The role of bacterial virulence factors in the clinical course of urinary tract infections

Ph.D. Thesis

Béla Köves M.D.

Supervisor:

Prof. Péter Tenke M.D., Ph.D.

Department of Urology, Jahn Ferenc South-Pest Hospital, Budapest
Department of Microbiology, Immunology and Glycobiology, Institute of Laboratory Medicine, Lund University, Lund, Sweden

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Publications directly related to the Ph.D. thesis

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List of Abbreviations

ABU	asymptomatic bacteriuria
CFU	colony forming unit
CNF1	Cytotoxic necrotizing factor 1
CRP	C reactive protein
CXCR	Chemokine (C-X-C motif) receptor
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
ExPEC	extraintestinal pathogenic *E. coli*
FCS	fetal calf serum
GbO3	globotriaosylceramide
GSL	glycosphingolipid
IBC	intracellular bacterial community
IL	Interleukin
LB	lysogeny broth
LPS	Lipopolysaccharide
PCR	polymerase chain reaction
PBS	Phosphate Buffered Saline
PMN	polymorphonuclear neutrophil
RNA	Ribonucleic acid
TcpC	TIR containing protein C
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UPEC	uropathogenic *Escherichia coli*
UTI	urinary tract infection

1. INTRODUCTION

Urinary tract infections (UTIs) are among the most common bacterial infections worldwide, as about 50% of women will experience at least one episode of UTI during their lifetime [1]. UTIs are also one of the leading causes of antibiotic consumption [2] and they represent about 40% of hospital acquired infections [3] with substantial financial implications and significant consequences to morbidity and mortality.

In the era of rapidly increasing antibiotic resistance the better understanding of the pathogenesis of UTIs and the role of virulence factors in the different clinical manifestations of urinary tract infections is of utmost importance. Despite the extensive research and major efforts that have been made in the field, there are still many questions regarding the molecular background of disease diversity and the interactions between bacteria and host.

1.1. Urinary tract infections

In healthy individuals the bladder and the upper urinary tract are sterile. Pathogens predominantly reach the urinary tract by ascending through the urethra, the other possible route is by haematogenous or lymphatic spread, usually to the kidneys. In women UTIs can be classified as lower urinary tract infections when the infection is restricted to the urethra (urethritis) or bladder (cystitis) and upper UTIs, when the kidneys are affected (pyelonpehritis). In case of an uncomplicated UTI there are no anatomical or functional complicating factors. In male patients or if one or more of these factors are present UTIs are considered as complicated.

1.1.1. Uropathogens

In most of the cases UTIs are caused by Gram-negative bacteria from the intestinal flora. The most important pathogen is *Escherichia coli*, being responsible for 70-95% of uncomplicated lower UTIs. *Proteus mirabilis* and *Klebsiella pneumonie* may be causative pathogens as well (2-5%). *Pseudomonas aeruginosa* is also an important Gram-negative bacterium, which can be found mostly in case of hospital acquired infections. The role of Gram-positive pathogens, such as *Enterococcus faecalis* shows an increasing trend due to the modern endourological practice and increased use of urinary foreign bodies. Rare pathogens such as *Corynebacterium urealyticum* or *Mycobacterium tuberculosis* can also be involved in UTIs.

1.1.2. Bacterial virulence factors

Virulence factors refer to the properties that enable a microorganism to establish itself and replicate on or within a specific host species, and that enhance the microbe's potential to cause disease [4]. Crucial virulence factors of uropathogenic *E. coli* (UPEC) confer resistance to the effects of the host defense and in addition, virulent bacteria are able to produce molecules that actively inhibit the immune response of the host, thereby enhancing bacterial persistence and tissue damage. The genes encoding virulence factors of UPEC are localized to chromosomal gene clusters called "pathogenicity islands" [5, 6]. The different virulence factors act in concert, their expression may be turned on or off during the course of the infection and can be regulated by environmental signals [7]. Many different factors have been implicated in UPEC pathogenesis, however, the specific factors that differentiate UPEC strains responsible for the different clinical manifestations of UTIs remain unclear.

Bacterial adherence to mucosal surfaces is considered to be a critical virulence factor. Fecal isolates, strains causing asymptomatic bacteriuria (ABU), cystitis or pyelonephritis differ in their adherence capacity to uroepithelial and vaginal cells [8]. The uropathogenic clones are usually fimbriated and may express several adhesive surface organelles, such as P, type 1 and S fimbriae [9, 10] [11, 12]. Adherence factors other than the P and type 1 fimbriae are less well studied, and their potential role as virulence determinants has not yet been convincingly shown. The most important virulence factors will be discussed below.

1.1.2.1. P fimbriae

P fimbriae are encoded by the *pap* gene cluster. The adhesin, PapG is located on the tip of the fibrillum, and it mediates the attachment to the uroepithelium. The host cell receptors for P fimbriae are globoseries of glycosphingolipids (GSLs), which are expressed on uroepithelial cells. These receptors are abundant on the uroepithelial [13], but their expression varies depending on the P blood group [14].

P fimbriae are classified according to their iso-receptor specificity. Class I P fimbriae carry $PapG_{J96}$ adhesin, which binds to globotriaosylceramide (GbO3). The association of this allele to UTIs is unknown [15], and it is uncommon in clinical isolates. Class II P fimbriae have been shown to have a strong association with acute pyelonephritis in both children [16] and adult women [17]. The $PapG_{IA2}$ adhesin binds to most members of the globoseries of GSLs or GbO4, and recognize all P blood group determinants. Class III G adhesins, encoded by the

$PrsG_{J96}$ sequences, binds to sheep erythrocytes or GbO5, and recognize P blood group determinants with a terminal blood group A residue [16, 18]. This allele is the predominant variant in women with first-episode or recurrent acute cystitis [19]. A fourth PapG allele was recently discovered, the receptor and the significance in UTIs are still unknown [20].

In epidemiological studies P fimbriae have shown the strongest association with acute disease severity, with at least 90% of acute pyelonephritis but less than 20% of ABU strains expressing this phenotype [13, 21, 22].

1.1.2.2. Type 1 fimbriae

Type 1 fimbriae are encoded by the *fim* gene cluster, and are the most abundant adhesion factors on *E. coli*. The adhesin FimH is situated along the shaft and at the top of the fimbriae. Type 1 fimbriae recognize mannose on secreted and cell bound proteins, and bind to β-1 and α-3 integrins [23], Tamm-Horsfall protein, secretory immunoglobulin A and the mannose-containing CD48 receptor on mast cells. In vitro studies have shown that upon type 1 fimbrial binding to uroplakins the bacteria invade the underlying immature cells, and form intracellular bacterial communities [24].

Studies on the role of type 1 fimbriae as a colonization factor in the human urinary tract have provided contradictory results. As most *E. coli* isolates carry the *fim* operon regardless of their source [25], the expression of the type 1 fimbriae, or possession of the *fim* gene cluster has not convincingly shown to correlate with uro-pathogenicity in humans [8, 13, 22]. On the other hand, the expression of type 1 fimbriae was shown to characterize the most virulent members of a single clone (O1:K1:H7) and a deletion of the *fim* gene cluster from that background was shown to attenuate virulence in the murine UTI model [26].

1.1.2.3. S fimbrial family

Members of the S-fimbrial family of adhesins consists of S-fimbriae (*sfa*), with its subtypes *sfaI* and *sfaII*; F1C-fimbriae (*foc*); S/F1C-related fimbriae (*sfr*) [27] [28] and AC/I-fimbriae (*fac*). S-fimbrial adhesins recognize α-sialyl-2-3-β-lactose-containing receptors and are predominantly expressed by strains responsible for meningitis and sepsis but they have been described in strains causing UTIs as well [28, 29], whereas F1C-fimbrial adhesins bind to β-GalNac-1,4-β-Gal-containing structures [30] and are preferentially expressed by UTI isolates [28].

1.1.2.4. Flagella

Flagella are filamentous structures attached to the surface of the bacteria. The flagellar filament is a polymer of flagellin subunits encoded by the *fliC* gene [31]. The filament is rotated by a motor apparatus in the plasma membrane [32] thus increasing bacterial motility. Flagella have been proposed to increase bacterial virulence by providing a selective advantage in the fight for nutrients in the urine and enhance bacterial dissemination to the upper urinary tract [33].

1.1.2.5. Biofilm

A biofilm is a structured community of microorganisms encapsulated within a self-developed polymeric matrix adherent to a surface [34]. In the urinary tract the formation of biofilm may protect bacteria against environmental stress, phagocytosis and antibiotics. Curli are bacterial surface organelles that bind several host extracellular matrix and contact phase proteins. These adhesive fibers enhance bacterial biofilm formation on various abiotic surfaces. In vitro, isolates causing asymptomatic bacteriuria formed biofilm more readily than isolates from acute pyelonephritis [35].

1.1.2.6. Lipopolysaccharide

Lipopolysaccharides (LPS) can be found in the outer membrane of Gram-negative bacteria. LPS is composed of 3 covalently linked components: outer carbohydrate chains of 1-50 oligosacharide units called the O antigen or O-specific side-chain; a core oligosaccharide; and an interior disaccharide with multiple fatty acids, called lipid A, which is responsible for much of the toxicity of Gram-negative bacteria (endotoxin). Some O antigen serotypes (i.e. O1, O2, O4, O6, O7, O8, O16, O18, O25, O50 and O75) were shown to be frequent among UPEC strains [36]. LPS interact with other virulence factors (e.g. LPS-dependent targeting of HlyA to host cell membranes) and may also play a role in the protection against the human immune system [37].

1.1.2.7. Siderophore systems

Bacteria need iron ions to successfully colonize the urinary tract. Bacteria express siderophores that scavenge iron from the environment to overcome iron limitation in the host [37]. *E. coli* strains may express different types of siderophores. Aerobactin is frequent in UPEC isolates, as it was shown to be present in about 45% of symptomatic isolates [38], but multiple systems may be expressed during colonization. Siderophore receptors may have dual functions, as the salmochelin siderophore receptor IroN also functions as an internalization factor promoting the invasion of urothelial cells by UPEC in vitro [39].

1.1.2.8. Toxins

Many UPEC secrete toxins that facilitate infection by damaging host tissues or by disabling the host immune system. The α-hemolysin (HlyA) is a pore-forming toxin, which is encoded by about 30-50% of UPEC isolates [37, 40]. The expression of α-hemolysin was shown to increase the clinical severity of urinary tract infections [40]. In high concentrations, α-hemolysin leads to cell lysis [41, 42], however, sublytic concentrations seem to be more physiologically relevant, when α-hemolysin was proposed to inhibit chemotaxis and phagocytosis as well as stimulation of host apoptotic and inflammatory pathways [37, 43, 44].

Cytotoxic necrotizing factor 1 (CNF1) is present in about 30% of UPEC strains [40]. CNF1 activates Rho GTPases in the host cell [45], promotes apoptosis of bladder epithelial cells [46] and counteracts phagocytic activity and chemotaxis of polymorphonuclear neutrophils (PMNs) [47].

1.1.2.9. TIR domain containing proteins

Toll/interleukin-1 receptor (TIR) domain containing proteins (Tcps) are soluble proteins which inhibit Toll-like receptor (TLR) signaling. Tcps are homologues of the Toll/Interleukin-1 receptor domain, and are secreted by virulent bacteria. TcpC promotes bacterial survival by inhibiting the innate host response and specifically MyD88 dependent signaling pathways. TcpC was shown to be clinically relevant as a virulence factor in UTIs with severe kidney infections, and promoted renal tissue damage in murine models of UTI [48].

1.1.3. Host response induction

The urinary tract relies primarily on innate immunity for its defense [49]. UPEC pathogenesis initiates with bacterial attachment to superficial bladder epithelial cells. The attachment is recognized by the cells, and triggers intracellular signaling proteins, transcription of target genes and release of effector proteins [50, 51] (Figure 1). TLR4 signaling is crucial for recognition of *E. coli* in the urinary tract, and may be activated via binding P fimbriae or type 1 fimbriae to uroepithelial receptors, which trigger different signaling pathways [51, 52]. The activated epithelial cells secrete chemokines (Interleukin [IL]-8), cytokines (IL-6, tumor necrosis factor [TNF]) and antimicrobial peptides (LL-37) depending on the activated signaling pathway. IL-8 is a strong chemoattractant for PMNs, its attachment to its receptors CXCR1 and CXCR2 on PMNs results in neutrophil recruitment and migration across the uroepithelium, and eventually the clearance of infection.

The same cells respond poorly to asymptomatic carrier strains and proinflammatory pathways are not activated but suppressed [53]. This unresponsiveness is probably essential to protect the host from constant innate immune activation and to permit the symbiotic relationship between bacteria and host to develop into the commensal like and protective state of ABU.

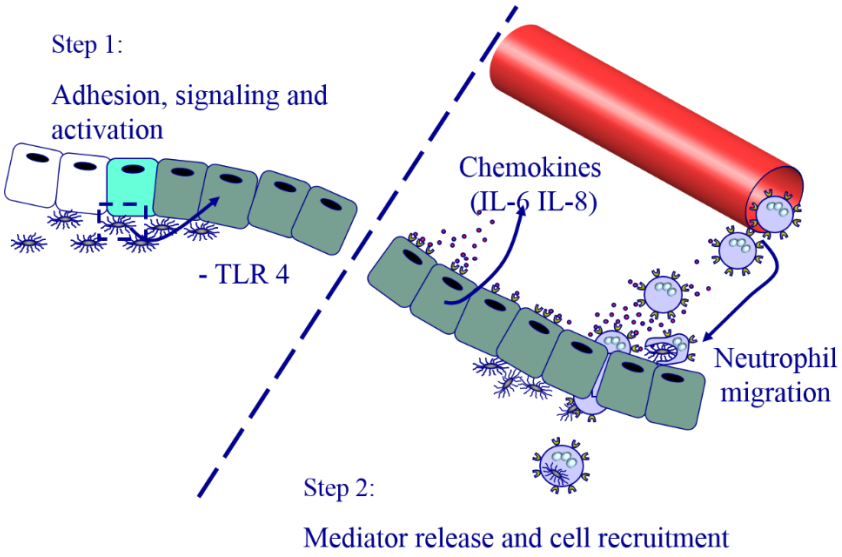

Figure 1. Host response induction in the urinary tract

1.2. Deliberate establishment of asymptomatic bacteriuria

1.2.1. Asymptomatic bacteriuria

Patients with asymptomatic bacteriuria (ABU) may carry more than 10^5 cfu/ml of bacteria in their urine for months or years without developing symptoms or sequels [54]. ABU was generally considered as a harmful state until the 1970's, and it was aggressively treated accordingly. In the next decades, more and more publications showed that ABU is actually a harmless condition in patients without risk factors [55]. Furthermore, Hansson and co-workers showed in pediatric populations that ABU may be protective against recurrent episodes of UTIs [56], as children with long time asymptomatic bacteriuria developed symptomatic infections more often, when they were treated from ABU. This beneficial effect has been attributed to bacterial interference by competition for nutrients and by bacterial production of toxic molecules [57]. ABU creates a special form of colonization resistance in the urinary tract, similarly to the intestinal or vaginal microflora, thus it can prevent superinfections with more virulent strains.

1.2.2. *Escherichia coli* 83972

Bacteria causing ABU differ from bacteria causing symptomatic infections. *E. coli* 83972 was first isolated in schoolgirls with ABU [55]. It is a non-fimbriated strain belonging to a non-pathogenic OKH serogroup (OR:K5:H-)[55] and a phylogenetic lineage B2 of *E. coli*, indicating a close relatedness to the UPEC strains which cause symptomatic UTI [58].

The strain has been fully sequenced and extensively studied. *E. coli* 83972 carries the different virulence genes, but does not express them, and never has been shown to express functional adhesion properties. It has a large deletion in the *fim* gene cluster and several point mutations in the papG adhesin, rendering both type 1 and P fimbriae unable to adhere, thus capable of colonizing the urinary tract for a long period. It carries a 1.6 kb plasmid, which is stable, and can be used for strain identification [59, 60]. *E. coli* 83972 was initially classified as O- and K antigen negative, due to weak surface antigen expression. Based on genome sequence analysis [61], an O antigen determinant which in part represents so far unknown DNA sequences and a group II capsule determinant coding for the K5 capsular type was identified. Analysis of the LPS O side-chain pattern demonstrated that *E. coli* 83972 lacks long O antigen side chains, explaining why it was initially classified as O-negative.

1.2.3. Inoculation studies

The idea of deliberately established bacteriuria of the lower urinary tract was born based on the observations regarding the protective effect of ABU in Lund, Sweden. *E. coli* 83972 was chosen for the purpose of colonization. The first colonization studies showed that the deliberate establishment of asymptomatic bacteriuria is a safe procedure without side effects, and long term asymptomatic bacteriuria can be achieved in patients with residual urine [59]. The presence of residual urine facilitates the development of stabile bacteriuria, and it is usually required for the successful colonization of the bladder.

In 2010 a prospective randomized, controlled study of bacterial colonization was carried out [62]. In this study the authors compared the time to the first UTI and the total number of UTIs in during 12 months in the same patients with and without *E. coli* 83972 bacteriuria (inoculated with saline only). The authors found that the time to the first UTI was significantly longer with than without *E. coli* 83972 bacteriuria (median 11.3 vs 5.7 months, $p < 0.0129$). Also, there were significantly fewer UTI episodes during 12 months in the bacteriuria group compared to the placebo group (13 vs 35 episodes, $p < 0.009$). There was no febrile UTI episode in either of the study arms and no significant side effects of intravesical inoculation were reported.

1.2.4. Deliberately established asymptomatic bacteriuria as a research model

The deliberate establishment of asymptomatic bacteriuria is not only a method for prevention, however. It creates a unique situation, where we have an extensive knowledge of both the pathogen and the host, and we also control the time of the infection. With this model of controlled uroinfection we can monitor the pathogen-host interactions in the human urinary tract, which gives us countless research opportunities regarding the molecular basis of UTIs.

The results of the colonization studies showed that colonized asymptomatic patients have a slightly elevated levels of neutrophils and cytokines in their urine, representing a low, but significant local host response. Interestingly, the rate of the host response varies between patients, they can be grouped as low or high responders. However, the same individual characteristic response can be observed for each patient during repeated colorizations [63].

Analysis of the bacterial isolates regained from colonized patients in different time points after colonizations provided the first, genome-wide example of a single bacterial strain's

evolution in different human hosts. The results showed different bacterial genetic changes in case of each colonized patients, proving that each host “personalizes” their microflora, and that this adaptive bacterial evolution points towards commensalism rather than virulence [61].

2. AIMS

Our major aim was to analyze the role of bacterial virulence factors in the clinical course and outcome of urinary tract infections caused by *Escherichia coli*.

With the analysis of *Escherichia coli* 83972 strains isolated from symptomatic episodes during deliberately established *E. coli* 83972 bacteriuria we aimed to (*Paper I*):

- Investigate if a reversion to a functional virulence gene repertoire by these isolates may account for the switch from asymptomatic carrier state to symptomatic lower urinary tract infections.
- Identify changes in the bacterial genome of *E. coli* 83972 strains isolated during symptomatic episodes.
- Assess the safety of the method of deliberately established ABU by investigating the potential of the colonizing bacteria to reacquire virulence.

