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Does *Escherichia coli* have pathogenic potential at a low level of bacteriuria in recurrent, uncomplicated urinary tract infection?

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ABSTRACT

Background: In recurrent uncomplicated lower urinary tract infection (uLUTI), bacteriuria below 10^3 colony-forming units (CFU)/mL is not usually investigated.
Objective: To determine the molecular-genetic characteristics of the pathogenic potential of Enterobacteriaceae isolated between episodes of recurrent uLUTI at bacteriuria of 10^2 CFU/mL.
Materials and methods: Midstream urine samples taken from 169 women between episodes of recurrent uLUTI were examined on six media. Fifteen Enterobacteriaceae strains at a low level of bacteriuria (10^2 CFU/mL) were analysed by polymerase chain reaction for the presence of the following virulence factor genes (VFGs): *pap*A, *pap*E/F, *pap*GII, *afa, bma*E, *iut*A, *feo*B, *fyu*A, *kps*MTII and *usp*.
Results: All 169 patients had leukocyturia and asymptomatic bacteriuria, and 62 of these cases were due to enterobacteria: 10^2 CFU/mL, 15 strains (24.2%); 10^3 CFU/mL, 10 strains (16.1%); and $>10^3$ CFU/mL, 37 strains (59.7%). VFGs were verified in all 15 strains at a low level of bacteriuria (10^2 CFU/mL): one VFG (*pap*GII) was found in two strains of *Klebsiella* spp., three VFGs were found in one strain of *Enterobacter* spp.xst, and between four and nine VFGs were found in the remaining 12 strains. Among the VFGs, *pap*GII (93.3%), *usp* (86.7%), *feo*B (80.0%), *fyu*A (80.0%) and *kps*MTII (73.3%) predominated. A significant correlation was found between the presence of *pap*E (P=0.02) and *usp* (P=0.025) and a relapse rate of more than four recurrences in 1 year. An association was found between leukocyturia and the presence of *pap*E (P=0.031) and *pap*GII (P=0.031).
Conclusion: Enterobacteria isolated from the urine of female patients with recurrent uLUTI at a low level of bacteriuria (10^2 CFU/mL) have a wide spectrum of VFGs, which may play a role in maintaining chronic inflammation of the lower urinary tract.

1. Introduction

Recurrent uncomplicated lower urinary tract infection (uLUTI) in women is characterized by the occurrence of two or more symptomatic episodes of urinary tract infection (UTI) within 6 months, or three or more such episodes within 12 months [1]. Recurrent uLUTI significantly reduces the quality of life of patients, and leads to approximately three medical consultations per year. Almost half of patients with recurrent uLUTI have at least six episodes of UTI per year and 14.4% have more than 12 episodes [2].

Members of the Enterobacteriaceae family, such as *Escherichia coli*, are recognized as the main aetiological agents of recurrent uLUTI worldwide [3]. In most cases, these bacteria are normal symbionts of almost all biotopes of a macro-organism [4]. However, under certain conditions, they can initiate the infectious and inflammatory process and lead to the manifestation of UTI.

In urological practice, quantification of bacteriuria has been widely used to diagnose UTI and to define microbiological success following treatment. Appropriate criteria have been developed for this purpose [5,6]. However, the complexity and severity of the clinical course of recurrent uLUTI, as well as the often-noted inefficiency of existing approaches in the management of such patients, indicate insufficient knowledge about the role of even low-level bacteriuria with uropathogens in the interval between acute relapse episodes. Therefore, further investigation of their aetiology

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and clinical significance may be relevant to improve the current therapeutic strategies for these patients.

The pathogenic potential of micro-organisms is dependent on the presence and/or appearance of specific properties for interaction with the macro-organism. Most important are virulence factors enhancing epithelial adhesion and colonization, which are necessary for pathogens to adapt to and persist in the new environment. These properties have an appropriate genetic determination. Approximately 40 genes have been described for *E. coli* which are associated with the virulence of these bacteria [7,8]. Many of these virulence factor genes (VFGs) are grouped into clusters called 'pathogenicity islands', which are distinguished by a high level of expression and, in some cases, correlation with the production of β-lactamases and antibiotic resistance [9].

Considering that bacterial phenotypes, including pathogenicity, are highly variable, there is a need to determine the genes of 'pathogenicity islands' in such uropathogenic strains. Therefore, the molecular-genetic aetiological assessment of *E. coli* strains isolated from patients with a low level of bacteriuria (10^2 CFU/mL) between episodes of recurrent uLUTI is of importance to evaluate their clinical significance.

