)	10[ad] (52.6)
Phylogeny					
A	14[a] (21.9)	7[a] (13.0)	5[a] (19.2)	1[a] (10)	5[a] (26.3)
B1	6[a] (9.4)	0[b] (0.0)	4[a] (15.4)	1[ab] (10)	3[a] (15.8)
B2	16[a] (25.0)	30[b] (55.5)	9[ab] (34.6)	2[ab] (20.0)	9[ab] (47.4)
D	28[a] (43.8)	17[ab] (31.5)	8[ab] (30.8)	6[a] (60.0)	2[b] (10.5)

The designation with different letters indicates that number of strains with the particular trait differs significantly among patients from different hospital wards at $P \leq 0.05$.

strains isolated from patients of the neurological (61.5%), obstetric (60%), and dialysis (52.6%) wards (Table 4).

No association of the degree of biofilm formed with adhesin and toxin genes and expression of the mannose-resistant or mannose-sensitive fimbriae was found. The strains that differed in degree of biofilm formation on catheter surfaces were not associated with any of the phylogenetic groups. Only the aerobactin gene was highly significantly associated with strong biofilm-producing *E. coli* strains (Table 5).

4. Discussion

Antimicrobial resistance among *E. coli* causing UTIs is increasing in many countries around the world (13). In this study, 55.5% of strains were resistant to ampicillin and this was similar to results of other authors, who found that 56% of *E. coli* isolates from hospitalized patients in Turkey showed resistance to this antibiotic (14). We found that resistance rates to ampicillin were significantly higher than to other antibiotics but among strains isolated from pregnant women were significantly lower than among strains isolated from patients of internal, neurological, or pediatric wards. A low level of antimicrobial resistance among strains from pregnant women may be associated with reduced use of antibiotics in pregnancy and relatively short stay in the hospital. In our research, a high level of resistance to tetracycline (above 39%) was also found. An even higher percentage (67.3%) of *E. coli* strains resistant to this antibiotic was isolated at the same time from people with UTIs from different parts of India (15).

The resistance to ciprofloxacin among our isolates was higher (19.1%) than among isolates from Turkey (15%) (14). Sanchez et al. (16) suggested that the increasing of resistance to ciprofloxacin among *E. coli* strains was a result of widespread use of this antibiotic in the treatment of uncomplicated UTIs in the early 2000s. In the present study over 21% of strains showed resistance to trimethoprim plus sulfamethoxazole and our result is consistent with previous reports regarding increases in antimicrobial resistance of urinary *E. coli* isolates to this agent (14,16,17). This trend has continued for decades and the increasing resistance of *E. coli* to trimethoprim plus sulfamethoxazole can be explained by frequent use of this antimicrobial agent because it is recommended as the second-line drug in treating acute uncomplicated cystitis in women. The level of resistance of *E. coli* strains to first-generation cephalosporins (cephalothin) was highly significantly higher than those for second- (cefuroxime) and third-generation cephalosporins (cefotaxime, ceftazidime) and results of our investigation were consistent with those obtained by other authors (16). Authors reported low resistance rates of *E. coli* to nitrofurantoin (0.85%–1.6%) (14,16) or that all strains were sensitive to this antimicrobial agent (15) and that in the last decade no increase in resistance was observed. In our research, the resistance rates to nitrofurantoin were higher and amounted to 3.5%.

Among 7 virulence genes studied, the *fim* gene, which encodes type 1 fimbriae, was the most frequently detected. The role of type 1 fimbriae in human disease is difficult to reconcile because they are present in both pathogenic and commensal isolates (18). The *pap* gene (pyelonephritis-associated pili) was present in about 39% of *E. coli* strains but among strains isolated from pregnant women the percentage of strains with this gene was 70%. Similar results were obtained by Naveen and Mathai (19), who showed that P fimbriae in isolates from pregnant women were identified significantly more frequently than in isolates from other groups of patients. In this study, only 10.5% of strains isolated from dialysis patients had the *pap* gene and the frequency of this gene among these strains was significantly less than among strains isolated from other groups of patients. This might have resulted from the fact that adherence mediated by P fimbriae might be less important when the defenses of the urinary tract against infection are compromised by anatomic abnormality. About 50% of uropathogenic *E. coli* isolates show the α-hemolysin gene and its expression is associated

Table 4. Ability of *E. coli* strains to produce biofilm on urinary catheters.