With the virulence factor analysis of clinical *Escherichia coli* isolates from urinary tract infections we aimed to investigate (*Paper II*):

- If *Escherichia coli* strains causing acute cystitis can be characterized by a distinct virulence factor repertoire.
- If the virulence factor profile of *E. coli* strains causing acute cystitis can be distinguished from the virulence factor profile of the strains causing acute pyelonephritis.

3. MATERIAL AND METHODS

3.1. Analysis of *E. coli* 83972 strains isolated from symptomatic episodes during deliberately established bacteriuria

We investigated *E. coli* 83972 re-isolates from symptomatic UTI episodes of patients colonized in a previously published human inoculation study (*Trial ID number*: RTP-A2003 - International Committee of Medical Journal Editors [*https://register.clinicaltrials.gov*]. [62]. Patients with incomplete bladder emptying due to spinal or lower motor neuron lesions who had recurrent lower UTIs were included in this placebo controlled study of intravesical inoculation with *E. coli* 83972. The human ethics committee at Lund University approved the study and patients gave their informed consent. The study design, patient characteristics and clinical results are described in detail in the paper by Sundén et al [62].

3.1.1. Inoculation protocol

Before inoculation pre-existing bacteriuria was eliminated by antibiotic treatment, and after an antibiotic-free interval urine was cultured to confirm sterility. The patients were catheterized (14 Ch. Low-Fric - Astra), and after complete evacuation of the bladder, 30 ml of the bacterial suspension of *E. coli* 83972 (10^5 cfu/ml) was injected, and the catheter removed. The procedure was repeated once daily for 3 days.

3.1.2. Symptomatic urinary tract infection episodes

We examined the subset of colonized patients in whom symptomatic UTI episodes developed. Patients defined UTI episodes with a previously developed self-reported method [64], and UTI were also determined in a structured interview and by urine culture yielding greater than 10^5 cfu/ml of a single organism. Symptomatic episodes were defined by at least 2 symptoms, including suprapubic pain, dysuria and/or frequency as well as increased spasticity in patients with a spinal cord lesion.

3.1.3. Bacteria, cytokines and DNA Techniques

E. coli 83972 re-isolates were identified by polymerase chain reaction (PCR) using primer pairs that matched the cryptic 1.6 kb plasmid and the internal 4,253 bp fim deletion. For

in vitro analysis strains were grown in lysogeny broth (LB) or in pooled human urine with or without 1.5% agar (Difco™).

Neutrophils were quantified in uncentrifuged urine using a hemocytometer chamber. We quantified IL-6 and 8 concentrations by Immulite® assay.

Qiagen® products were used for genomic DNA isolation. Primers were obtained from Eurofins MWG/Operon, Ebersberg, Germany. Restriction enzymes were obtained from New England Biolabs®. Genomic DNA was analyzed by pulsed field gel electrophoresis.

Phylogenetic classification of the re-isolates was done according to the results of a triplex PCR using a method described by Clemont et al [65].

3.1.4. Bacterial genotypes

The detection of fitness- and virulence-associated genes of extraintestinal pathogenic *E. coli* (ExPEC) included type 1 fimbrial (*fim*), P fimbrial (*pap*), F1C fimbrial (*foc*) gene clusters, the genes coding for the toxins alpha-hemolysin (*hlyA*) or cytotoxic-necrotizing factor (*cnf*1), the yersiniabactin siderophore receptor (*fyuA*), the salmochelin siderophore receptor (*iroN*), the aerobactin siderophore (*iuc*) as well as the K5 capsule (*kpsMT* K5) determinant and the pathogenicity island marker *malX*. Genotyping was performed by PCR using primers that matched unique regions of the gene sequences [28, 58, 66].

3.1.5. Phenotypic assays

Type 1 fimbrial expression was detected by hemagglutination of guinea pig and human erythrocytes after in vitro passage in Luria broth. Agglutination was performed both in the presence and absence of α-methyl-D-mannoside. Strains causing mannose sensitive agglutination were defined as type 1 fimbriated [67].

P- and S/F1C fimbriae were detected by hemagglutination of defibrinated human and bovine erythrocytes, respectively. Aliquots of bacterial overnight cultures in LB or pooled human urine were incubated with a suspension of human or bovine blood (Elocin lab, Munich). Hemagglutination was compared after incubation for some minutes on ice. UPEC strain 536 was used as a positive and *E. coli* strain HB101 as a negative control.

Hemolytic activity was detected on sheep blood agar plates (Oxoid) after overnight incubation at 37 °C as the formation of clear halos around the colonies. UPEC strain 536 was used as a positive and *E. coli* strain HB101 as a negative control.

The aerobactin siderophore was detected by the aerobactin cross-feeding bioassay [68]. 10^9 cells of aerobactin-requiring indicator *E. coli* strain LG1522 were cultured in M9 soft agar containing 200 mM 2'-2'-dipyridyl (Sigma, Deisenhofen, Germany). Aerobactin production by the test strains was indicated by a zone of enhanced growth of *E. coli* LG1522 around the colonies of the test strains. *E. coli* ABU strain 83972 was used as a positive and *E. coli* strain HB101 as a negative control.

Morphotype analysis on Congo red and Calcoflour plates was used to study curli fimbria and cellulose expression [69].

3.1.6. Biofilm formation

Biofilm formation was assessed in a microtiter plate assay modified after O'Toole and Kolter [70]. Bacteria were grown overnight in LB medium at 37 °C with agitation. Filter-sterilized pooled human urine was then inoculated (1:100) with the overnight bacterial culture and 160 µl of this inoculum was pipetted into 96-well U-bottom flexible microtiter plates (8 wells per strain). Microtiter plates were incubated statically at 37 °C for 48 h. Afterwards, the medium was removed and the microtiter plates were washed twice with 1% PBS followed by drying at 65 °C for 10 min. The plates were then stained with 0.1% crystal violet for 10 min. Next, plates were washed twice with 1% PBS and dried at 65 °C for 10 min. Absorbed crystal violet was eluted using 180 µl acetone-ethanol (1:5), pooled and diluted 1:10. Finally, optical density was measured at 580 nm. Biofilm assays were performed at least in triplicate.

3.1.7. O antigen side chain analysis

Isolation of LPS from the *E. coli* strains used in this study was performed as previously described by Grozdanov et al [71].

3.1.8. Motility

Overnight cultures were stabbed into the middle of motility agar plates (LB supplemented with 0.3% w/v agar). Plates were incubated for 16 h at 37 °C. Motility was then assessed by inspection of the migration zone of the bacteria. Three independent experiments were performed with three individual colonies per strain.

3.1.9. Bacterial growth in pooled human urine

Growth of the bacterial isolates was compared to *E. coli* 83972 wild type by growing them without agitation in pooled human urine overnight and inoculating 30 ml fresh medium the following day with the overnight culture. Optical density was measured at 600 nm every hour for 8 h and overnight. The experiment was repeated three times using different batches of pooled human urine.

3.1.10. Gene expression profiling

RNA preparation and microarray hybridisation was performed as previously reported [61]. For total RNA isolation, the strains were grown statically in pooled human urine at 37 °C until they reached mid-logarithmic phase. Samples were then treated with RNAprotect (Qiagen) and extracted using the RNeasy mini kit (Qiagen). DNA traces were removed by RNase-free DNase I (New England Biolabs).

For expression profiling, custom-tailored oligonucleotide microarrays (Operon Biotechnologies) were used. 10 μg of total RNA were reverse transcribed (SuperScript III, Invitrogen) with direct incorporation of fluorescently labelled (Cy3- or Cy5-) dCTP (GE Healthcare). 160 pmol of each Cy-3 and Cy-5 labelled probe were used for hybridisation. For each experiment, at least three independent hybridizations were performed. Hybridized and washed slides were scanned using a GenePix 4000B Microarray Scanner (GE Healthcare) with a resolution of 10 μm pixel size.

The data was further analyzed with Acuity 4.0 (Molecular Devices) including normalization by a linear ratio-based method. For statistical significance, one sample t-test was applied with Bonferroni correction. For data analysis, a cut-off value of 1.7 (ln2) was used with $p \leq 0.09$. Hierarchical clustering and visualization of expression patterns was performed with CLUSTER and TREEVIEW [72], respectively.

3.1.11. In vitro cell experiments

The human kidney carcinoma A498 (ATCC HTB-44) and T24 bladder carcinoma cell lines were grown in RPMI 1640 supplemented with 1 mM sodium pyruvate, 1 mM non-essential amino acids, 50 mM/ml gentamicin, and 10% fetal calf serum (FCS - PAA

Laboratories, Pasching, Austria). Cells were maintained at 37 °C + 5% CO2 in a humidified atmosphere and split weekly.

For adhesion studies 10^9 cells were exposed to 10^5 bacteria (*E. coli* 83972 wild type, the symptomatic re-isolates from the patients and CFT073 as positive control) for 45 minutes. After washing adhesive bacteria were counted and microscopic pictures were taken.

For host response experiment cells were exposed to *E. coli* 83972 wild type, the symptomatic re-isolates or CFT073 (10^8 cfu in 0,01 mL) diluted in 1 mL media with 5% FCS or without FCS. Supernatants were collected 6 and 24 hours after stimulation and the secreted cytokines (IL-6 and IL-8) were quantified by Immulite 100 (Siemens, Bad Nauheim, Germany).

3.1.12. Experimental animal infections

Experiments were performed with the permission of the animal experimental ethics committee, Lund District Court, Sweden. Female C3H/HeN mice bred at the MIG animal facility were used at age 6 to 12 weeks. After anesthesia (Isofluorane), mice were infected by intravesical inoculation with *E. coli* 83972 wild type and the symptomatic re-isolates (10^9 cfu in 0.1 mL) through a soft polyethylene catheter (outer diameter 0.61 mm; Clay Adams, Parsippany, NJ, USA). Animals were sacrificed at 6 hours, 24 hours and 7 days while under anesthesia, and the kidneys and bladders were removed. Viable counts in homogenized tissues were determined after overnight growth on tryptic soy agar plates at 37 °C. Urine samples collected prior to and daily after infection were cultured and recruited neutrophils were quantified in uncentrifuged urine by use of a hemocytometer.

3.2. Virulence factor analysis of clinical *Escherichia coli* isolates from urinary tract infections

We examined the virulence factor repertoire of *Escherichia coli* strains prospectively isolated from women with community-acquired acute cystitis. The course of UTIs and upper urinary tract involvement were documented.

3.2.1. Patients, UTI episodes

Women > 18 years of age with symptomatic UTI were enrolled in the analysis. They had significant bacteriuria, defined as $\geq 10^4$ cfu/ml. Patients were diagnosed with acute cystitis

based on the following symptoms: frequency, dysuria and/or suprapubic pain, a temperature <38.0 °C and no flank pain. Patients who also had flank pain and/or fever were diagnosed as having acute cystitis with upper urinary tract involvement. The UTI episode was classified as sporadic (<two episodes during the previous six months or <three during the previous 12 months) or recurrent. The history of previous UTI, concomitant disease and medical treatment were recorded. Blood samples were obtained at diagnosis and examined for C reactive protein (CRP, cut off ≥ 10 mg/l) and white blood cell counts (cut off ≥ $10x10^9$/l).

3.2.2. Urine cultures

Midstream urine samples were obtained at diagnosis. Quantitative urine cultures identified 247 *E. coli* growing as monocultures, and the isolates were stored in deep agar stabs. For analysis, bacteria were grown overnight on tryptic soy agar plates at 37 °C.

3.2.3. Bacterial genotypes, phenotypes and hemolysin production

We genotyped the gene sequences coding the virulence factors (*pap* gene cluster - $papG_{IA2}$, $prsG_{J96}$*; fim; TcpC).* The genotypes were defined by PCR, using primer pairs that matched unique regions of the adhesin sequences [48, 73].

The expression of type 1 and P fimbriae, curli and cellulose, as well as biofilm formation was determined as described previously.

Hemolytic strains were identified in nutrient agar with 5% washed horse erythrocytes after overnight incubation. A hemolytic zone larger than the overlying colony was considered positive [74].

3.2.4. Statistical analysis

Chi-square test or the Fisher's exact test was used. P <0.05 was considered statistically significant (two-tailed).

4. RESULTS

4.1. Analysis of *E. coli* 83972 strains isolated from symptomatic episodes during deliberately established bacteriuria

4.1.1. Symptomatic UTI episodes during *E. coli* 83972 bacteriuria

In the placebo controlled colonization study by Sundén et al, the number of symptomatic UTI episodes was significantly reduced in patients inoculated with *E. coli* 83972 compared to the placebo control group (inoculated with saline only) or to the patients previous condition before the study. A small group of the patients with *E. coli* 83972 bacteriuria developed symptomatic UTI, however. Before the symptoms developed these patients carried *E. coli* 83972 asymptomatically without discomfort. The reported UTI episodes were non-febrile lower urinary tract infections and antibiotic treatment resulted in prompt symptom relief.

Out of the 20 patients, who spent a total of 202 months (mean 10.1) with *E. coli* 83972 bacteriuria 13 symptomatic episodes were reported in nine patients. In 10 cases super-infection was caused by a different *E. coli* strain (n=7), *Pseudomonas aeruginosa* (n=1), *Enterococcus faecalis* (n=1) or *Proteus mirabilis* (n=1). *E. coli* 83972 infection was verified in three cases, two of which occurred in one patient. In patients R4 and R15 only *E. coli* 83972 was recovered during 1 and 2 symptomatic episodes, respectively, suggesting that the symptomatic episodes were caused by the colonizing strain. The 3 symptomatic episodes were accompanied by elevated urine cytokine levels (IL-6 and IL-8), and increased urine polymorphonuclear leukocyte numbers indicating a significant host response.

To examine if a change in bacterial properties had precipitated the symptomatic episode, the three *E. coli* 83972 isolates were further examined. In addition, we included two *E. coli* 83972 isolates from symptomatic episodes in patients not included in the previously published study. One from patient R10, who carried *E. coli* 839972 after the closure of the study for a total of 104 days, when he had a symptomatic infection, and one from patient Sp10, who was successfully colonized with *E. coli* 83972, and who developed symptoms after 67 days of *E. coli* 839972 bacteriuria, but who was excluded from the study due to steroid treatment. Thus, a total of 5 symptomatic re-isolates were included in the analysis (Table 1).

The re-isolates were verified as *E coli* 83972 by identification of the cryptic 1.6 kb plasmid and the internal 4,253 bp *fim* deletion. Genomic restriction patterns of all re-isolates were defined by pulse field electrophoresis and were found to be identical to *E coli* 83972.

	Pt R4	Pt R15 (episode No.) 1	Pt R15 (episode No.) 2	Pt R10	Pt Sp10
Gender	F	F	F	F	M
Age	60	46	46	47	67
Diagnosis	Detrusor insufficiency, post-void residual urine	Detrusor insufficiency, post-void residual urine		Detrusor insufficiency, post-void residual urine	Spinal lesion, neurogenic bladder disorder
UTI episode:					
Days after inoculation	192	20	19	104	67
Symptoms	Suprapubic pain, dysuria, frequency	Local discomfort, dysuria, frequency		Suprapubic pain, dysuria, frequency	Local discomfort, dysuria, increased spasticity

F= female M=male

Table 1. Characteristics of 4 patients with *E. coli* 83972 asymptomatic bacteriuria and total of 5 proven UTI episodes caused by *E. coli* 83972

4.1.2. Virulence properties of the symptomatic re-isolates

The re-isolates were characterized regarding virulence genes associated with extraintestinal pathogenic *E. coli*. All of the re-isolates carried the examined gene sequences. The re-isolates were also examined for the expression of virulence factors. The strains did not express functional P, type 1, or F1C fimbriae. To exclude that *E. coli* 83972 had acquired new adhesins the re-isolates were incubated with human uroepithelial cells (A498 kidney cells and T24 bladder cells). The re-isolates, such as the wild type failed to adhere to the human cells (Figure 2). There was no consistent change in biofilm, curli or cellulose formation. Re-isolates expressed an O antigen pattern identical to that of *E. coli* 83972.

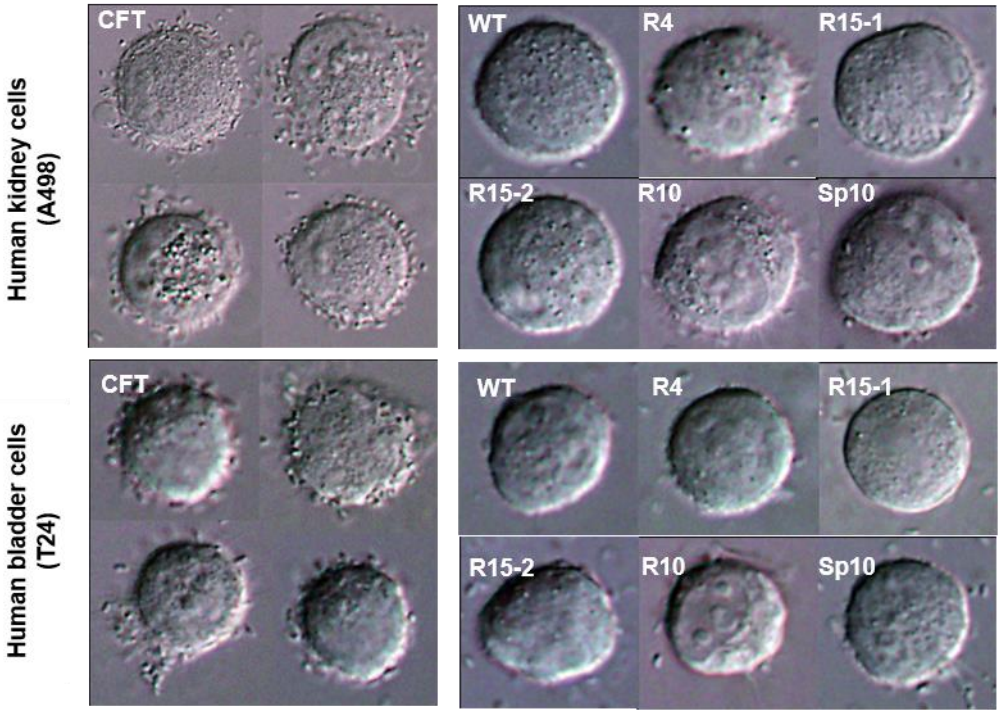

Figure 2. Adhesion to T-24 bladder epithelial cells and A498 kidney epithelial cells. No difference in adhesion capacity between the wild type and the re-isolates.

4.1.3. The presence of heterogeneous phenotypes

Further phenotypic assays were performed to identify differences in the expression of bacterial traits that might influence survival in the urinary tract. Growth rates in urine were monitored and compared to growth in Luria broth. No increase was observed in growth rates compared to the *E. coli* 83972 wild type, however re-isolates of samples R15-1 and R15-2 showed heterogeneous phenotypes and comprised colonies of different sizes or motility.