The aim of this study was molecular-genetic characterization of the pathogenic potential of *E. coli* isolated between episodes of recurrent uLUTI at a bacteriuria level of 10^2 CFU/mL.

2. Materials and methods

This study included 169 female patients with recurrent uLUTI examined in 2016–2017. The mean age of patients was 36.2 (standard deviation 4.7) years. All patients were premenopausal, and the duration of disease was >10 years in 53.2% of cases.

Inclusion criteria were as follows: history of symptoms of recurrent uLUTI (at least three exacerbations within 1 year or two exacerbations within 6 months); presence of leukocyturia in urinalysis; absence of sexually transmitted diseases; and patient consent to participate in the study.

Midstream urine samples were obtained following an appropriate hygiene procedure, and collected in a sterile disposable container (Sterile Uricol; HiMedia, Mumbai, India) between episodes of recurrent uLUTI. Samples were transported to the laboratory under normal temperature conditions within 30–60 min.

Urine culture for the detection of Enterobacteriaceae and determination of the level of bacteriuria was carried out in accordance with clinical recommendations using MacConkey, HiCrome Klebsiella Selective Agar Base, Blood Agar Base, HiCrome Enterococci Agar, HiCrome Aureus Agar Base HiCrome and Candida Differential Agar (HiMedia) media. Samples were incubated at 37°C for 24 h. Cultures were differentiated by staining (Gram Stains-Kit, HiMedia) and cultural techniques, and identified by biochemical properties (Lachema, Brno, Czech Republic). Haemolytic activity (HA) was determined using a Blood Agar Base.

Extended-spectrum β-lactamase (ESBL) production was determined using ESBL detection kits with various cartridges: Kit for ESBL Identification, Cephotaxime; Kit for ESBL Identification, Cefepime; and Kit for ESBL Identification, Ceftazidime (HiMedia).

The presence of gene fragments from *E. coli* pathogenicity islands in Enterobacteriaceae clinical strains isolated from the urine of patients with recurrent uLUTI was determined using polymerase chain reaction (PCR).

2.1. Sample preparation for polymerase chain reaction

Before DNA extraction, strains were incubated in Muller-Hinton broth (HiMedia) at 37°C for 16–18 h until the bacterial culture density was 0.5 (McFarland standard) with a concentration of approximately 1–2 × 10^8 CFU/mL. A broth culture (1 mL) was concentrated by centrifugation (10 000 ***g*** for 10–15 min). The precipitate was washed two to three times by alternating mixing and subsequent centrifugation (3000 ***g*** for 3–5 min) in a vortex centrifuge (Microspin FV-2400; BIOSAN, Riga, Latvia) with removal of the supernatant. The washed centrifugate was resuspended before lysis in 1 mL of sterile distilled water and used to isolate DNA.

Table 1
Sequences of the primers and amplification method used in the study.

Name	Sequence, 5'-3'	Amplicon size	Source
papAFor	ATGGCAGTGGTGTTTTGGTG	720	[10]
papARev	CGTCCCACCATACGTGCTCTTC		
papE/FFor	GCAACAGCAACGCTGGTTGCATCAT	336	[11]
papE/FRev	AGAGAGAGCCACTCTTATACGGACA		
papGIIFor	GGGATGAGCGGGCCTTTGAT	190	[11]
papGIIRev	CGGGCCCCCAAGTAACTCG		
afaFor	GGCAGAGGGCCGGCAACAGGC	559	[10]
afaRev	CCCGTAACGCGCCAGCATCTC		
bmaEFor	ATGGCGCTAACTTGCCATGCTG	507	[10]
bmaERev	AGGGGGACATATAGCCCCCTTC		
iutAFor	GGCTGGACATCATGGGAACTGG	300	[11]
iutARev	CGTCGGGAACGGGTAGAATCG		
feoBFor	AATTGGCGTGCATGAAGATAACTG	470	[11]
feoBRev	AGCTGGCGACCTGATAGAACAATG		
fyuAFor	TGATTAACCCCGCGACGGGAA	880	[10]
fyuARev	CGCAGTAGGCACGATGTTGTA		
kpsMTIIFor	GCGCATTTGCTGATACTGTTG	272	[10]
kpsMTIIRev	CATCCAGACGATAAGCATGAGCA		
uspFor	ATGCTACTGTTTCCGGGTAGTGTGT	1000	[12]
uspRev	CATCATGTAGTCGGGGCGTAACAAT		

Amplification mode: (1) denaturation, 5 min, 94°C; (2) 35 cycles – (a) 1 min, 94°C; (b) 1 min, 55 °C; (c) 2 min, 72°C; (3) elongation, 10 min, 72 °C.