Origin of strains (no.)	No. (%) of strains			
	Specific biofilm formation			
	None	Weak	Moderate	Strong
Internal (64)	1 (1.6)	19 (29.7)	32 (50)	12 (18.7)
Pediatric (54)	0	9 (16.7)	25 (46.3)	20 (37)
Neurological (26)	0	1 (3.8)	9 (34.6)	16 (61.5)
Obstetric (10)	0	0	4 (40)	6 (60)
Dialysis (19)	0	1 (5.3)	8 (42.1)	10 (52.6)

Table 5. Distribution of virulence determinants and phylogenetic groups according to ability of biofilm formation by *E. coli*.

Trait	No. (%) of strains		P-value
	Strong biofilm producers (n = 64)	Moderate, weak, and no biofilm producers (n = 109)	
Fimbriae			
MSHA	53 (82.8)	95 (87.2)	0.347
MRHA	30 (46.8)	52 (47.7)	0.909
Adhesin genes			
pap	28 (43.7)	39 (35.8)	0.315
sfa	31 (48.4)	62 (56.9)	0.386
fim	59 (92.2)	101 (92.6)	0.919
afa	5 (7.8)	3 (2.7)	0.118
Toxin genes			
hly	16 (25.0)	16 (14.7)	0.077
cnf	6 (10.6)	15 (19.4)	0.439
Aerobactin gene			
aer	41 (64.1)	49 (44.9)	0.028
Phylogeny			
A	14 (16.9)	18 (19.4)	0.387
B1	5 (7.8)	9 (8.25)	0.763
B2	21 (32.8)	45 (41.3)	0.371
D	22 (34.4)	39 (35.8)	0.871

with increased clinical severity in patients (1). Our results showed that the *hly* gene was highly significantly more often identified in *E. coli* strains isolated from dialysis and neurological patients and from children than among strains from patients hospitalized in the internal ward. Approximately one-third of uropathogenic *E. coli* strains encode CNF1, which can promote apoptosis of bladder epithelial cells and stimulate their exfoliation, which enhances bacterial access to underlying tissue (1). In this study, the presence of the *cnf* gene was found only in over 12% of strains, which was consistent with the results obtained by Tiba et al. (20). Urine is poor in iron, and uropathogenic *E. coli* produces a number of iron-chelating factors (siderophores), including aerobactin (1). Our results revealed that 52.6% of strains had the *aer* gene. In this study, we also investigated the presence of genes encoding S fimbriae. The *sfa* gene was detected in above 54% of strains, as similarly observed by other authors (21).

Most strains belonged to the B2 and D phylogenetic groups and our results agree with other studies concerning phylogenetic lines in uropathogenic *E. coli* (22). In this study, phylogenetic group B2 of strains was highly significantly associated with children, while strains belonging to the D group were associated with pregnant women and internal medicine patients. The strains belonging to the A phylogenetic group were not associated with any group of patients, as similarly observed by Clermont et al. (11).

In our study, an association between the degree of biofilm formed and the presence of adhesion, toxin genes, or expression of MSHA and MRHA was not found. However, we found that the presence of the *aer* gene was significantly associated with our strains producing strong biofilm. This indicates that high potential of biofilm formation is related to the possession of an iron acquisition system and production of siderophore aerobactin that maintains metabolic activity and makes proliferation of bacteria in biofilm possible.

In conclusion, analysis of the antimicrobial resistance of *E. coli* strains showed a high level of resistance to ampicillin and tetracycline. We observed association of resistance to antibiotics with the origin of the strains. No significant association between the degree of biofilm formed and the presence of adhesion and toxin genes was found. A high potential for biofilm formation seems to be associated with the presence of the *aer* gene encoding siderophore aerobactin. Further studies involving larger numbers of clinical strains are required with regard to the influence of the aerobactin-mediated iron uptake system on the potential for biofilm formation.

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