For colonies from urine sample R15-1 about 75% the colony size and morphology resembled those of strain *E. coli* 83972 (R15-1 clone I). The remaining colonies (R15-1 clone II) were small, and these variants grew more slowly in liquid medium than *E. coli* 83972 wild type. These colonies were also identified as *E. coli* 83972 with the analysis of the specific plasmid, *fim* deletion, restriction pattern and virulence genes. The slow growth and reduced colony size were reminiscent of small colony variants associated with persistent infection. Individual colonies of urine sample R15-2 differed in motility and flagella expression.

4.1.4. Motility

Motility has been proposed to be an important virulence factor, as flagella enhance the ascent of bacteria from the lower urinary tract to the kidneys [75]. Motility of the strains was therefore screened on swarming agar plates and compared with the *E. coli* 83972 wild type. We observed an increase in motility in two of the re-isolates, R15-2 and Sp10. While Sp10 was a 100% motile re-isolate, R15-2 appeared as a phenotypically heterogeneous population with individual cells displaying increased motility (R15-2 clone I), while the motility of the remaining cells (R15-2 clone II) did not differ from the *E. coli* 83972 wild type (Figure 3). The increase in motility reflected increased expression of flagella as shown by Western Blot analysis.

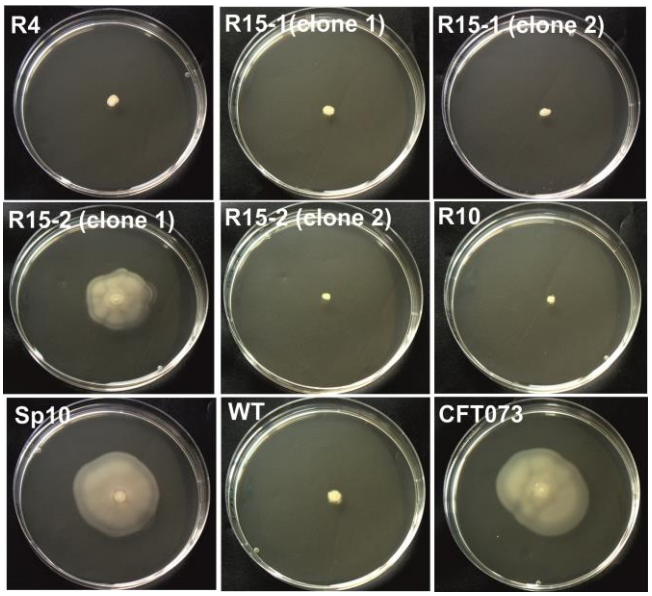

Figure 3. Motility of *E. coli* 83972, the symptomatic re-isolates and CFT073 as positive control on urine swarm agar plates

Whole genome transcription analysis was conducted to compare the transcriptional profile of *E. coli* 83972 to the motile re-isolates (R15-2 clone I and Sp10). There were 95 de-regulated genes in case of R15-2 clone I. 80 genes were up-regulated and 15 were down-regulated. Most up-regulated genes encoded bacteriophage components, and genes were also involved in the stress response, including *recA, recN, lexA, ruvB, dinI, dinB, sulA, yebG* and *umuD,* and sigma factor expression (*rpoA, rpoE and rpoS*). In addition, acid stress response genes (*gadA, gadB, hdeAB, cadB and slp*) were up-regulated. In contrast, a group of genes

involved in phosphotransferase transport were down-regulated. In this re-isolate flagellar gene expression was less pronounced.

In Sp10 from the 98 de-regulated genes 81 were up-regulated and 17 were down-regulated. In this 100% motile re-isolate genes involved in flagella biosynthesis and assembly (*flgA, flgDEFG, flhA, fliA, fliG* and *fliO*) was found to be significantly up-regulated. Other upregulated genes were involved in heat shock response (*groEL* and *groES*), LPS biosynthesis (*rfaGPIJY*, *waaV*, *waaW*, *lpxAB*), amino sugar utilization (*glmUS*), and iron-uptake (*chuATUWXY* and *entCA*). Significantly down-regulated genes were involved in N-acetyl-D-galactosamine (*aga*), sorbitol (*srl*) and galactonate (*dgo*) transport.

Few de-regulated genes were shared by the two re-isolates. Genes up-regulated in both R15-2 and Rp10 were involved in biofilm formation (*yjbE, yqjD*), D-glucarate utilization (*gudD, gudP*) and in the synthesis of proteins of the 30S and 50S ribosomal subunits. Ribose ABC transporter genes were down-regulated in both re-isolates relative to the wild type (Figure 4).

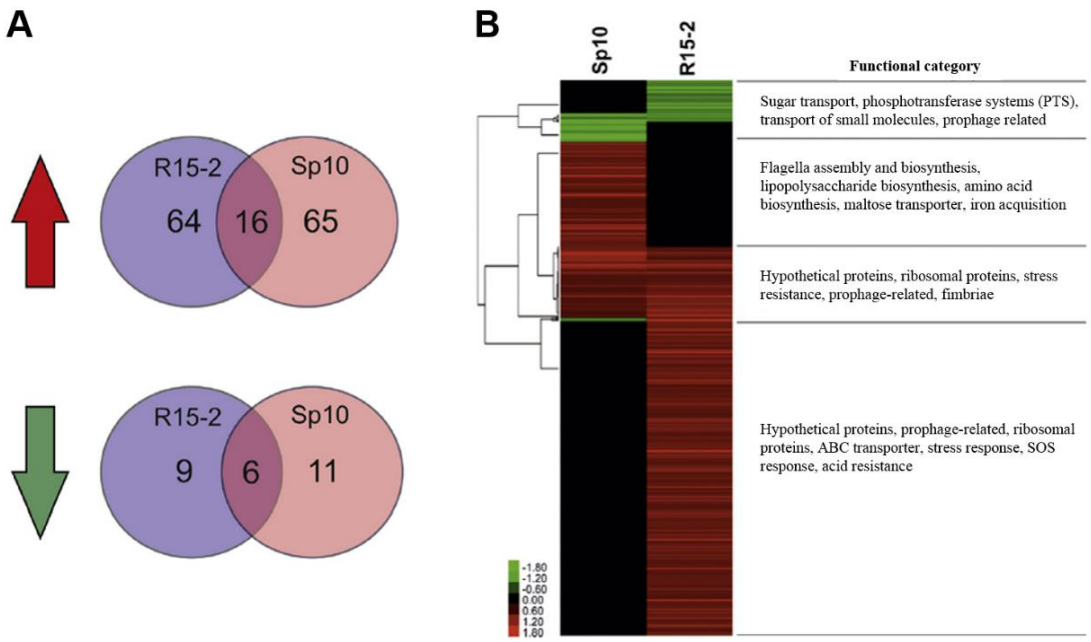

Figure 4. Gene expression profiling of the motile re-isolates. A, gene expression in re-isolates relative to *E. coli* 83972. Most regulated genes were unique to each isolate. B, hierarchical cluster analysis of deregulated genes in re-isolates relative to *E. coli* 83972. Values represent mean expression ratio of at least 3 independent microarray experiments. Green areas indicate significantly regulated (log twofold change, $p < 0.05$) suppressed genes. Red areas indicate significantly regulated (log twofold change, $p < 0.050$) up-regulated genes. Black areas indicate genes without statistically significant change ($p > 0.05$).

4.1.5. Host response induction by the re-isolates

To analyze the re-isolates capacity to induce host response, we performed in vitro host response experiments in A498 human kidney cells. The IL-6 and IL-8 secretion in A498 cells exposed to the symptomatic re-isolates for 24 hours did not differ significantly from the response of the cells exposed to *E. coli* 83972 wild type (Figure 5).

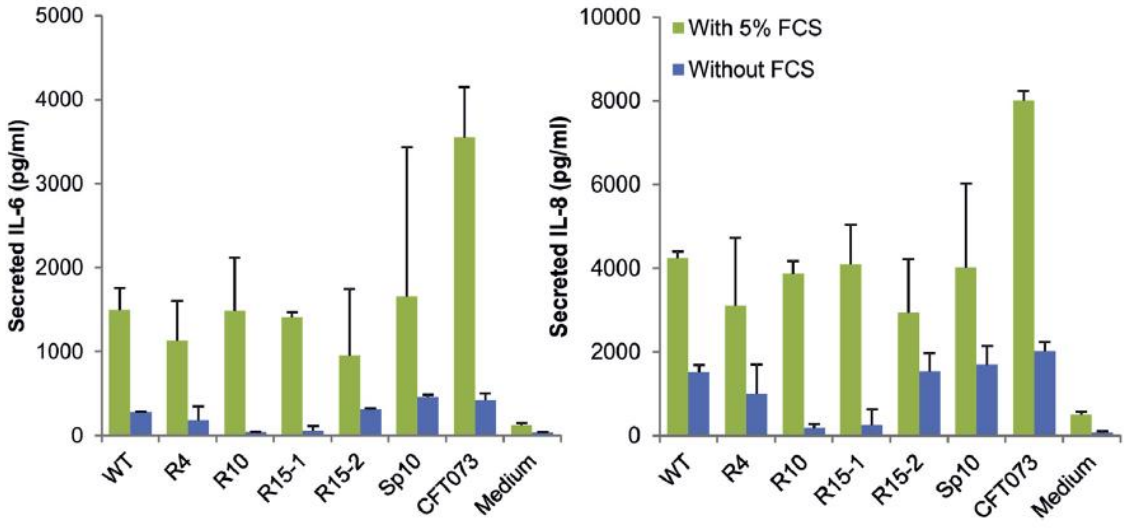

Figure 5. Epithelial response to in vitro infection of A498 cells with *E. coli* 83972 wild type, symptomatic re-isolates and uropathogenic *E. coli* strain CFT073. Geometric means ± SEM of two independent experiments.

Next we performed experimental urinary tract infections with the re-isolates and *E. coli* 83972 wild type in C3H/HeN mice to further investigate if the symptomatic re-isolates reacquired increased virulence. There were no major difference in the bacterial clearance between the strains compared to the wild type, in respect of the bacterial counts in urine, kidneys and bladders or mortality. No symptoms appeared in any group. The kinetics of neutrophil recruitment did not differ between the groups. The level of neutrophils in the urine reached its maximum at 6 hours, and then decreased and remained low until day 7, reflecting a low acute

inflammatory response to *E. coli* 83972. Motility of the re-isolates did not influence bacterial numbers or urine neutrophil counts (Figure 6).

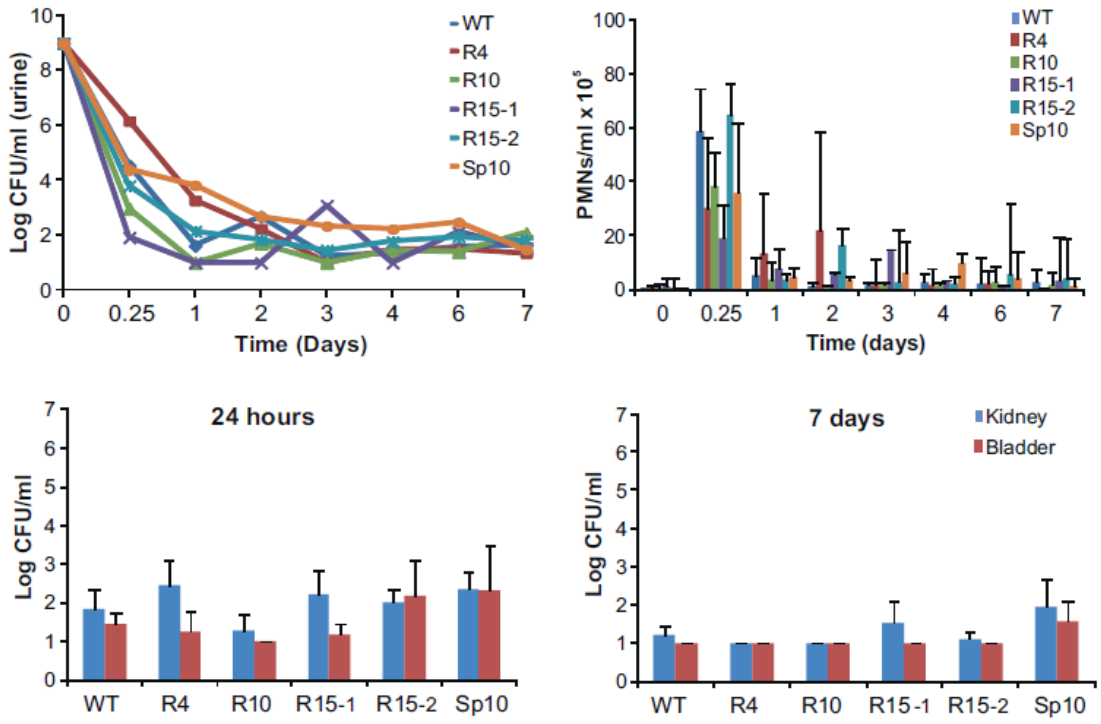

Figure 6. Experimental infection of C3H/HeN mice by intravesical inoculation with 10^9 cfu in 0.1 ml *E. coli* 83972 or re-isolates from symptomatic episodes. Bacterial number in urine, kidneys and bladders, and neutrophil response revealed no difference in virulence.

4.2. Virulence factor analysis of clinical *Escherichia coli* isolates from urinary tract infections

4.2.1. Patient characteristics

247 women with microbiologically proven uncomplicated urinary tract infections were included in the analysis (mean age 51 years, range 18 - 91) and their infecting *E. coli* strains were saved. 242 patients (98%) had bacteriuria $\geq 10^5$ cfu/ml, in 5 cases patients had 10^4 cfu/ml of urine. 215 patients were diagnosed with acute cystitis only, while 32 patients (13%) also had

upper urinary tract involvement. This group had significantly increased CRP levels and white blood cell counts compared to the acute cystitis group ($p = 0.01$ and $p = 0.01$ respectively). The infection was sporadic in 180 cases, while 67 women had recurrent infection.

4.2.2. Virulence factor genotypes and expression

Fim sequences coding type 1 fimbriae were present in 96% of the isolates and type 1 fimbrial expression was detected in 80%. There was no significant difference between isolates from patients with acute cystitis (81%) and patients with upper urinary tract involvement (71%) (Figure 7A).

Hemolysin expression was only detected in 28% in the total sample and the frequency did not differ between the two groups.

Curli fimbriae were detected in 75% of all the cases (73% in case of acute cystitis and 89% in patients with upper tract involvement) and 13% of the strains formed cellulose (14% vs. 10%). There were no significant differences between the subgroups. However, only 16% of all patients formed biofilm, 15% of cystitis patients and 24% of patients with upper UTI, with no significant difference between the subgroups (Figure 7B).

The *pap* gene cluster was detected in 43% of all isolates (*papG*$_{IA2}$ 24%, *prsG*$_{J96}$ 20%, both 3%). The *pap* genotype was more common in the isolates from patients with upper urinary tract involvement (56%) compared to the acute cystitis group (41%), although the difference was not significant (Figure 7C and D). P fimbrial expression (Class II + III) was present in 42% of the isolates. Among those, Class II fimbriae -*papG*$_{IA2}$- were more common (77%) than Class III fimbriae -*prsG*$_{J96}$- (23%) (Table 2). P fimbrial expression was more common in case of upper tract involvement (50%) compared to isolates from patients with acute cystitis (41%), however the difference was not significant (p=0.332). There was no difference in Class II distribution among patients with acute cystitis with or without upper tract involvement (76% versus 81%, p = 0.75).

TcpC was expressed by 33% of the isolates, and it was significantly more common in the upper urinary tract involvement group (32% vs. 42%, p<0.01). TcpC was also significantly more common in case of *papG+/prsG+* strains compared to those lacking these sequences (Figure 7E and F).

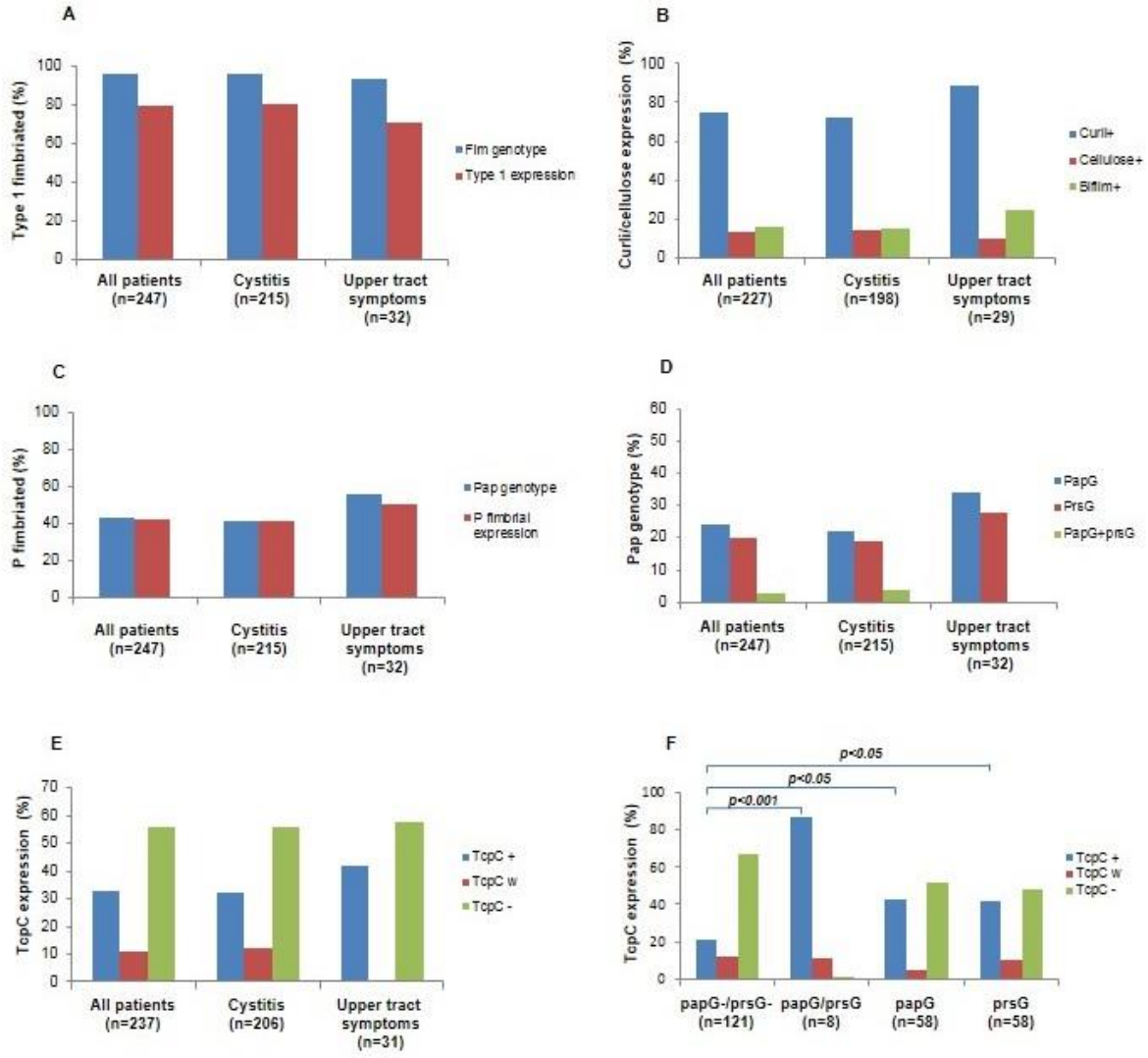

Figure 7. Virulence factor repertoire of *Escherichia coli* isolates from women with acute cystitis. (A) *Fim* genotype and type 1 fimbrial expression (B) Curli, cellulose expression and biofilm formation (C) and (D) *Pap* genotype and P fimbrial expression (E) TIR homologous *TcpC* sequences in the different patient groups, (F) and in relation to the *pap* genotype. 23 isolates were weakly positive and are not included. Significantly higher TcpC frequency in patients with *papG*+ and/or *prsG*+ strains.