2.2. DNA extraction

A suspension of washed bacterial cells (20 µL) was lysed in 300 µL of lysis solution (5M guanidine thiocyanate, 1% Triton X100). Samples were mixed thoroughly in a vortex centrifuge and heated for 5 min at 65°C (TS-100; BIOSAN, ELMI Ltd, Riga, Latvia). Following centrifugation for 5 s at 5000 rpm in a microcentrifuge (SM-50; ELMI Ltd, Riga, Latvia), 25 µL of resuspended sorbent was added to each tube using a separate tip. The mixture was resuspended in a vortex, incubated at room temperature (2 min), resuspended again, and incubated at room temperature (5 min). The sorbent was precipitated by single centrifugation at 5000 rpm (30 s). The supernatant was removed with a vacuum aspirator (medical aspirator OM-1, OAO Utyos, Ulyanovsk, Russia) using a separate tip for each of the test samples. Three hundred microlitres of solution was added to each tube for washing sorbed DNA (5 M guanidine thiocyanate). The precipitate was carefully resuspended and precipitated by centrifugation at 5000 rpm (30 s), and the supernatant was removed. Nine hundred and fifty microlitres of washing solution (70% ethanol) was added to the samples, and the precipitate was mixed in a vortex centrifuge until a homogeneous suspension was obtained. Sorbent was precipitated by centrifugation at 10 000 rpm (30 s), and the supernatant was removed. Open-lid tubes were incubated at 65°C (5–10 min) to dry the sorbent. Next, 50 µL of elution DNA TE buffer was added, mixed and incubated at 65°C (5 min), with periodic shaking in a vortex centrifuge. Following elution, the sorbent was precipitated by centrifugation at 12 000 rpm (1 min). The supernatant containing purified DNA of the studied samples was collected and used for PCR.

2.3. Polymerase chain reaction

Amplification was carried out using Tertsik MS-2 DNA thermal cyclers (DNA-Technology, Moscow, Russia). The primers (Table 1) were synthesized at Bioscreen OOO (Ufa, Russia) and Syntol

(Moscow, Russia). The reaction mixture (20–30 µL, total volume of the mixture) included 67 mM Tris-HCl, 16.6 mM (NH4) $2SO_4$, 1.5 mM $MgCl_2$, 0.01% Tween-20, 0.1 µg of genomic DNA, primers - each 10pM, dATP, dTSP, dTTP, dGTP - each 200 μM with the addition of 1 unit of Taq polymerase (Sileks, Moscow, Russia). In some cases, dimethyl sulfoxide up to 0.1% and $MgCl_2$ up to 3 mM was added to the PCR mixture to increase the yield of the amplification product. In some cases, 20 µL of the following reaction mixture was used for amplification: 1.0 µL of test DNA, 10 µM primers; 2.0 µL of 10xTaq buffer; 2.5 mM deoxynucleotide triphosphate mixture; and 2.5 mM Taq polymerase.

Amplification products were separated by gel electrophoresis and visualized under ultraviolet light on a transilluminator.

The main objective of this study was to detect gene fragments associated with the presence of the following virulence factors in clinical strains of uropathogenic bacteria.

2.4. Adhesive structure coding

P pili group

- *pap*A – structural subunit of P pili (colonization factor in extraintestinal infections)
- *pap*E/F – apical adhesion of P pili
- *pap*GII – apical adhesion of P pili (II allele)
- *afa* – afimbrial adhesin (adhesin binds to the DAF receptor on the epithelium of the cell surface and enables haemagglutination)
- *bma*E – M pili

2.5. Coding of iron absorption systems

- *fyu*A, *iut*A, *feo*B – synthesis of siderophores

2.6. General pathogenicity

- *kps*MII – capsule synthesis
- *usp* – uropathogenic-specific protein

Statistical analysis included the calculation (quartiles, structural averages, mode and median) of detection frequencies and the number of micro-organisms isolated from the urine. The eta coefficient (or empirical correlation ratio) was used to analyse the relationship between detection frequencies of VFG fragments and HA and ESBL production. The eta coefficient is used to evaluate the relationship between a variable expressed in an interval scale (quantitative) and a variable expressed in an ordinal or nominal scale. The relationships between the detection frequencies of VFGs were analysed using Pearson's contingency coefficient (PCC). Statistical analysis was undertaken using SPSS Version 23 (IBM Corp., Armonk, NY, USA).