Pap genotype and P fimbrial expression	No. of isolates (%)			P values
	All isolates	Cystitis	Upper Tract	
Pap genotype[a], total[b]	247	215	32	
Positive	106 (43)	88 (41)	18 (56)	n.s.
*Pap*G alleles, total	247	215	32	
papG_{IA2}	59 (24)	48 (22)	11 (34)	
prsG_{J96}	50 (20)	41 (19)	9 (28)	
papG_{IA2}+*prs*G_{J96}	8 (3)	8 (4)	0 (0)	
P fimbrial expression, total	247	215	32	
Positive[c]	104 (42)	88 (41)	16 (50)	n.s.
P fimbrial subtypes, total	104	88	16	
Class II[d] (PapG)	80 (77)	67 (76)	13 (81)	n.s.
Class III[e] (PrsG)	24 (23)	21 (24)	3 (19)	n.s.

[a] Analysis based on restriction fragment length polymorphism.
[b] Total = number of isolates examined for each parameter.
[c] Agglutinated human P_1 but not p erythrocytes.
[d] Class II P fimbriated strains defined by agglutination of human A_1P_1, OP_1 but not p erythrocytes.
[e] Class III P fimbriated strains defined by agglutination of human A_1P_1 but not OP_1 or p erythrocytes.

Table 2. *Pap* genotype and P fimbrial expression in *E. coli* isolates

4.2.3. The presence of a combined virulence profile

The *E. coli* isolates were assigned a virulence profile based on their expression of virulence factors (Figure 8). The complete virulence factor repertoire (*fim*, *papG/prsG*, *TcpC* genotypes and curli) was present in 18% of the isolates. Strains expressing the complete virulence factor profile were significantly more common in patients with upper tract involvement compared to acute cystitis only (15% vs. 37%, $p<0.01$). 35% of all the strains had a combined virulence factor with the *fim*, *papG/prsG* sequences and curli, while 76% of the strains were *fim*+ and expressed curli. Both combinations were more common in patients with upper tract involvement (p =0.001 and p <0.05 respectively). There were no significant differences between strains from sporadic or recurrent UTIs.

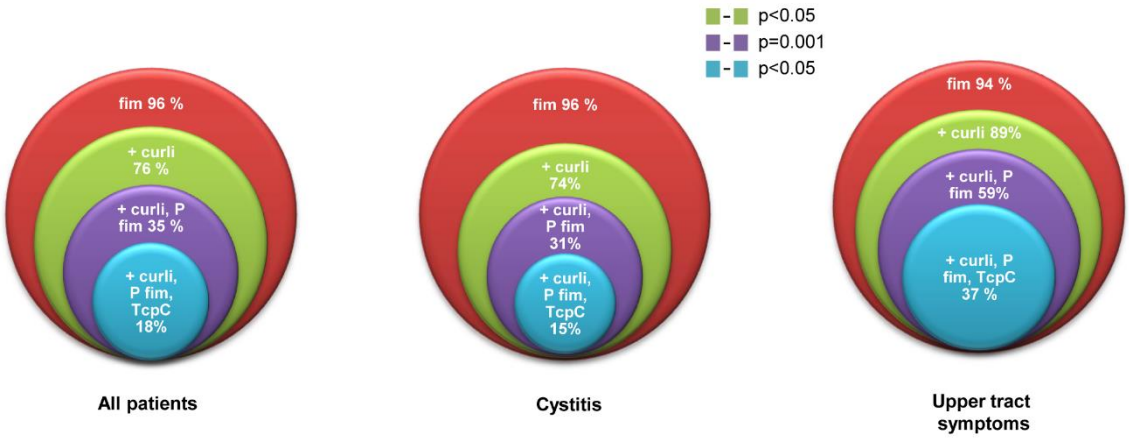

Figure 8. Combined virulence repertoire of the strains. Strains with the combined virulence repertoire were more common in the subgroup of patients with acute cystitis and upper tract involvement compared to patients with acute cystitis alone.

5. DISCUSSION

Virulence factors are instrumental in bacterial infections, as they enhance the ability of the microorganisms to disseminate and overcome host defenses. Pathogens causing urinary tract infections, unlike most commensal bacteria may possess many different virulence factors, which influence the site and severity of urinary tract infections. The different virulence factors play role in different steps of the UTI pathogenesis, and their expression can be variable depending the environment and the host. Despite the extensive research the specific virulence factors responsible for the different clinical manifestations of UTIs have not been convincingly identified.

In our investigation we aimed to analyze the role of the different bacterial virulence factors in the clinical course and outcome of urinary tract infections caused by *Escherichia coli*. With the analysis of *E. coli* 83972 strains isolated from symptomatic episodes during deliberately established asymptomatic *E. coli* 83972 bacteriuria we wanted to identify if there is a change in the virulence profile of these strains responsible for the transition from the stable asymptomatic state to symptomatic lower urinary tract infections. Next, we analysed clinical *E. coli* isolates from urinary tract infections to determine if *Escherichia coli* strains causing acute cystitis can be characterized by a distinct virulence factor repertoire, and if their virulence factor

profile can be distinguished from the strains causing acute pyelonephritis.

5.1. Analysis of *E. coli* 83972 strains isolated from symptomatic episodes during deliberately established bacteriuria

The method of deliberately established asymptomatic *E. coli* 83972 bacteriuria is an effective alternative method for preventing recurrent urinary tract infections. Furthermore it creates a unique opportunity to investigate how the bacteria and the host interact during asymptomatic colonization of the urinary tract. Many asymptomatic bacteriuria strains have lost the ability to express virulence factors due to point mutations and deletions and these strains do not trigger the mucosal inflammatory response that characterizes infection with fully virulent strains. In particular, although *E. coli* 83972 carries the different virulence genes, it does not express functional virulence factors. A personalized bacterial adaptation was observed during previous colonization studies, and this adaptive bacterial evolution pointed towards commensalism rather than virulence during asymptomatic bladder colonization [61]. Evolution toward virulence in colonizing *E. coli* 83972 strains have not been reported.

Patients colonized with *E. coli* 83972 developed symptoms of urinary tract infections significantly less frequent compared to controls in the randomized controlled inoculation study by Sundén et al. Most of the symptomatic episodes were triggered by superinfections caused by other uropathogens, only five cases of symptomatic UTI episodes caused by the colonizing *E. coli* 83972 strains were documented. To detect if changes in bacterial virulence are responsible for the rare development of symptoms during asymptomatic carriage we investigated genotypic and phenotypic changes in the bacterial re-isolates that might have triggered the symptomatic episodes.

We could not prove a reacquisition of virulence factors as a cause of symptoms in our analysis, however. The ExPEC virulence-associated gene set was identical in the *E. coli* 83972 wild type and symptomatic re-isolates, suggesting that the overall pathogenicity island structure remained largely intact. Also, the expression of classical virulence factors, such as fimbrial adhesins, LPS and capsule as well as biofilm formation were not changed by the re-isolates, and we did not observe increased growth rates either.

Since bacterial adhesion is a key-step in the pathogenesis of urinary tract infections, we performed further adhesion studies on uroepithelial cells to rule out the possibility that the re-isolates acquired new adhesins that has not been analysed earlier. However, we did not find any difference between the adhesion capacity of the symptomatic re-isolates and the wild type.

Increased motility compared to the wild type was the only shared feature we found in two of the re-isolates. Flagella have been proposed to provide a selective advantage in the early colonization of the urinary tract [33] and flagellum-driven motility is proposed to enhance bacterial dissemination to the upper urinary tract and facilitate bacterial spread to sites more advantageous for colonization [76]. Sp10 was a 100% motile re-isolate, whereas R15-2 contained a mixture of motile and non-motile cells. Transcriptomic analysis identified individually de-regulated genes in these two isolates mainly involved in stress responses, metabolism and LPS biosynthesis, but no common expression pattern was detected. Flagella biosynthesis and assembly was found to be upregulated only in one of the re-isolates, Sp10. However, we did not observe any difference during the in vitro host response induction experiment between the motile re-isolates and the wild type strain, or any motility-related differences in bacterial persistence during the in vivo murine infection model.

As flagella were found to be involved not just in motility, but in bacterial adhesion as well in case of Enteropathogenic *E. coli* [77] and Salmonella spp. [78] [79] their possible role as adhesive organelles was also proposed in the urinary tract. However, in a study by Wright et al the authors did not find flagellation to be a significant factor in the adhesion and invasion of uroepithelial cells [75]. Our data support this finding, as flagella did not to promote adhesion in the case of the re-isolates. The results imply that although two of the re-isolates were motile, their motility did not contribute to an increased virulence in the urinary tract.

To exclude the possibility that the symptomatic re-isolates acquired any unidentified virulence factors not measured by the previous investigations, we compared the virulence of the re-isolates with the *E. coli* 83972 wild type using an in vitro cell host response experiment and in vivo experimental murine UTI model. We did not observe any difference in the host response induction in A498 human kidney cells by the re-isolates compared to the wild type measured by IL6 and IL-8 secretion. Also, there were no significant differences in the bacterial virulence between the re-isolates compared to the wild type in the murine UTI model in respect of bacterial counts in urine, kidneys and bladders, kinetics of neutrophil recruitment, or mortality. If the development of symptoms were due to changes in bacterial virulence, we would have expected the re-isolates to trigger an increased host response in vitro and show an increased fitness in the in vivo UTI model. This would have been reflected by higher counts in bladders and kidneys and in parallel, we would have expected these strains to trigger an inflammatory response not observed after infection with the wild type strain. Such changes were not observed, however. Thus, our results suggest that the symptomatic episodes during long-term asymptomatic carriage of *E. coli* 83972 do not reflect regained expression of

established virulence factors.

The individual changes in the pattern of phenotypic traits and the transcriptional profile of the re-isolates may suggest that these changes may be attributed to the involvement of response mechanisms of the hosts and not only on the characteristics of the bacteria. For example up-regulation in the genes coding for acid stress response in R15-2 clone I may indicates that this re-isolate has undergone a drastic change in environmental pH.

Moreover, since there were no distinct general changes in the virulence among the re-isolates which could explain the occurrence of symptoms, it may seem reasonable to speculate whether such symptomatic episode was not triggered by bacterial changes in virulence but changes within the host itself. Even if the patients have been stably colonized with *E. coli* 83972 without triggering any sign of infection for many months, there may have been changes in the homeostasis of the patients during the course of time (i.e. older age, temporary decrease in the general immune status) which could have increase their susceptibility to environmental stressors which aided the immune system to recognize *E. coli* 83972, and could have led to a host driven break in the tolerance of asymptomatic colonization.

The method of deliberately established asymptomatic *E. coli* 83972 bacteriuria is an effective non-antibiotic based alternative approach for preventing recurrent urinary tract infections. Although previous colonization studies concluded that deliberate establishment of asymptomatic bacteriuria is a safe procedure without side effects [59, 80], it is reasonable to be peculiarly careful when inoculating patients with bacteria. Our results provide strong evidence that even in the rare case of symptomatic episodes caused by *E. coli* 83972, colonizing bacteria did not reacquire virulence, and did not regain potential to cause serious infections to the patients, thus underlining the safety of the method.

5.2. Virulence factor analysis of clinical *Escherichia coli* isolates from urinary tract infections

Though the molecular background of acute cystitis has been extensively studied in the past, while pyelonephritis-associated molecular traits have been defined, virulence factors specific for acute cystitis strains have not been identified. In the second part of our investigation, we examined if we can characterize clinical *Escherichia coli* isolates causing acute cystitis by a distinct set of virulence factors, and if their virulence profile can be distinguished from the strains causing acute pyelonephritis.

We found type 1 fimbriae to be the most characteristic virulence factor for *E. coli* strains

causing acute cystitis, as 96% of the strains carried the *fim* gene cluster and 81% expressed functional type 1 fimbriae, supporting their role in bladder infection. Although curli were expressed by 73% of cystitis strains, only 15% of them formed biofilm. The expression of P fimbriae and TcpC were more characteristic to the strains causing upper UTIs. The presence of a complete or a combined virulence profile for the tested virulence factors (*fim, papG, prsG, TcpC* and curli) were not characteristic in the acute cystitis group. On the other hand, strains expressing the complete or combined virulence factor profile were significantly more common in patients with upper tract involvement.

The presence of *fim* sequences and type 1 fimbrial expression were the most common features of the cystitis isolates in our analysis. Type 1 fimbriae are ubiquitously expressed by uropathogenic *E. coli* as well as other Gram-negative bacteria. Although type 1 fimbriae have been implicated in cystitis pathogenesis and shown to be essential virulence factors in the murine UTI model [81], as most *E. coli* isolates carry the fim operon regardless of their source [25], their role as independent virulence factors has been debated [67].

The expression of type 1 fimbriae was shown to characterize the most virulent members of a single clone, as the disease severity of UTI was greater in children infected with *E. coli* O1:K1:H7 isolates expressing type 1 fimbriae than in those infected with type 1 negative isolates of the same serotype and a deletion of the *fim* gene cluster from that background was shown to attenuate virulence in the murine UTI model [26]. Type 1 fimbriae were also shown to promote bacterial attachment and trigger a partially TLR4 dependent innate immune response in the murine model [81]. Type 1 fimbriae are also required for UPEC-induced urothelial apoptosis [82, 83]. In vitro studies have shown that upon type 1 fimbrial binding to uroplakin complexes on the uroepithelial surface the disruption of superficial epithelium by type 1 pilus-dependent apoptosis enables bacteria to invade the underlying immature cells [84], and form intracellular biofilm-like structures, called intracellular bacterial communities (IBCs) [24, 85]. Type 1 fimbriae were also proposed to have an important function in the intracellular aggregation and maturation of IBCs [24]. IBCs act as intracellular bacterial reservoirs and have been proposed to play a key role in recurrent UTIs [86].

Previous human inoculation studies provided somewhat contradictory results, however, as transformation of *E coli* 83972 with functional *fim* gene cluster followed by human inoculation did not trigger a higher innate immune response than the wild type strain and there was no difference in the establishment of bacteriuria [67]. In our analysis of *E. coli* 83972 re-isolates from symptomatic episodes during deliberately established bacteriuria we did not find re-acquisition of type 1 fimbrial expression to be involved in the transition from asymptomatic

state to symptomatic episodes either.

The high frequency of type 1 fimbrial expression in the present analysis of *E. coli* strains from patients with acute cystitis is consistent with a contribution of type 1 fimbriae to acute cystitis pathogenesis supporting their role in bladder infection either during the colonization phase or by enhancing inflammation and symptoms.

Biofilm consists of microorganisms and their extracellular products forming a structured community on a surface. The low frequency of biofilm forming strains in the cystitis group compared to the isolates from upper urinary tract involvement in our data suggests that biofilm formation is more associated with the pathogenesis of acute pyelonephritis rather than acute cystitis. Similar results were obtained by other groups in adults [87] and children [88]. On the other hand, Mabbett et al found biofilm formation to be less pronounced in pyelonephritis compared to ABU or acute cystitis [89], while Soto et al observed no differences between cystitis and pyelonephritis strains regarding biofilm formation [90]. Biofilm formation was also found to be associated with recurrent pyelonephritis in children recently [88].

The *pap* gene cluster is strongly associated with acute pyelonephritis and urosepsis but in acute cystitis strains reported frequencies have been below 50%, suggesting a less strong effect on bladder infections than in the kidneys [22, 91]. Our results correspond to these data, as P fimbrial expression was more dominant in the upper urinary tract infection than in acute cystitis strains.

TcpC is a TIR domain homologous protein secreted by UPEC, which promotes bacterial survival by inhibiting the innate host response. Cirl et al described TcpC as a novel virulence factor in 2008 [48]. They found *TcpC* sequences to be present in about 40% of acute pyelonephritis isolates and 21% of cystitis isolates. TcpC was also shown to be more associated with acute pyelonephritis and urosepsis in a recent publication by Vejborg et al [91]. Our results confirmed the strong association of TcpC with disease severity.

We could not characterize the strains causing acute cystitis with a distinct set of virulence factors. In view of the variability in virulence profile, we speculate that acute cystitis may be triggered by a convergent host response, allowing bacteria with different virulence profiles to cause the characteristic clinical symptoms.

However, the presence of a complete or a combined virulence profile was significantly more common in the isolates causing upper urinary tract infections in women compared to the isolates form acute cystitis. The same tendency was shown in children [92] and men [93].

This theoretically means that with virulence factor profiling of the pathogens we can gain information about the clinical course of UTIs. In the traditional management of urinary

tract infections urologists focus mainly on the patients (host side) and try to make risk assessments of the possible disease severity based on patient characteristics, such as co-morbidity, the presence of complicating factors, immunosuppression, etc. [94]. The investigation of the pathogens is superficially included the decision making, and is practically reduced to the results of urine cultures, and antibiotic susceptibility. However, with the characterization of the bacterial virulence factor profile it is possible to make risk assessments about disease severity with the investigation of the bacteria itself.

According to the current guidelines asymptomatic bacteriuria only needs to be treated before an invasive genitourinary procedure and in case of pregnancy [95], because ABU can lead to the development of pyelonephritis and also has been associated with low birth weight and prematurity [96, 97]. In every other condition (diabetes, postmenopausal women, urinary foreign bodies, etc.) treatment of asymptomatic bacteriuria is not recommended, as the low risk of a severe urinary tract infection to evolve does not counterweight the cost meant by the vast amount of antibiotic usage. If we could predict the possibility of a severe infection in case of a clinical asymptomatic bacteriuria by identifying bacteria with a virulence potential, we could selectively treat patients who are in risk of a serious infection.

In the era of increasing antibiotic resistance and multidrug-resistant bacteria deeper understanding of the causative bacteria and the analysis of bacterial virulence profile can be a valuable asset. Urologists need to widen their diagnostic arsenal from the traditional urological methods to a more microbiology-centered aspect in the future in order to be able to successfully manage the increasing threat of urinary tract infections.

6. CONCLUSIONS

1. Our results suggest that symptomatic episodes caused by *E. coli* 83972 during deliberately established asymptomatic bacteriuria do not reflect regained expression of established virulence factors by the colonizing strain.

2. The individual changes in the pattern of phenotypic traits and the transcriptional profile of the re-isolates suggest that these changes may be attributed to the involvement of response mechanisms of the hosts and not only on the characteristics of the bacteria.

3. Our results verify that the deliberately established asymptomatic bacteriuria for preventing recurrent urinary tract infection is a safe method, as even in the rare case of symptomatic episodes caused by *E. coli* 83972 colonizing bacteria did not reacquire virulence, and did not regain potential to cause serious infections to the patients.

4. Clinical strains causing acute cystitis could not be characterized with a distinct virulence factor repertoire. The most characteristic virulence factor was the expression of type 1 fimbriae.

5. The presence of a complete or a combined virulence profile was significantly more common in the isolates causing upper urinary tract infections.

7. ÖSSZEFOGLALÁS

A húgyúti fertőzések klinikai jelentősége több szempontból is kiemelkedő, hiszen miközben a kórokozók antibiotikumokkal szembeni rezisztenciája világszerte növekedő tendenciát mutat, a húgyúti fertőzések képezik az antibiotikum felhasználás egyik vezető okát, továbbá a nozokomiális fertőzések egyik legfontosabb forrását szerte a világon. A téma fontossága ellenére a húgyúti fertőzések molekuláris alapjairól, illetve a kórokozók és a szervezet közötti interakcióról csak korlátozott ismeretek állnak rendelkezésünkre.

A húgyúti kórokozók fertőzőképességét, illetve a kialakuló fertőzés súlyosságát nagymértékben befolyásolja, hogy az adott baktériumtözs milyen virulencia faktorokkal rendelkezik. Számos különböző virulencia faktort írtak le, melyek befolyásolják a húgyúti fertőzések patogenezisének különböző szakaszait, azonban nem tisztázott, hogy a különböző klinikai formák (tünetmentes bacteriuria, cystitis, pyelonephritis, stb.) kialakulásában a szervezeti tényezőkön túl pontosan mely virulencia faktorok és milyen módon játszanak szerepet.

Jelen kutatás célja a leggyakoribb húgyúti kórokozó, az *Escherichia coli* virulencia faktorainak tanulmányozása volt a húgyúti fertőzések klinikai lefolyásában. Vizsgálatunk első szakaszában azt a kérdést kívántuk megválaszolni, hogy az *Escherichia coli* 83972 törzsek virulenciájában bekövetkezett változás áll-e a mesterségesen kialakított tünetmentes *E. coli* 83972 bacteriuria során kialakult, a kolonizáló törzs által okozott alsó húgyúti tünekkel járó epizódok hátterében.

A mesterségesen kialakított tünetmentes bacteriuria módszerének lényege, hogy egyéb kezelésre nem reagáló, visszatérő húgyúti fertőzésben szenvedő betegek húgyhólyagját a tünetmentes bacteriuriát okozó, avirulens *E. coli* 83972 törzsekkel kolonizáljuk, így egyfajta kolonizációs rezisztenciát hozva létre. Sundén és munkatársai 2010-ben egy randomizált, kontrollált kolonizációs vizsgálatban igazolták a módszer klinikai hatékonyságát. A vizsgálat során az *E. coli* 83972 törzsekkel kolonizált betegeknél szignifikánsan ritkábban alakultak ki húgyúti fertőzéses epizódok a fiziológiás sóoldattal kolonizált kontrollokkal összehasonlítva. Ezen epizódok többségét más baktériumokkal való felülfertőződés okozta, mindössze 5 esetben (4 betegben) igazolódott a kolonizáló *E. coli* 83972 törzs a tünetek hátterében. Vizsgálataink során ezen izolátumok virulenciáját analizáltuk.

A baktériumtörzseken először genotípus meghatározást végeztünk az extraintesztinális fertőzést okozó *E. coli* törzsekhez köthető legfontosabb virulencia génekre vonatkozóan. Nem

találtunk különbséget a vad típushoz képest a *fim* (1-es típusú fimbria), *pap* (P fimbria), *foc* (F1C fimbria), *hlyA* (α-hemolysin), *cnf*1 (citotoxikus nekrotizáló faktor), *fyuA* (yersiniabactin sziderofór receptor), *iroN* (salmochelin sziderofór receptor), *iuc* (aerobactin sziderofór), *kpsMT* K5 (K5 tok) és a *malX* (patogenitási sziget marker) szekvenciákkal kapcsolatban.

A fenotípus meghatározás során nem találtunk különbséget a virulencia faktorok expressziójával kapcsolatban sem. Az izolátumok nem expresszáltak 1-es típusú, P, vagy F1C fimbriákat. Az esetleges egyéb adhezinek jelenlétét A498 vese és T24 húgyhólyag sejteken végzett adhéziós kísérlettel zártuk ki. Az izolátumok biofilm képző képessége, curli, illetve cellulóz, valamint O antigén expressziója szintén megegyezett a vad típussal.

Két izolátum esetében találtunk fokozott motilitást a vad törzshöz képest. A két izolátum génexpressziós vizsgálata során leginkább a stressz választ, metabolizmust, illetve az LPS szintézist érintő egyedi változásokat észleltünk, a flagella (ostor) szintézisének fokozódása csak az egyik izolátum esetén volt kimutatható.

Az izolátumok virulenciájának további tesztelésére, és valamilyen esetlegesen jelen lévő ismerelten virulencia faktor kiszűrésére in vitro és in vivo virulencia kísérleteket végeztünk. Az izolátumok által A498 vesesejteken kiváltott in vitro immunválasz nem különbözött a vad törzs által kiváltott választól a sejtek interleukin 6 és 8 szekréciója tekintetében. Hasonlóképpen nem találtunk különbséget az izolátumokkal, illetve a vad típussal kolonizált C3H/HeN egereknél a vizeletben, húgyhólyagban és vesékben mért baktériumszám, a neutrophil granulocyták kinetikája, illetve a mortalitás tekintetében.

Vizsgálatunk második szakaszában azt a kérdést kívántuk megválaszolni, hogy az akut cystitist okozó *Escherichia coli* törzsek karakterizálhatóak-e valamilyen jellegzetes virulencia faktor repertoárral, illetve, hogy ez megkülöböztethető-e az akut pyelonephritist okozó törzsek virulencia profiljától. Akut húgyúti fertőzésben szenvedő nők vizeletéből izolált 247 *E. coli* törzsnél végeztünk virulencia faktor analízist az akut cystitisben felmerült legfontosabb faktorok jelenlétére, illetve azok kombinációjára vonatkozóan, összehasonlítva az eredményeket a fertőzés klinikai lefolyásának függvényében, azaz, hogy felső húgyúti fertőzés kialakult-e, vagy sem.

Az 1-es típusú fimbriák jelenléte volt a leggyakoribb az akut cystitist okozó törzseknél, a fimbriát kódoló *fim* szekvenciák az izolátumok 96%-ában voltak jelen, a fimbriákat az izolátumok 80%-a expresszálta. Ugyan curli-fimbriák 73%-ban voltak jelen a cystitis csoportban, biofilm képzést csak az izolátumok 15%-ánál észleltünk. Nem találtunk szignifikáns különbséget sem az 1-es típusú fimbriák, sem a biofilm képzés tekintetében az akut cystitist és az akut pyelonephritist okozó törzsek között.

A P fimbriákat kódoló *pap* génklaszter az összes törzs 43%-ban volt azonosítható (*papG* $_{IA2}$ 24%, *prsG*$_{J96}$ 20%, mindkettő 3%), akut cystitis esetén 41%-ban, felső húgyúti érintettség esetén 56%-ban. A P fimbriák expresszióját (Class II+III) az összes izolátum 43%-ában, a cystitist okozó törzsek 41%-ban, a felső húgyúti érintettséggel járó csoport esetén 50%-ban észleltük. A pap gének és a P fimbriák tekintetében észlelt különbség azonban nem volt szignifikáns a két csoport között. A TcpC expressziója szignifikánsan gyakoribb volt felső húgyúti érintettség esetén (42%) a csak cystitist okozó törzsekhez képest (32%) (p<0.01). A teljes, ill. halmozott virulencia profilt (*fim, pap, TcpC* és curli-fimbria) expresszáló törzsek szignifikánsan gyakoribbak voltak felső húgyúti érintettség, mint csak cystitis esetén (37% vs. 17%, p<0.01). Nem találtunk szignifikáns különbséget a visszatérő ill. sporadikus húgyúti fertőzésekből izolált törzsek virulencia profiljában.

Összefoglalva, a mesterségesen kialakított tünetmentes *Escherichia coli* 83972 bacteriuria során kialakult, a kolonizáló törzs által okozott alsó húgyúti tünekkel járó epizódok hátterében a törzsek fokozódott virulenciája nem volt igazolható. Eredményeink arra utalnak, hogy a tünetek létrejöttében valószínűleg a gazdaszervezet állapotában kialakult változások játszhattak döntő szerepet, továbbá megerősítik a módszer klinikai alkalmazásának biztonságos voltát.

A húgyúti fertőzésekből izolált *Escherichia coli* törzsek vizsgálata során az akut cystitist okozó *E. coli* törzsek az 1-es típusú fimbriák expressziójával voltak leginkább jellemezhetők. Az akut cystitist okozó törzsek jellegzetes virulencia faktor repertoárral nem voltak karakterizálhatóak, azonban felső húgyúti érintettség kialakulása esetén a kombinált virulencia faktor profil szignifikánsan gyakrabban volt jelen.

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I.

Rare Emergence of Symptoms during Long-Term Asymptomatic Escherichia coli 83972 Carriage without an Altered Virulence Factor Repertoire

Béla Köves,* Ellaine Salvador,* Jenny Grönberg-Hernández, Jaroslaw Zdziarski, Björn Wullt,† Catharina Svanborg and Ulrich Dobrindt‡

From the Department of Microbiology, Immunology and Glycobiology, Institute of Laboratory Medicine, Lund University (BK, JG-H, BVV, CS), Lund, Sweden, and Institute for Molecular Biology of Infectious Diseases, University of Würzburg (ES, JZ, UD), Würzburg and Institute for Hygiene, University of Münster, Münster, Germany (UD)

Purpose: Asymptomatic bacteriuria established by intravesical inoculation of Escherichia coli 83972 is protective in patients with recurrent urinary tract infections. In this randomized, controlled crossover study a total of 3 symptomatic urinary tract infection episodes developed in 2 patients while they carried E. coli 83972. We examined whether virulence reacquisition by symptom isolates may account for the switch from asymptomatic bacteriuria to symptomatic urinary tract infection.

Materials and Methods: We used E. coli 83972 re-isolates from 2 patients in a prospective study and from another 2 in whom symptoms developed after study completion. We phylogenetically classified the re-isolates, and identified the genomic restriction patterns and gene expression profiles as well as virulence gene structure and phenotypes. In vivo virulence was examined in the murine urinary tract infection model.

Results: The *fim*, *pap*, *foc, hlyA*, *fyuA, iuc, iroN*, *kpsMT* K5 and *malX* genotypes of the symptomatic re-isolates remained unchanged. Bacterial gene expression profiles of flagellated symptomatic re-isolates were unique to each host, providing no evidence of common deregulation. Symptomatic isolates did not differ in virulence from the wild-type strain, as defined in the murine urinary tract infection model by persistence, symptoms or innate immune activation.

Conclusions: The switch from asymptomatic E. coli 83972 carriage to symptomatic urinary tract infection was not explained by reversion to a functional virulence gene repertoire.

Abbreviations and Acronyms

ABU = asymptomatic bacteriuria
*cnf*1 = cytotoxic-necrotizing factor1
fyuA = yersiniabactin receptor
hlyA = α-hemolysin
IFNγ = interferon-γ
IL = interleukin
iroN = salmochelin receptor
iuc = aerobactin
kpsMT K5 = K5 capsule
LPS = lipopolysaccharide
PMN = polymorphonuclear leukocyte
UTI = urinary tract infection
wt = wild type

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* Equal study contribution.

† Financial interest and/or other relationship with OM Pharma.

‡ Correspondence: Institute of Hygiene, University of Münster, Robert Koch-Str. 41, 48149 Münster, Germany (telephone: +49 (0)251 9802875; FAX: +49 (0)251 9802868; e-mail: dobrindt@uni-muenster.de).

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BACTERIA invading the urinary tract may cause symptomatic disease or give rise to ABU, a symptom-free carrier state resembling commensalism.[1] ABU is even more common than symptomatic UTI.[1,2] Epidemiological studies show that asymptomatic carriage protects the patient against symptomatic superinfections[2,3] compared to patients in whom bacteriuria is eradicated by antibiotic therapy.[3] This protective effect has been used as a rationale to deliberately establish ABU in patients prone to UTI.[4–6] The therapeutic efficacy of this approach was demonstrated in randomized clinical trials.[5,7] Observational studies established that therapeutic inoculation is safe and decreases UTI morbidity.[8,9]

The prototype ABU Escherichia coli strain 83972[3,6] is extensively used for human inoculation since it produces no adverse effects, fails to express virulence factors associated with symptomatic UTI and lacks conjugative plasmids.[4] E. coli 83972 and other ABU strains have a smaller genome size than uropathogenic strains. This is due in part to virulence gene deletions that abolish fimbrial expression and adherence, suggesting that ABU strains adapt to the human urinary tract by undergoing reductive evolution.[9]

After therapeutic E. coli 83972 inoculation in a series the number of symptomatic episodes decreased during E. coli 83972 bacteriuria and patients experienced a longer infection-free interval than a placebo group.[6] While most symptomatic UTI episodes were caused by superinfection with other E. coli or nonE. coli strains, we identified a few patients in whom symptoms developed during E. coli 83972 bacteriuria, suggesting a transition from ABU to symptomatic UTI.

In the current study we examined whether E. coli 83972 evolves toward virulence during asymptomatic carriage in the urinary tract. We compared phenotypic and genotypic traits of E. coli 83972 to those of re-isolates from patients with symptomatic episodes. We found no evidence of increased expression of traditional virulence factors by E. coli 83972 in hosts with symptomatic UTI during asymptomatic carriage.

METHODS

Patients and Study Design

Patients with incomplete bladder emptying due to spinal or lower motor neuron lesions who had recurrent lower UTIs were included in a placebo controlled study of intravesical inoculation with E. coli 83972. In all patients optimal treatment, including clean intermittent catheterization, had been tried but failed. Study exclusion criteria were upper urinary tract dilatation, febrile UTI episodes or pyelonephritis, corticosteroid treatment and significant comorbidity. The study was approved by the Lund University human ethics committee and patients provided informed consent (RTP-A2003, www.ClinicalTrials.gov). Sundén et al previously reported patient characteristics and identification numbers, diagnostic criteria and study design.[6]

Before inoculation preexisting bacteriuria was eliminated by antibiotic treatment. After an antibiotic-free interval E. coli 83972 bacteriuria was established by intravesical inoculation of 10^5 cfu/ml in saline. The procedure was repeated once daily for 3 days. After bacteriuria was established the effect on UTI morbidity was quantified as the total number of symptomatic UTI episodes during 10 to 12 months compared to UTI morbidity after a crossover period of similar duration without E. coli 83972 bacteriuria.

Symptomatic UTI Episodes

We examined the subset of patients in whom symptomatic UTI episodes developed during the study (see table). UTI episodes were self-reported, a method previously shown to be reliable in select patient groups.[10] UTI was also determined in a structured interview by the study physician and by urine culture yielding greater than 10^5 cfu/ml of a single organism. Symptomatic episodes were defined by at least 2 symptoms, including suprapubic pain, dysuria and/or frequency as well as increased spasticity in patients with a spinal cord lesion. Antibiotic treatment initiated by the study physician resulted in prompt relief of symptoms.

Bacteria and Cytokines

Urine samples were semiquantitatively cultured and the antibiotic susceptibility pattern was recorded. Isolated bacteria were maintained as deep agar stabs or frozen glycerol cultures. For E. coli species verification of isolates 16S rRNA sequencing was performed with phenotypes different from E. coli 83972. E. coli 83972 re-isolates were identified by polymerase chain reaction, which detected the cryptic 1.6 kb plasmid and the internal 4,253 bp *fim* deletion. For in vitro analysis strains were grown in lysogeny broth or in pooled human urine with or without 1.5% agar (Difco™).

Neutrophils were quantified in uncentrifuged urine using a hemocytometer chamber. IL-6 and 8 concentrations were quantified by Immulite® assay. The MILLIPLEX® MAP Human Cytokine/Chemokine Panel was used to screen for additional cytokines.

DNA Techniques

QIAGEN® products were used for genomic DNA isolation. Primers were obtained from Eurofins MWG/Operon, Ebersberg, Germany. Restriction enzymes were obtained from New England Biolabs®. Genomic DNA was analyzed by pulsed field gel electrophoresis. Phylogenetic

Data on 4 patients with E. coli 83972 asymptomatic bacteriuria and total of 5 proven UTI episodes caused by E. coli

		Pt R15 (episode No.)			
	Pt R4	1	2	Pt R10	Pt Sp10
		Pt data			
Gender	F	F		F	M
Yr born	1944	1958		1957	1937
Diagnosis	Detrusor insufficiency, post-void residual urine	Detrusor insufficiency, post-void residual urine		Detrusor insufficiency, post-void residual urine	Spinal lesion, neurogenic bladder disorder
UTI episode:					
Days after E. coli 83972 inoculation	192	20	19	104	67
Symptoms	Suprapubic pain, dysuria, frequency	Local discomfort, dysuria, frequency		Suprapubic pain, dysuria, frequency	Local discomfort, dysuria, increased spasticity
		Urine inflammatory response			
During preceding E. coli 83972 bacteriuria:					
IL-6 (pg/ml)	39.6	145.2	235.4	Mean 2.1 (range 2—2)	Mean 6.9 (range 2—24)
IL-8 (pg/ml)	4,706	3,268	551	Mean 20.2 (range 5—62)	Mean 518 (range 37—407)
Neutrophils ($\times 10^4$)	240	960	800	Mean 0.6 (range 0—2)	Mean 25 (range 8—24)
At UTI episode:					
IL-6 (pg/ml)	18	145	235	8	300
IL-8 (pg/ml)	4,706	3,268	551	43	7,500
Neutrophils ($\times 10^4$)	240	960	800	Not determined	20,000

classification of re-isolates, and ExPEC virulence genes were determined as previously described.[11]

Virulence Factor Expression

Functional type 1, P, F1C fimbriae, hemolysis and motility as well as O antigen, aerobactin expression and biofilm formation were detected.[11,12] Adhesion to the human urinary tract cell lines A498 and T24, and curli and cellulose expression were determined as described previously.[13] Experiments were performed in triplicate. We determined growth rates at 600 nm optical density in triplicate experiments using different batches of pooled human urine.

Gene Expression Profiling

RNA preparation and microarray analysis were performed as previously reported.[9] For statistical significance the 1-sample t-test was applied with the Bonferroni correction. A cutoff of 1.7 (ln2) was used at $p \leq 0.09$.

Experimental Infection

Experiments were performed with the permission of the animal experimental ethics committee, Lund District Court, Sweden. Female C3H/HeN mice bred at the MIG animal facility were infected at age 6 to 12 weeks by intravesical inoculation with E. coli 83972 wt or re-isolates from each symptomatic episode.[14] The mice were sacrificed at 6 or 24 hours, or 7 days, and the kidneys and bladders were removed. Infection was quantified by viable counts on kidney and bladder homogenates. Neutrophils were quantified in uncentrifuged urine using a hemocytometer chamber. For statistical analysis the groups were compared by the paired t-test or Mann-Whitney test.