3. Results

Leukocyturia and asymptomatic bacteriuria were detected in all 169 female patients. In 107 (63.7%) cases, Gram-positive microbiota were isolated from the urine: *Corynebacterium* spp. (82.8%), *Enterococcus* spp. (62.7%), coagulase-negative staphylococci [*S. epidermidis* (33.1%), *S. lentus* (11.3%), *S. warneri* (10.6%)], and yeast-like fungi from the *Candida* genus [*C. albicans* (8.3%), *C. tropicalis* (5.9%), *C. krusei* (5.3%)]. Enterobacteria with varying levels of bacteriuria were isolated from urine in 62 (36.7%) women: 10^2 CFU/mL, 15 strains (24.2%); 10^3 CFU/mL, 10 strains (16.1%); and $>10^3$ CFU/mlL, 37 (59.7%).

At a bacteriuria level of 10^2 CFU/mL, *E. coli* (n=11) was dominant in the urine; *Enterobacter* spp. (n=2) and *Klebsiella* spp. (n=2) were detected less frequently.

VFG fragments were found in all 15 enterobacteria strains isolated at a bacteriuria level of 10^2 CFU/mL. A single VFG (*pap*GII) was found in two strains of *Klebsiella spp.*, three VFGs were found in one strain of *Enterobacter* spp., and four to nine VFGs were found in the remaining 12 strains cultured (Table 2).

It is noteworthy that eight of the nine VFGs studied were found in 46.6% of the strains tested.

*pap*GII (93.3%), *usp* (86.7%), *feo*B (80.0%), *fyu*A (80.0%) and *kps*MTII (73.3%) were most common at a bacteriuria level of 10^2 CFU/mL (Table 3).

The PCCs of the detection frequencies of the VFGs at a bacteriuria level of 10^2 CFU/ml were analysed (Table 4).

Nine significant PCCs were found between different pairs of VFGs at a bacteriuria level of 10^2 CFU/mL.

HA was observed in eight (53.3%) Enterobacteriaceae strains isolated from urine. Two significant PCCs were found between the detection frequencies of *bma*E and *iut*A and HA (PCC=0.519, P=0.019).

ESBLs were produced by eight (53.3%) Enterobacteriaceae strains. Significant PCCs were found between *bma*E (PCC=0.519, P=0.019) and *feo*B (PCC=0.471, P=0.038) and ESBL production.

More than four relapses of recurrent uLUTI per year were observed in eight (53.3%) patients with a bacteriuria level of 10^2 CFU/mL between episodes of uLUTI. A relationship was found between the detection of *pap*E (eta coefficient =0.6, P=0.02) and *usp* (eta coefficient =0.9, P=0.025) and more than four relapses per year.

Leukocyturia was significantly associated with the presence of *pap*E (eta coefficient =0.165, P=0.031) and *pap*GII (eta coefficient =0.628, P=0.031).

4. Discussion

The debate about whether relapses of recurrent uLUTI are related to infection with a new strain or the same pathogen that caused the previous episode of LUTI has been underway for decades [13–18]. The latest pulsed-field gel electrophoresis-based *E. coli* assay showed that relapses of LUTI were associated with a previously detected strain in 77% of cases, and with a new strain in 23% of cases in patients treated with pivmecillinam [19], and 96% of *E. coli* strains isolated from patients receiving placebo were identical to the strain that caused the infection. Similar results were obtained in other studies [20–22]. The present study shows that leukocyturia of varying severity may persist in patients between episodes of LUTI. In all cases, leukocyturia was associated with bacteriuria; this was at a low level (10^2 CFU/mL) in 24.2% of cases. The presence of leukocyturia with a low level of bacteriuria is evidence of a chronic course of inflammation in the lower urinary tract. A significant relationship between leukocyturia and the presence of two VFGs, *pap*E and *pap*GII, in enterobacteria emphasizes the role of adhesion factors in the formation of chronic latent inflammation. Prior to this study, research on virulence factors of *E. coli* isolated between episodes of recurrent uLUTI have only been performed at bacteriuria levels $\geq 10^3$ CFU/mL, recognized as clinically significant [3,8,19,23]. However, the present study shows that lower levels of bacteriuria, such as 10^2 CFU/mL, should also be regarded as clinically significant; this has been proposed in previous clinical studies [24–26].

The *E. coli* strains showing persistence were characterized by a high prevalence of haemolysis and 12 VFGs, some of which were independently associated with LUTI relapse. However, no combination of presence or absence of VFGs could be identified as a relapse marker of UTI.

All of the 15 Enterobacteriaceae strains studied contained VFGs. Among them, *E. coli* had a maximal set (8–9) of VFGs in 46.6% of cases. *pap*GII, *usp, feo*B and *fyu*A genes were identified most

Table 2
Fragments of virulence factor genes (VFGs) (number of primer pairs) detected in 15 Enterobacteriaceae strains cultured from midstream urine at a low level of bacteriuria (10^2 colony-forming units/mL) from female patients with recurrent, uncomplicated lower urinary tract infection.