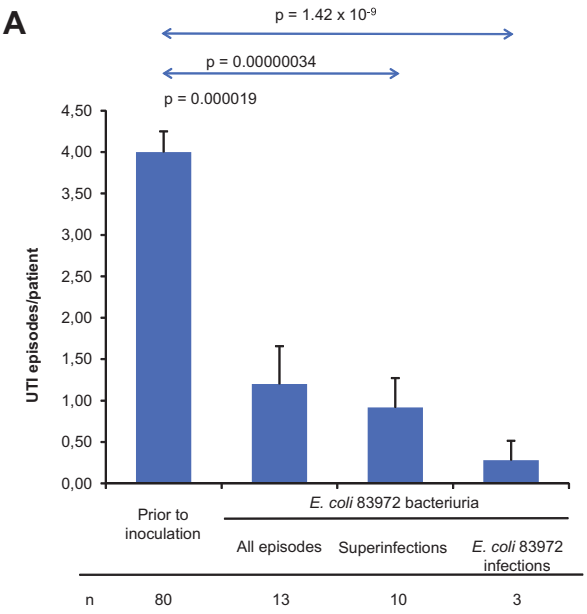
RESULTS

E. coli 83972 Bacteriuria Delayed UTI Recurrences and Decreased Number of Symptomatic UTI Episodes

Re-isolates of E. coli 83972 were obtained from patients who participated in a placebo controlled crossover study of the protective effect of E. coli 83972 bacteriuria after deliberate inoculation of this strain into the urinary tract.[6] Patients were protected from symptomatic UTI, as defined by the number of episodes, before study entry and while in the placebo arm of the study. The mean number of symptomatic episodes per patient-year was 1.2 during ABU and 4 before the study (paired t-test $p = 0.000019$, fig. 1, *A*). In the E. coli 83972 bacteriuria arm with 202 months of observation a total of 13 symptomatic UTI episodes developed in 9 patients (0.8 per patient-year). This was significantly lower than in the placebo arm with 168 months of observation time during which 4 of 20 patients had a total of 35 UTI episodes (2.5 per patient-year, $p = 0.009$). Median time to the first symptomatic episode was also significantly less in the placebo group than during ABU (11.3 vs 5.7 months, $p < 0.013$).

The 13 UTI episodes recorded during E. coli 83972 bacteriuria were further characterized. Ten episodes were superinfections. E. coli 83972 was replaced by a different E. coli strain in 7 episodes, and by Pseudomonas aeruginosa, Enterococcus faecalis and Proteus mirabilis in 1 each. In patients R4 and R15 only E. coli 83972 was recovered during 1 and 2 symptomatic episodes, respectively, suggesting that symptoms were caused by this strain (see table and fig. 1, *B*). Before symptoms developed these patients carried E. coli 83972 asymptomatically without discomfort. The 3 symptomatic episodes were accompanied by increased urine polymorphonuclear leukocyte numbers and increased urine cytokine levels (see table, fig. 2, *A* and supplementary fig. 1, http://jurology.com/). In patient R4 an increase in RANTES, IP-10, sIL-2Ra, MCP-1, IL-1a, IL-1RA and IFNγ was observed and in patient R15 IL-6, sIL-2Ra and IL-1α were increased.

A



B

Patient ID	Days after inoculation	IL-6 (ng/L)	IL-8 (ng/L)	PMN x10⁴/mL	Culture	Isolate
E. coli 83972						
R4	192	39.6	4706	240	*E.coli* 83972	R4
R15	20	145.2	3268	960	*E.coli* 83972	R15-1
R15	19	235.4	551	800	*E.coli* 83972	R15-2
Other bacteria						
R2	69	19.8	2627	nd	*E.coli*	
R15	116	206.8	1626	562	*Enterococcus faecalis*	
R16	103	4.4	9	0	*E. coli; Proteus mirabilis*	
R16	12	nd	nd	nd	*E.coli*	
Sp2	332	nd	nd	28	*Pseudomonas aeruginosa*	
Sp5	88	nd	nd	nd	*E.coli*	
Sp5	95	4.4	7156	nd	*E.coli*	
Sp8	119	4.4	62	1	*E.coli*	
Sp12	272	nd	nd	nd	*E.coli*	
Sp13	193	nd	nd	nd	*E.coli*	

Figure 1. Symptomatic UTI episodes during E. coli 83972 bacteriuria. *A,* mean ± SEM frequency (*n*) of symptomatic UTI episodes during year before inoculation and during year with E. coli 83972 bacteriuria in 20 patients (paired t-test). *B,* innate immune response of patients to symptomatic E. coli 83972 episodes quantified as urine cytokine and PMN levels.

However, these isolates did not stimulate a higher cytokine response in human uroepithelial cells (fig. 2, *B* and supplementary fig. 1, http://jurology.com/). In all patients the peak mucosal response was several fold higher during the symptomatic episode compared to the preceding ABU period. In patient R10 the preceding ABU response was low or absent.

Properties of E. coli 83972 Re-Isolates from Symptomatic Episodes

To examine whether a change in bacterial properties precipitated the symptomatic episodes we examined the 3 E. coli 83972 re-isolates. We also included E. coli 83972 re-isolates from symptomatic episodes in 2 patients. Patient R10 participated in the therapeutic study but symptoms developed after study completion. Patient Sp10, who received E. coli 83972 inoculation in a separate open study protocol, was excluded from analysis due to corticosteroid treatment (see table).

To identify E. coli 83972 re-isolates we screened 20 randomly chosen colonies for the presence of the cryptic 1.6 kb plasmid and the internal 4,253 bp *fim* deletion (fig. 3, *A*). Like the wt strain, re-isolates were phylogenetically classified into the B2 lineage and shared an identical genomic restriction pattern (supplementary fig. 2, http://jurology.com/).

E. coli 83972 carries the type 1 (*fim*), P (*pap*), F1C (*foc*) fimbrial genetic determinants and genes coding for *hlyA* or *cnf*1, *fyuA*, *iroN*, *iuc*, *kpsMT* K5 and the pathogenicity island marker *malX*.[11] These genes were present in all re-isolates, suggesting that the overall pathogenicity island structure remained largely intact (fig. 3, *B*).

Re-isolates did not express functional P, F1C or type 1 fimbriae (fig. 3, *C*). To exclude other adhesins we monitored adherence to A498 kidney cells and T24 bladder cells but it was not detected (fig. 4, *A*). We observed no consistent change in biofilm, curli or cellulose formation (figs. 3, *C* and 4, *B*).

Re-isolates expressed an O antigen pattern identical to that of E. coli 83972 (supplementary fig. 2, http://jurology.com/). They had a growth rate in pooled human urine similar to that of *E. coli* 83972 except re-isolates R10 and R15-1 clone II, which grew more slowly (fig. 4, *C*).

Re-Isolate Population Heterogeneous Phenotypes

Although urine samples from symptomatic episodes were E. coli 83972 monocultures, re-isolates of samples R15-1 and R15-2 showed heterogeneous phenotypes and comprised colonies of different sizes or motility. For colonies from urine sample R15-1 about 75% the colony size and morphology resembled those of strain E. coli 83972 (R15-1 clone I). The remaining colonies (R15-1 clone II) were small and grew slowly (fig. 4, *C*). However, they had the same 1.6 kb cryptic plasmid, *fim* deletion, restriction pattern and virulence gene content as E. coli 83972. The slow growth and reduced colony size were reminiscent of small colony variants associated with persistent infection.[15] Furthermore, individual colonies of urine sample R15-2 differed in motility and flagella expression.

A

Parameter*	Patient R4		Patient R15		
	ABU	Symptomatic episode	ABU	Symptomatic episode 1	2
IL-6	3 ± 0.00	18	101.2 ± 22.30	145.2	235
IL-8	325 ± 88.3	4706	2093 ± 283.9	3268	551
PMNs	13 ± 2.84	240	862.5 ± 212.5	960	800
RANTES	4 ± 0.51	12	3 ± 0.490	3	7
IP-10	19 ± 5.60	130	111 ± 63.80	36	195
sIL-2Rα	59 ± 26.6	193	62 ± 9.300	148	128
IL-1α	2 ± 0.20	23	34 ± 14.90	56	97
IL1RA	3 ± 0.00	95	644 ± 257.9	575	1074
IFNϒ	3 ± 0.00	106	17 ± 9.700	3	29

*pg/μl for proteins and x10⁴/ml for PMNs, in urine. For ABU, average and SEM of four samplings.

B

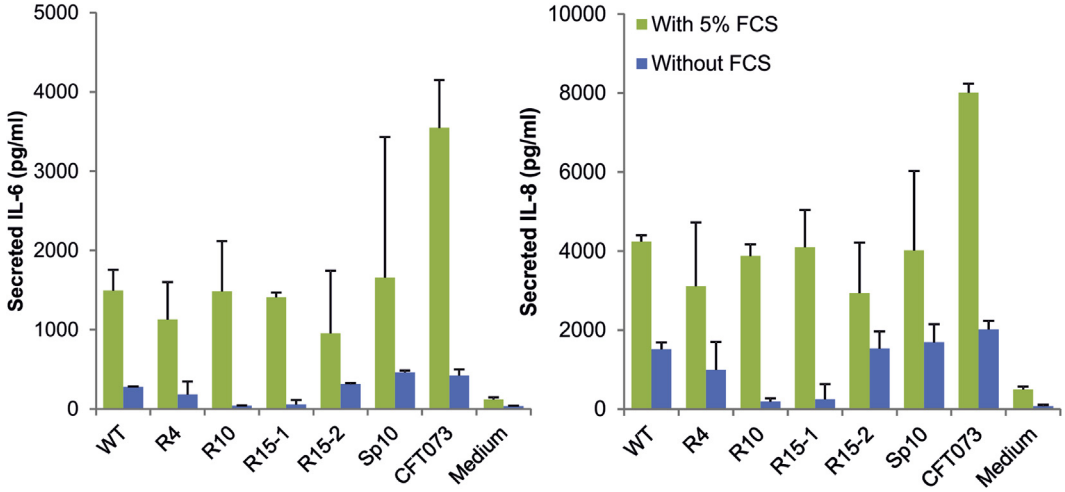


Figure 2. Host response to ABU in vivo and in vitro. *A*, host response to symptomatic E. coli 83972 episodes. *B*, geometric mean ± SEM epithelial response to in vitro infection of A498 cells with E. coli 83972 wt, symptomatic E. coli 83972 re-isolates and uropathogenic E. coli strain CFT073 in 2 independent experiments. Fetal calf serum (FCS) (5%) was used to provide soluble CD14.

Re-Isolate Increased Motility

Since flagella were proposed to facilitate ascending UTI,[16,17] we compared the motility of E. coli 83972 and the re-isolates. Increased motility was observed for re-isolates R15-2 and Sp10. R15-2 appeared as a phenotypically heterogeneous population with increased motility of individual cells (R15-2 clone I) and flagellar expression relative to E. coli 83972 (fig. 4, *D* and supplementary fig. 3, http://jurology.com/). The remaining re-isolates (R15-2 clone II) were as motile as E. coli 83972.

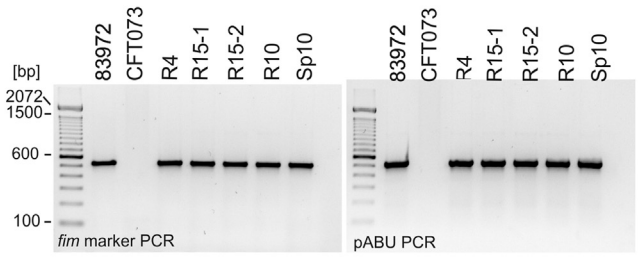
Motile Re-Isolate Gene Expression Analysis

To analyze differences in the gene expression of re-isolates with phenotypes that markedly deviated from the wt we compared the transcriptome between E. coli 83972 and the motile re-isolates (R15-2 clone I and Sp10). Of 95 de-regulated genes in R15-2 clone I 80 were up-regulated and 15 were down-regulated while 81 and 17 of 98 genes in Sp10 were increased and decreased, respectively (fig. 5).

Most up-regulated genes in R15-2 clone I encoded bacteriophage components. In addition, activated genes were involved in the SOS or stress response (*recA*, *recN*, *lexA*, *ruvB*, *dinI*, *dinB*, *sulA*, *yebG*, *osmB* and *umuD*), σ factor expression (*rpoA*, *rpoE* and *rpoS*) and acid resistance (*gadA*, *gadB*, *hdeAB*, *cadB* and *slp*). *mglAB* genes that code for a galactose transporter and some phage related genes were down-regulated. In E. coli R15-2, representing a heterogeneous group of motile and less motile colonies, increased flagellar gene expression was less pronounced.

In contrast, in re-isolate Sp10, in which all colonies showed increased motility, flagella biosynthesis and assembly genes (*flgA*, *flgDEFG*, *flhA*,

A



B

Strain	ExPEC virulence and fitness-associated genes
83972 wt	*fimH*, *hlyA*, *sfa/focDE*, *papA*, *papC*, *papEF*, *papG*II/III, *cnf*1, *fyuA*, *iroN*, *iucD*, *kpsMT* (K5), *malX*
R4	*fimH*, *hlyA*, *sfa/focDE*, *papA*, *papC*, *papEF*, *papG*II/III, *cnf*1, *fyuA*, *iroN*, *iucD*, *kpsMT* (K5), *malX*
R15-1 (clone I)	*fimH*, *hlyA*, *sfa/focDE*, *papA*, *papC*, *papEF*, *papG*II/III, *cnf*1, *fyuA*, *iroN*, *iucD*, *kpsMT* (K5), *malX*
R15-1 (clone II)	*fimH*, *hlyA*, *sfa/focDE*, *papA*, *papC*, *papEF*, *papG*II/III, *cnf*1, *fyuA*, *iroN*, *iucD*, *kpsMT* (K5), *malX*
R15-2 (clone I)	*fimH*, *hlyA*, *sfa/focDE*, *papA*, *papC*, *papEF*, *papG*II/III, *cnf*1, *fyuA*, *iroN*, *iucD*, *kpsMT* (K5), *malX*
R15-2 (clone II)	*fimH*, *hlyA*, *sfa/focDE*, *papA*, *papC*, *papEF*, *papG*II/III, *cnf*1, *fyuA*, *iroN*, *iucD*, *kpsMT* (K5), *malX*
R10	*fimH*, *hlyA*, *sfa/focDE*, *papA*, *papC*, *papEF*, *papG*II/III, *cnf*1, *fyuA*, *iroN*, *iucD*, *kpsMT* (K5), *malX*
Sp10	*fimH*, *hlyA*, *sfa/focDE*, *papA*, *papC*, *papEF*, *papG*II/III, *cnf*1, *fyuA*, *iroN*, *iucD*, *kpsMT* (K5), *malX*

C

Strain	pABU	*fim* del	Auto-aggre-gation	Motility	Biofilm	K5 capsule	Cellulose	Rdar morphotype
83972 wt	+	+	+	+	+	+	30 °C	30 °C
R4	+	+	+	-	<	+	-	-
R15-1 (clone I)	+	+	+	-	-	+	37 °C	37 °C
R15-1 (clone II)	+	+	+	-	-	+	37 °C	37 °C
R15-2 (clone I)	+	+	+	>	>	-	30 °C, 37 °C	-
R15-2 (clone II)	+	+	+	-	>	-	30 °C, 37 °C	-
R10	+	+	+	-	-	+	30 °C, 37 °C	37 °C
Sp10	+	+	+	>	-	-	-	-

Figure 3. Virulence gene repertoire and characteristics of E. coli 83972 and symptomatic re-isolates. *A,* 1.6 kb plasmid and *fim* deletion were amplified by polymerase chain reaction (*PCR*) to verify E. coli 83972 identity in patient urine. *B,* identical virulence gene set was identified in E. coli 83972 and symptomatic re-isolates. *C,* E. coli 83972 and re-isolate phenotypic characteristics.

fliA, fliG and *fliO*) were significantly up-regulated together with genes involved in heat shock response (*groEL* and *groES*), LPS biosynthesis (*rfaGPIJY*, *waaV*, *waaW* and *lpxAB*), amino sugar use (*glmUS*) and iron uptake (*chuATUWXY* and *entCA*). Transport genes were significantly down-regulated, including *aga*, *srl* and *dgo*.

The *yjbE* and *yqjD* genes involved in biofilm formation were commonly up-regulated in R15-2 clone I and Sp10 compared to E. coli 83972, as were *gudD* and *gudP* involved in D-glucarate use, and genes coding for 30S and 50S ribosomal subunit components. Transcription of ribose transporter genes was repressed in each re-isolate relative to the wt.

Accordingly, transcriptional regulation was unique for bacteria recovered from each host. It provided no evidence of commonly deregulated genes in re-isolates from different symptomatic hosts.

Bacterial Persistence and Host Response Activation In Vivo

To investigate whether the re-isolates showed increased virulence we established in vivo infections in C3H/HeN mice. There was no significant difference in the bacterial number in kidneys and bladders 24 hours and 7 days after inoculation (fig. 6, *A*). Urine neutrophil counts reflected the low acute inflammatory response to E. coli 83972. Motility of the re-isolates did not influence bacterial numbers or urine neutrophil counts (fig. 6, *A*).

E. coli 83972 was compared to the strain 83972*ΔfliC* mutant, which does not express functional flagella. SN25, the most motile asymptomatic

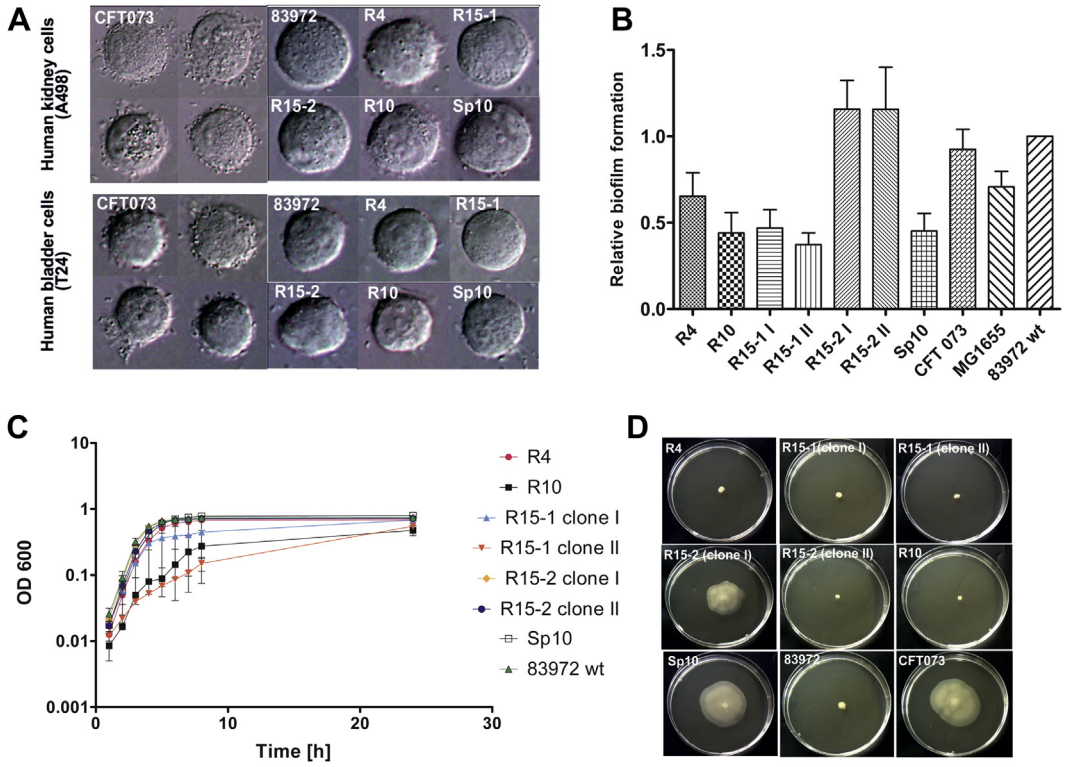


Figure 4. Phenotypic characterization of E. coli 83972 re-isolates from symptomatic episodes. *A*, adhesion to T-24 bladder epithelial cells and A498 kidney epithelial cells. *B*, biofilm formation in pooled human urine. *C*, growth kinetics in pooled human urine in vitro. *D*, motility on urine swarm agar plates.

re-isolate, served as the positive control (fig. 6, *B*). We observed a biphasic infection pattern. Five hours after infection E. coli 83972 and SN25 reached significant numbers while the kidneys of mice colonized with E. coli 83972*ΔfliC* remained sterile, consistent with a role for flagellation in the early phase of ascending infection.[16] At 7 days persistent bacteriuria (greater than 10^5 cfu/ml) developed in mice infected with E. coli 83972 and 83972*ΔfliC*. The highly motile isolate was eliminated more rapidly than the wt and no increase in the urine neutrophil number was related to flagellation (fig. 6, *B*).

DISCUSSION

After comparing the genome of E. coli 83972 re-isolates from different inoculated human hosts we previously suggested that evolution toward commensalism is favored during asymptomatic bladder colonization.[9] The current study was designed to address whether evolution toward virulence may occur in parallel in specific hosts. To detect changes in bacterial properties associated with the rare development of symptoms during asymptomatic carriage we investigated phenotypic or genotypic changes in re-isolates that might have precipitated the symptomatic episodes.

After 202 patient-months of E. coli 83972 ABU only 3 symptomatic UTI episodes with E. coli 83972 were recorded. Analysis of these isolates and an additional 2 re-isolates from symptomatic episodes excluded regained expression of virulence factors as a cause of symptoms. LPS and capsule as well as biofilm formation and adherence properties remained unchanged. Deregulated genes were mainly involved in different stress responses, metabolic versatility and LPS biosynthesis but no common expression pattern was detected. Flagellation was perturbed but differences in virulence were not observed in the murine UTI model. Results suggest that the occasional symptomatic UTI episode does not reflect regained expression of established virulence factor in E. coli 83972 during long-term carriage.

Interestingly, we observed phenotypic variation in the E. coli 83972 monoculture populating the bladder. This behavior mirrored adverse and stress conditions, and it may ensure the fitness and survival of a subset of cells in this niche. The presence of 2 phenotypes in a clonal population[18] suggests bistable gene expression facilitating the exploitation of dynamic host environments and promoting gene

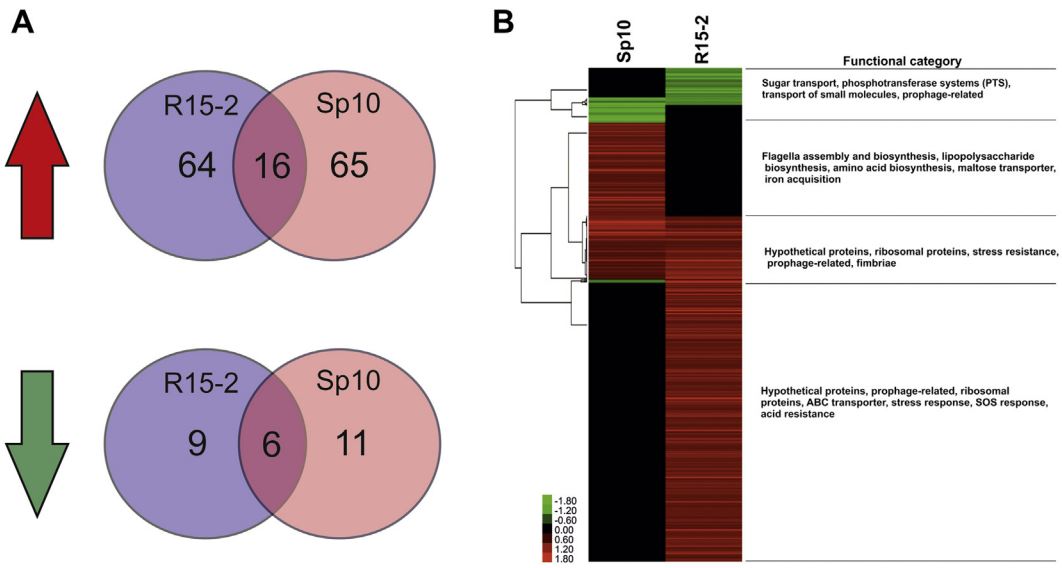


Figure 5. Gene expression profiling of individual re-isolates. *A*, gene expression in re-isolates relative to E. coli 83972. Most regulated genes were unique to each isolate. *B*, hierarchical cluster analysis of deregulated genes in re-isolates relative to E. coli 83972. Strains were grown in vitro at 37C in pooled human urine. Values represent mean expression ratio of at least 3 independent microarray experiments. Green areas indicate significantly regulated (log twofold change, $p < 0.05$) suppressed genes. Red areas indicate significantly regulated (log twofold change, $p < 0.050$) up-regulated genes. Black areas indicate genes without statistically significant change ($p > 0.05$).

expression changes, eg those favoring chronic infection.[19] Examples of such heterogeneous phenotypes correlated with increased fitness of Vibrio cholerae[20] and E. coli.[21] Flagellin expression of Salmonella typhimurium also underlies bistable gene regulation[22] but host environmental factors driving these changes remain poorly understood. Small colony variant formation in Staphylococcus aureus or P. aeruginosa[23,24] has correlated with chronic infection and in Campylobacter jejuni it is considered a survival strategy relying on stress fit individuals in a heterogeneous population.[25] Therefore, bacterial adaptation to long-term in vivo growth in the urinary tract could include phenotype switching. Alternatively, the occurrence of heterogeneous populations at symptomatic episodes may represent spontaneous stochastic events, including minor transient populations.

If symptom development were due to changes in bacterial virulence, the re-isolates should have shown increased fitness in the murine UTI model, as reflected by a higher count in the bladders and kidneys. In parallel, we would have expected these strains to trigger an inflammatory response that was not observed after infection with the wt strain. However, such changes were not observed. The highly flagellated asymptomatic strain SN25 attained a significant number in kidneys 5 hours after infection. This suggests that flagellar motility may be important for initial ascent of bacteria to the upper urinary tract but this was cleared earlier than the wt strain. In contrast, the 83972*ΔfliC* mutant established persistent bacteriuria without upper urinary tract involvement, similar to human ABU. This implies that increased flagellar expression may be counterproductive for long-term persistence.

Symptomatic episodes were accompanied by an innate immune response with increased cytokine and chemokine levels in urine as well as pyuria. IL-6 and 8 responses in symptomatic UTI have been extensively studied and the concentrations reflect disease severity.[26] A recent experimental study suggested a potential role for noninflammatory host responses, showing distinct symptomatic responses to bacteriuria mediated by TLR4 that are independent of inflammation.[27] However, in our study all symptomatic UTI episodes were accompanied by increased cytokine levels. Additional proinflammatory cytokines included IL-1α, which was counterbalanced by the IL-1 receptor antagonist IL-1RA, as well as RANTES, which was associated with eosinophil/mast cell activation. The increase in soluble IL-2Rα and IFNγ confirmed a response profile previously observed in patients with E. coli 83972 bacteriuria.[28] These findings are consistent with local lymphocyte and dendritic cell activation, and infection dependent formation of lymphoid follicles in patients with long-term ABU. The follicles resolve after antibiotic eradication of bacteriuria, confirming that they are driven by infection. However, there was no evidence of follicle formation in patients who carried E. coli 83972.[6]

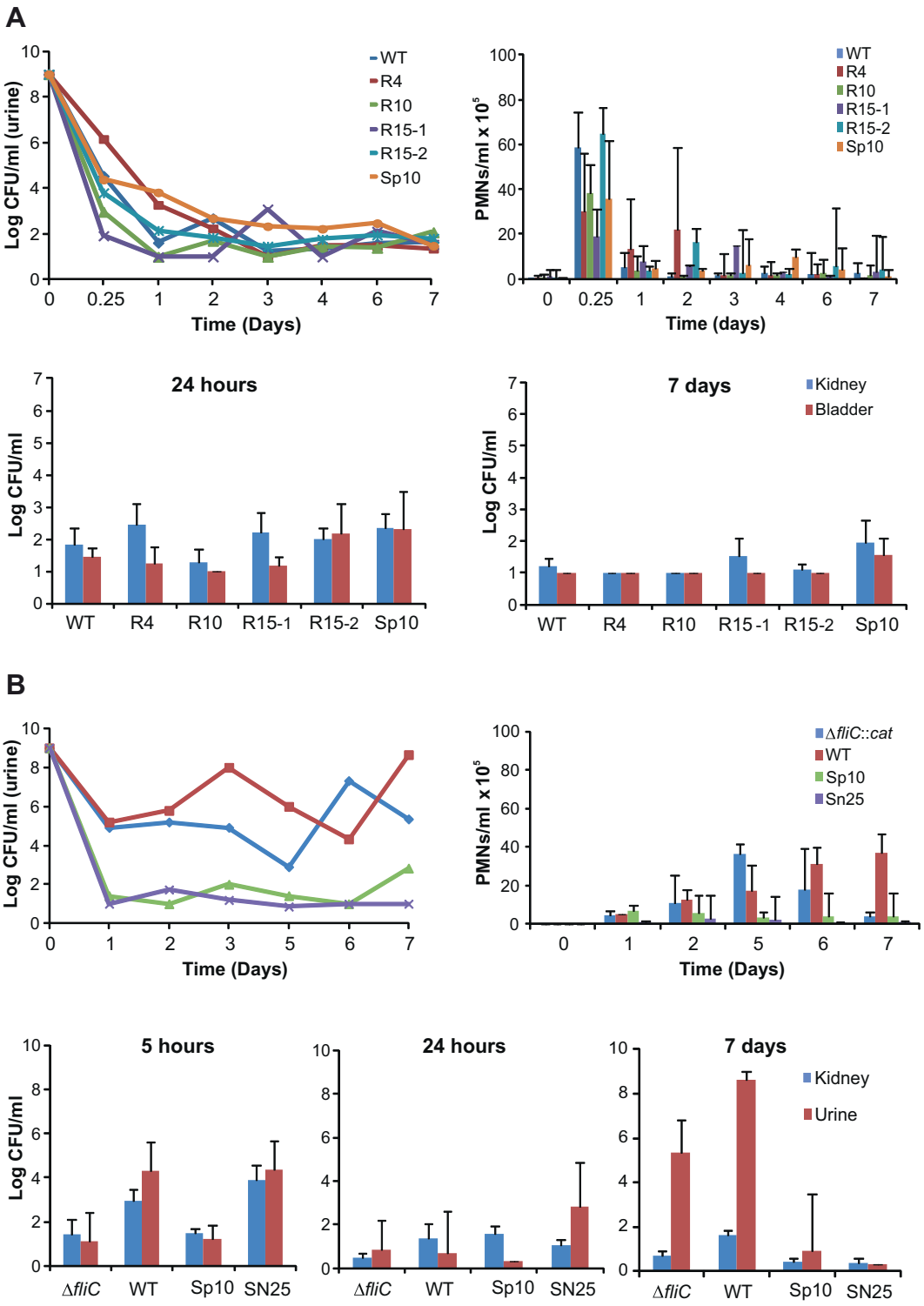


Figure 6. Geometric mean ± SEM values of re-isolate virulence, as defined by in vivo infection (Mann-Whitney test). *A*, experimental infection of C3H/HeN mice by intravesical inoculation with 10^9 cfu in 0.1 ml E. coli 83972 or re-isolates from symptomatic episodes. Bacterial number in urine, kidneys and bladders, and neutrophil response revealed no difference in virulence (Mann-Whitney test). *B*, role of increased flagellation and motility were compared for E. coli 83972 wt and highly motile re-isolate Sp10. Mutant strains E. coli 83972 *ΔfliC*::*cat* and SN25, highly motile re-isolate from asymptomatic carrier, served as negative and positive control, respectively. Bacterial numbers in urine, kidneys and bladders, and neutrophil influx revealed no long-term advantage for flagellated strains.

CONCLUSIONS

In the absence of functional virulence factors and shared molecular changes in bacteria the mechanism behind the emergence of symptoms remains unclear. A possibility is a host driven break in the tolerance of asymptomatic colonization, which triggers spurious pathogen recognition signaling. However, it is intriguing to speculate that the molecular events that precipitate cystitis symptoms have a different mechanistic nature than that of acute pyelonephritis. It is also intriguing that the few study patients in whom symptoms developed during ABU strain carriage may share what is to our knowledge an as yet undefined host response pattern that leads to immune overactivity and symptoms.

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II.

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Do *Escherichia coli* strains causing acute cystitis have a distinct virulence repertoire?

Birgit Stattin Norinder [a,b], Béla Köves [a], Manisha Yadav [a], Annelie Brauner [b], Catharina Svanborg [a,*]

[a] Department of Microbiology, Immunology and Glycobiology, Institute of Laboratory Medicine, Lund University, Sölvegatan 23, SE-22362 Lund, Sweden
[b] Division of Clinical Microbiology, Department of Microbiology, Tumor and Cell Biology, Karolinska University Hospital and Karolinska Institutet, Nobels väg 16, Box 280, SE-17177 Stockholm, Sweden

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ABSTRACT

Bacterial virulence factors influence the site and severity of urinary tract infections. While pyelonephritis-associated molecular traits have been defined, virulence factors specific for acute cystitis strains have not been identified. This study examined the virulence factor repertoire of 247 *Escherichia coli* strains, prospectively isolated from women with community-acquired acute cystitis. *Fim* sequences were present in 96% of the isolates, which also expressed Type 1 fimbriae. Curli were detected in 75%, 13% of which formed cellulose. *Pap* sequences were present in 47%, 27% were *papG*+, 23% were *prsG*+ and 42% expressed P fimbriae. TcpC was expressed by 33% of the strains, 32% in a subgroup of patients who only had symptoms of cystitis and 42% in patients with signs of upper urinary tract involvement; most frequently by the *papG*+/*prsG*+ subgroup. Strains with the full *fim*, *pap* and *TcpC* and curli virulence profile were more common in cystitis patients with than in patients without upper tract involvement ($p < 0.05$). The varied virulence profile of *E. coli* strains causing acute cystitis suggests that diverse bacterial strains, expressing Type 1 fimbriae trigger a convergent host response, involving pathways that give rise to the characteristic symptoms of acute cystitis.

1. Introduction

The severity of urinary tract infections (UTI) reflects the virulence and tissue specificity of the infecting strain. Acute pyelonephritis is caused by a restricted subset of uropathogenic *Escherichia coli* (UPEC) clones, distinguished for example by O:K:H serotypes or *E. coli* reference collection types combined with specific virulence factors with specific functions during the pathogenesis of infection [1]. Adhesins, including P and Type 1 fimbriae facilitate tissue attack and toxins perturb diverse cellular functions [1,2]. TcpC, a homolog of the Toll/Interleukin-1 receptor domain is a new type of virulence factor, which acts by inhibiting Toll-like receptor (TLR) signaling [3]. These virulence factors increase the fitness of UPEC for the renal environment and aid them to resist elimination by the host defense. Through their interactions with host cells, the virulence factors trigger the innate immune response, leading to symptoms like fever, general malaise and flank pain.

Acute cystitis is a more common but less well-defined disease entity than acute pyelonephritis, characterized by inflammation of the lower urinary tract with symptoms like dysuria, frequency and suprapubic pain. Acute cystitis strains form an intermediary group with respect to O:K:H serotype diversity, ECOR types and certain virulence gene frequencies [1,2,4,5]. Type 1 fimbrial expression alone has been discussed as major virulence factors in acute cystitis as these fimbriae enhance virulence in the murine urinary tract [6,7–9], through attachment to the bladder mucosa. Receptor epitopes are provided by mannosylated host cell glycoconjugates in sIgA [10], uroplakins on bladder cells, CD48 on mucosal mast cells [11], integrins β1 and α3 [12] and Tamm-Horsfall Protein (THP) [13] and diverse signaling pathways trigger bacterial internalization and innate immunity. On the other hand, human inoculation studies have so far not confirmed the role of Type 1 fimbriae for persistence and inflammation in the urinary tract [9,14,15]. Toxins such as hemolysin (hly) and cytotoxic necrotizing factor (CNF) enhance uroepithelial damage [16] and curli and cellulose support biofilm formation but there is no evidence that these properties are unique for acute cystitis strains or more abundant in this group [17]. Acute cystitis strains also express P fimbriae [4,5,18–20] and three PapG adhesin variants have been identified [21]. The reported frequencies of P fimbriated strains vary among acute cystitis isolates as

* Corresponding author.
E-mail addresses: catharina.svanborg@mig.lu.se, catharina.svanborg@med.lu.se (C. Svanborg).

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shown by binding assays and PCR-based genotyping [5,18–20,22] and thus, the contribution of the PapG adhesin variants to bladder infection remain unclear.

In this study, we have used molecular epidemiology to address if strains causing acute cystitis have a distinct virulence factor repertoire. The results show that Type 1 fimbriae and curli are common in acute cystitis isolates but analysis of multiple virulence factors did not define a cystitis-specific virulence profile. These findings raise the question if the symptoms of acute cystitis actually result from the action of specific virulence factors, especially Type 1 fimbriae which are most abundant among these strains, or if the pathogenesis of acute cystitis is fundamentally different from that of acute pyelonephritis, in terms of the variety of organisms that can give rise to a similar symptom profile. Understanding the pathogenesis of acute cystitis thus remains a major challenge.

2. Materials and methods

2.1. Patients

Women ≥ 18 years of age were enrolled in a controlled randomized treatment trial of symptomatic UTI in general practice [23]. They had significant bacteriuria ($>10^4$ cfu/mL) and were assigned a diagnosis of acute cystitis based on frequency, dysuria and/or suprapubic pain, a temperature $<38.0\,^{\circ}C$ and no flank pain. Patients who also had flank pain and/or fever ($>38.0\,^{\circ}C$) were diagnosed as having acute cystitis with upper urinary tract involvement. On admission, a history of previous UTI, concomitant disease and medical treatment were recorded. The UTI episode was classified as sporadic (<two episodes during the previous six months or <three during the previous 12 months) or recurrent and as uncomplicated or complicated if the patient had structural or functional abnormalities of the urinary tract.

2.2. Host response to infection

Blood samples were obtained at diagnosis and examined for C-reactive protein (CRP, cut off ≥ 10 mg/L), white blood cell counts (cut off $\geq 10 \times 10^9$/L) and erythrocyte sedimentation rate (ESR, cut off > 25 mm/h).