Micro-organisms	VGF fragments (number of primer pairs)										HA	ESBL
	papA (5–6)	*papE*/F (7–8)	*papG*II (9–10)	*afa* (11–12)	*bmaE* (13–14)	*iutA* (15–16)	*feoB* (17–18)	*fyuA* (19–20)	*kps*MTII (21–22)	*usp* (23–24)		
E. coli	-	-	-	-	-	-	+	+	+	+	-	+
E. coli	-	-	+	-	-	-	+	+	+	+	-	-
E. coli	-	-	+	+	-	-	+	+	-	+	-	-
E. coli	+	+	+	-	+	+	+	+	+	+	+	+
E. coli	+	-	+	+	-	+	+	+	+	+	+	-
E. coli	+	-	+	+	+	+	+	+	+	+	+	+
E. coli	+	-	+	+	+	-	+	+	+	+	+	+
E. coli	-	-	+	+	+	+	+	+	+	+	+	+
E. coli	-	-	+	+	+	+	+	+	+	+	-	-
E. coli	-	-	+	+	+	+	+	+	+	+	+	+
E. coli	-	-	+	+	-	-	+	-	-	-	-	-
Enterobacter spp.	+	-	+	-	-	-	+	-	+	-	-	-
Enterobacter spp.	-	-	+	-	-	-	-	-	-	-	+	-
Klebsiella spp.	-	-	+	-	-	-	-	-	-	-	-	-
Klebsiella spp.	-	-	+	-	-	-	-	-	-	-	-	-

E. coli, *Escherichia coli*; HA, haemolytic activity; ESBL, extended-spectrum β-lactamase.

Table 3
Detection frequencies of virulence factor gene (VFG) fragments in Enterobacteriaceae strains cultured from midstream urine at a bacteriuria level of 10^2 colony-forming units/mL.

VFG fragments	Detection frequency (%)
papA	40.0
papE/F	6.7
*papG*II	93.3[a]
afa	53.3
*bma*E	46.7
*iut*A	46.7
*feo*B	80.0[a]
*fyu*A	80.0[a]
*kps*MTII	73.3[a]
usp	86.7[a]

[a] $P<0.05$.

Table 4
Pearson's contingency coefficient (PCC) of detection frequencies of virulence factor gene (VFG) fragments in Enterobacteriaceae strains.

VGF pairs		PCC	p
afa	*bma*E	0.519	p=0.019
afa	*iut*A	0.519	p=0.019
afa	*feo*B	0.471	p=0.038
afa	*fyu*A	0.471	p=0.038
*bma*E	*iut*A	0.591	p=0.005
*feo*B	*fyu*A	0.707	p<0.001
*feo*B	*usp*	0.617	p=0.002
*fyu*A	*usp*	0.617	p=0.002
*kps*MTII	*usp*	0.545	p=0.012

frequently. A significant number of strains had paired genes, which indicates their mutual involvement in the formation of virulent properties. A high frequency of HA was found in Enterobacteriaceae strains with an association with some specific VFGs. Clinical attention should be paid to the association between high relapse frequency (more than four times per year) of UTI and *pap*E/F and *usp*.

As far as is known, this is the first study to investigate VFGs in Enterobacteriaceae, mainly *E. coli*, at a bacteriuria level of 10^2 CFU/mL between episodes in women with recurrent uLUTI. This study showed that, in the absence of an acute episode, women with persistent leukocyturia in one-quarter of cases had a bacteriuria level of 10^2 CFU/mL associated with *E. coli* in 73.3% of cases, and less often with *Klebsiella* spp. and *Enterobacter* spp. In all cases of bacteriuria of 10^2 CFU/mL, all Enterobacteriaceae strains had VFGs that encoded for adhesion (93.3%), iron uptake (80.0%), production of protectins (73.3%) and uropathogenic-specific protein (86.7%). The latter property was found in all strains of *E. coli*. These data enable the studied strains to be attributed to uropathogens.

5. Conclusion

The VFGs found in this study at 10^2 CFU/mL correlate well with other studies of recurrent UTI at bacteriuria levels $\geq 10^3$ CFU/ml [27–29]. This indicates that a bacteriuria level of 10^2 CFU/mL between episodes of recurrent uLUTI should be considered as a risk factor for the development of subsequent relapse under certain conditions, and the VFGs studied may play an important role in maintaining chronic inflammation in the lower urinary tract.

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