2.3. Urine cultures

Midstream urine samples were obtained at diagnosis. Quantitative urine cultures identified 247 *E. coli* growing as monocultures, and the isolates were stored in deep agar stabs. For analysis, bacteria were grown overnight on tryptic soy agar plates at 37 °C. The urinary tract is normally sterile, and urinary tract infections are usually caused by a single bacterial strain, originating from the fecal flora [24,25]. Infections by multiple organisms are associated with long-term catheterization or mechanical disorders affecting the urine flow [26].

2.4. Pap, fim, papG and TcpC genotypes

P fimbriae are encoded by the *pap* operon [27]. The *pap* genotype was determined by DNA–DNA hybridization with probes specific for the 5′ (*Hind*III) and 3′ (*Sma*I) fragments of the *pap* operon and derived from the *pap* gene cluster [22]. The *pap*G adhesin isotypes were defined by PCR, using primer pairs that matched unique regions of the *pap*G_{IA2}, *prs*G_{J96} sequences [28]. Whole bacterial cells provided template DNA and primers did not cross-amplify other *pap*G sequences, as shown by the recombinant strains containing a single known copy of *pap*G_{IA2} or *prs*G_{J96}. The P fimbriated *E. coli* IA2 and *E. coli* J96, and the *pap* positive recombinants *E. coli* HB101 (*pap*G_{IA2}) and *E. coli* HB101 (*prs*G_{J96}) were used as positive controls and *E. coli* HB101, *E. coli* AAEC (pPKL4) as negative controls. The *TcpC* genotype was defined by PCR, using specific primer pairs defining unique regions of the *TcpC* sequences [3].

The *fim*H genotype was defined by PCR, using primer pairs that matched unique regions of the adhesin sequences [28].

2.5. Bacterial phenotypes

Type 1 fimbrial expression was detected by hemagglutination of guinea pig and human erythrocytes after *in vitro* passage in Luria broth. Agglutination was performed both in the presence and absence of α-methyl-D-mannoside. Strains causing mannose-sensitive agglutination were defined as Type 1 fimbriated [15].

The P-fimbrial phenotype was defined by P blood group-dependent hemagglutination [22]. P-fimbrial expression was defined by agglutination of P_1 (receptor positive) but not p (receptor negative) erythrocytes. Class II strains agglutinated A_1P_1, OP_1 but not A_1p erythrocytes and Class III agglutinated only A_1P_1 and not OP_1 erythrocytes. Strains, which agglutinated A_1p erythrocytes were assigned to a group with “other mannose resistant adhesins”.

Morphotype analysis on Congo red and Calcoflour plates was used to study curli and cellulose expression [17]. After overnight culture, morphotypes were determined at daylight (Congo red) and UV-light (Calcoflour), as previously described. Reference strains were included and all strains were classified as curli+ and cellulose+, curli+ and cellulose−, curli− and cellulose− and curli− and cellulose+.

Biofilm formation was quantified by the crystal violet method [17]. Bacteria diluted in Luria-Bertani broth without salt were seeded into 96-well plates, incubated overnight at 37 °C without shaking, washed, air-dried and stained with crystal violet (3%). The dye was solubilized with ethanol (95%) and the optical density (OD) was measured at 570 nm. Ability to form biofilms was defined at an OD ≥ 0.5.

2.6. Hemolysin production

Hemolytic strains were identified in nutrient agar with 5% washed horse erythrocytes after overnight incubation. A hemolytic zone larger than the overlying colony was considered positive [4].

2.7. Statistical analysis

Chi-square test or the Fisher’s exact test was used. $p < 0.05$ was considered statistically significant (two-tailed).

3. Results

3.1. Characteristics of the patient population at inclusion

Women with cystitis symptoms and bacteriuria ($n = 247$, mean age 51 years, range 18–91) were included and their infecting *E. coli* strains were saved. All but five patients had bacteriuria defined as $\geq 10^5$ cfu/mL (98%); the remaining had 10^4 cfu/mL of urine. Most patients (83%) were healthy, except for the ongoing UTI episode, but 39 had hypertension and/or diabetes (Table 1). The UTI episode was sporadic in 73% while 16% had a history of childhood UTI, indicating UTI susceptibility. Most of the patients ($n = 215$) had only acute cystitis symptoms but a smaller group ($n = 32$, 13%) also had flank pain and/or fever, suggesting upper tract involvement (Table A.1). This group had increased circulating CRP levels and

Table 1
Host background variables in women with acute cystitis.

Host background variables	Patients No. (%)
Age, years median [range]	51.0 [18–91]
Medical events	
No illness[a]	205 (83)
Hypertension[b]	31 (13)
Diabetes	8 (3)
Diuretics[c]	29 (12)
UTI history	
Childhood UTI	39 (16)
Current UTI	
Cystitis	215 (87)
Upper tract involvement[d]	32 (13)
Type of symptomatic UTI[e,f]	
Sporadic uncomplicated	154 (62)
Sporadic complicated	26 (11)
Recurrent uncomplicated	56 (23)
Recurrent complicated	11 (4)
Total No. of patients	247

[a] Patients without any known illness other than UTI.
[b] One patient had both hypertension and diabetes, 26 patients with hypertension received diuretics and 3 additional patients received diuretic treatment without a diagnosis of hypertension.
[c] Diuretic treatments: tiazides ($n = 14$), loop-diuretics ($n = 6$), K-sparing drugs ($n = 1$), combinations of diuretics ($n = 8$).
[d] Patients with flank pain alone or in combination with cystitis symptoms and/or fever.
[e] Complicated UTI structural or functional abnormalities of the urinary tract including diabetes.
[f] Sporadic UTI < 2 UTI episodes during the last 6 months or <3 during the last 12 months.

white blood cell counts compared to the group with only acute cystitis symptoms ($p = 0.01$ and $p = 0.01$ respectively, Table 2).

3.2. Fim genotype, Type 1 fimbrial and hemolysin expression

As Type 1 fimbriae have been implicated in cystitis pathogenesis and shown to be essential virulence factors in the murine UTI model, we first defined the Type 1 fimbrial genotype by PCR using *fim* specific primers. The expression of Type 1 fimbriae was also detected by mannose-sensitive hemagglutination. Except ten isolates, all were *fim*+ (96%) and Type 1 fimbrial expression was detected in 80% of the isolates (Table 3). There was no significant difference in *fim* frequency between isolates from patients with acute cystitis (81%) and the subgroup which also had upper tract involvement (71%) (Fig. 1A). Hemolysin expression was only detected in 28% in the total sample and the frequency did not differ between the two groups (Table 3). The results confirm the high *fim* frequency among cystitis strains, consistent with these adhesins being essential for the pathogenesis of acute cystitis.

Table 2
Laboratory parameters in women with acute cystitis.

Laboratory parameter	Total No. (%)	Symptoms		*p* Values
		Cystitis No. (%)	Upper tract No. (%)	
C-reactive protein	247			
>10 mg/L	67	52 (24)	15 (47)	$p = 0.01$
White blood cell counts	242			
$>10 \times 10^9$/L	43	32 (15)	11 (35)	$p = 0.01$
Erythrocyte sedimentation rate	145			
>25 mm/hg	50	45 (21)	5 (16)	n.s.

Table 3
Fim genotype, type 1 fimbrial, curli/cellulose expression and biofilm formation.

Virulence typing, *E. coli* isolates	Total No. (%)	Symptoms		*p* Values
		Cystitis No. (%)	Upper tract No. (%)	
Fim genotype[a]	247	215	32	
Positive	237 (96)	207 (96)	30 (94)	n.s.
Type 1 expression[b,c]	226	198	29	
Positive	181 (80)	161 (81)	20 (71)	n.s.
Hemolysin expression[d]	245	213	32	
Positive	68 (28)	60 (28)	8 (25)	n.s.
Morphotypes[e]	227	198	29	
Curli+ and cellulose+	30 (13)	27 (14)	3 (10)	
Curli+ and cellulose−	140 (62)	117 (59)	23 (79)	$p = 0.036$
Curli− and cellulose−	57 (25)	54 (27)	3 (10)	
Curli− and cellulose+	0	0	0	
Biofilm formation[f]	225	196	29	
0.0–0.49	189 (83)	167 (85)	22 (76)	
0.5 ≥ 2	36 (16)	29 (15)	7 (24)	n.s.

[a] Analyzed by PCR.
[b] Analyzed by hemagglutination.
[c] Information from 21 patients was missing.
[d] 16 strains had weak hemolysin production.
[e] Information from 20 patients was missing.
[f] Information from 22 patients was missing.

3.3. Curli, cellulose and biofilm expression

Curli are bacterial surface organelles that bind several host extracellular matrix and contact phase proteins. These adhesive fibers enhance bacterial biofilm formation on various abiotic surfaces. To analyze curli expression as a virulence factor in acute cystitis isolates, curli expression was examined by morphotype analysis. Curli were detected in seventy-five per cent of the isolates; 73% in patients with acute cystitis compared to 89% of patients, who also had upper tract involvement. Only 13% of the strains formed cellulose (Table 3). The curli+ and cellulose− phenotype was more frequent in patients with upper tract symptoms ($p < 0.05$) (Fig. 1B). Biofilm, which consists of microorganisms and their extracellular products forming a structured community on a surface, was detected by the crystal violet method in <20% of all strains after growth at 37 °C, which was selected to resemble the conditions in the urinary tract. The results suggest that strains causing acute cystitis frequently express curli but biofilm formation was mostly not detected.

3.4. Pap/PapG genotypes and P-fimbrial expression

The pap gene cluster is strongly associated with acute pyelonephritis and urosepsis but in acute cystitis strains reported frequencies have been below 50%, suggesting a less strong effect on bladder infections than in the kidneys. The P-fimbrial G adhesin determines the receptor specificity is localized at the tip of the fimbrial organelle and at least 3 isotypes have been distinguished, based on receptor specificity of the G adhesin (Class I PapG, Class II PapG and Class III PapG or PrsG). Two P-fimbrial isotypes predominate among uropathogenic *E. coli*. Class II G adhesins, encoded by the $papG_{IA2}$ sequences, recognize all P blood group determinants. Class III G adhesins, encoded by the $prsG_{J96}$ sequences, recognize P blood group determinants with a terminal blood group A residue [22,27]. Class I P fimbriae ($papG_{J96}$) are uncommon in clinical isolates.

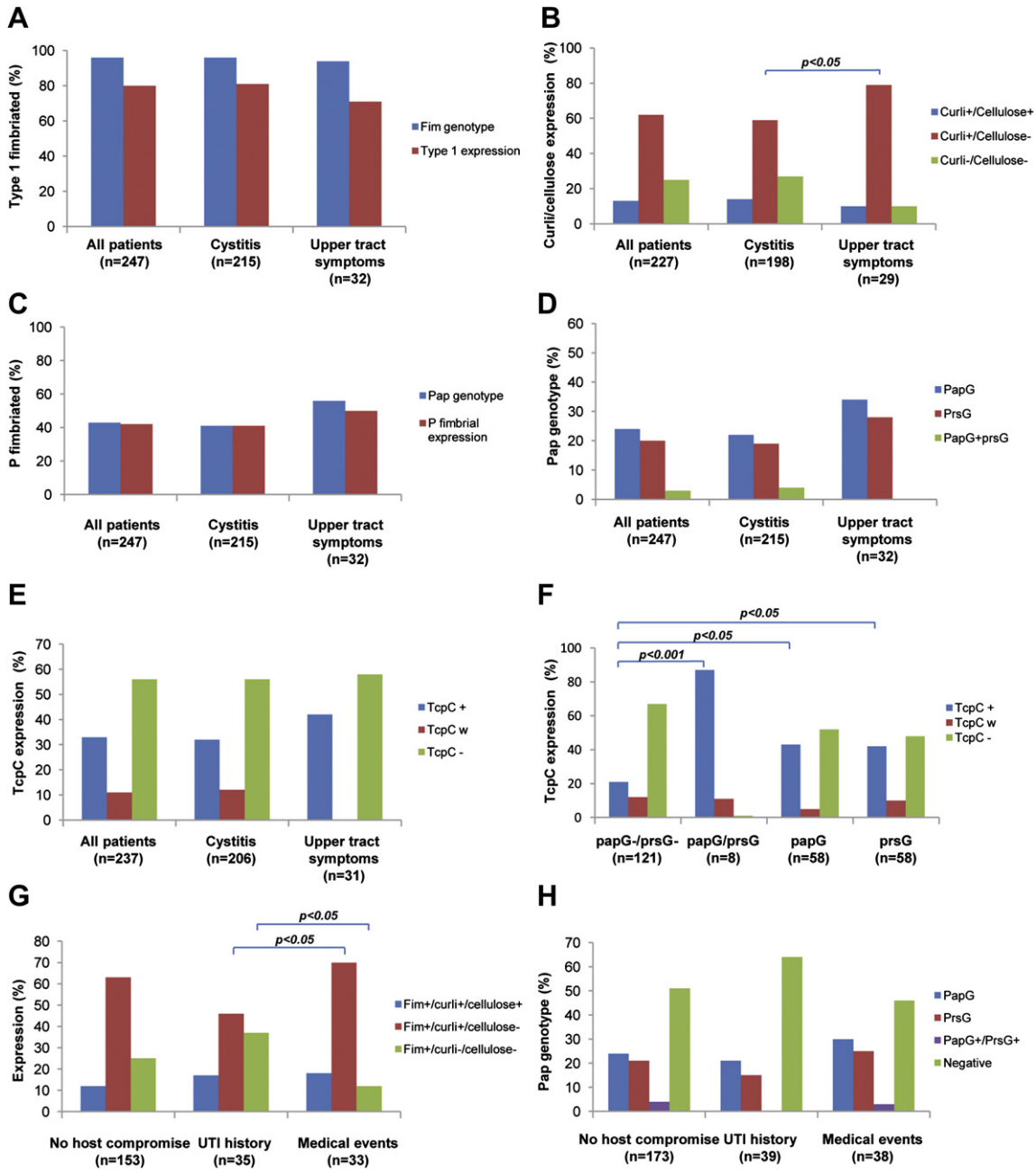

Fig. 1. Virulence factor repertoire of *Escherichia coli* isolates from women with acute cystitis. (A) *Fim* genotype and Type 1 fimbrial expression in isolates from 247 patients, all with symptoms of acute cystitis ($n = 215$) and a subgroup, who also had upper tract symptoms ($n = 32$). (B) Curli and cellulose expression of *Fim* genotype positive strains in the different patient groups. The curli+ and cellulose− phenotype was more frequent in the subset of patients with upper tract symptoms ($p < 0.05$). (C) and (D) *Pap* genotype and P-fimbrial expression in the different patient groups. (E) TIR homologous *TcpC* sequences in the different patient groups, and in relation to the *pap* genotype. (F) 23 isolates were weakly positive and are not included. Significantly higher *TcpC* frequency in patients with *papG*+ and/or *prsG*+ strains ($p < 0.001$ and $p < 0.05$). (F) *Fim* genotypes, curli/cellulose expression and *papG* genotypes (G) in patients with no host compromise, patients with history of UTI and patients with medical events. The frequency of fim+ and curli+ isolates was increased in patients with medical events compared to those with a history of UTI ($p < 0.05$).

To further clarify this question, the P-fimbrial gene cluster was detected by DNA hybridization and adhesin isotypes (*papG*/*prsG*) were identified by PCR, using specific primers. The *pap* gene cluster was present in 43% of all isolates (Table 4). The $papG_{IA2}$ adhesin sequences were present in 24% and $prsG_{J96}$ sequences in 20% of all isolates, while 3% of the isolates carried both adhesin genes (Fig. 1C and D).

The P-fimbrial phenotype is defined by hemagglutination, using erythrocytes specifically expressing the P blood group antigens in the presence or absence of the A blood group determinant and with P blood group deficient cells as a negative control. P-fimbrial expression (Class II + III) was detected by hemagglutination in 104 (42%) of the isolates (Table 4). Among those, Class II fimbriae ($papG_{IA2}$) were more common (77%) than Class III fimbriae ($prsG_{J96}$) (23%, $p < 0.001$). P blood group independent adhesins were found in 13% of the strains.

P-fimbrial expression was further examined as a function of the *papG* genotype. As expected, most strains expressing Class II P fimbriae were *papG*+ (80%) and isolates expressing Class III P fimbriae were *prsG*+ (96%), 30% of the strains agglutinating A_1p

Table 4
Pap genotype and P-fimbrial expression in *E. coli* isolates.

Pap genotype and P-fimbrial expression	No. of isolates (%)			*p* Values
	All isolates	Cystitis	Upper Tract	
Pap genotype,[a] total[b]	247	215	32	
Positive	106 (43)	88 (41)	18 (56)	n.s.
*Pap*G alleles,[c] total	247	215	32	
$papG_{IA2}$	59 (24)	48 (22)	11 (34)	
$prsG_{J96}$	50 (20)	41 (19)	9 (28)	
$papG_{IA2} + prsG_{J96}$	8 (3)	8 (4)	0 (0)	
P-fimbrial expression,[d] total	247	215	32	
Positive[e]	104 (42)	88 (41)	16 (50)	n.s.
P-fimbrial subtypes, total	104	88	16	
Class II[f] (PapG)	80 (77)	67 (76)	13 (81)	n.s.
Class III[g] (PrsG)	24 (23)	21 (24)	3 (19)	n.s.

[a] Analysis based on restriction fragment length polymorphism.
[b] Total = number of isolates examined for each parameter.
[c] Analyzed by PCR.
[d] Analyzed by P blood group specific hemagglutination.
[e] Agglutinated human P_1 but not p erythrocytes.
[f] Class II P fimbriated strains defined by agglutination of human A_1P_1, OP_1 but not p erythrocytes. There is a higher frequency of Class II P fimbriae compared to Class III in all three groups $p < .001$.
[g] Class III P fimbriated strains defined by agglutination of human A_1P_1 but not OP_1 or p erythrocytes.

erythrocytes were *prsG*+, suggesting that P-fimbrial expression might be masked in this group.

In patients with upper tract involvement, 56% of isolates were *pap*+ and 50% expressed P fimbriae compared to 41% and 41% of the isolates from patients without upper tract symptoms ($p = 0.102$ and $p = 0.332$ respectively). There was no difference in Class II distribution among patients with acute cystitis with or without upper tract involvement, however (76% versus 81%, $p = 0.75$).

The results suggest that about half of acute cystitis strains are *pap*+, that the *papG* genotype predominates over *prsG* and that most *pap*+ acute cystitis strains express functional P fimbriae.

3.5. TcpC genotype

TcpC is a TIR domain homologous protein secreted by UPEC, which promotes bacterial survival by inhibiting the innate host response and specifically MyD88 dependent signaling pathways [3]. The *TcpC* genotype of the cystitis isolates was defined by PCR, using specific primers. *TcpC* was detected in 33% of the isolates, in 32% of patients with acute cystitis compared to 42% in the subset of patients with upper tract symptoms (Fig. 1E). TcpC was more common in the *papG*+/*prsG*+ subset of the strains than in isolates lacking *papG* and/or *prsG* ($p < 0.01$ and $p = 0.01$, respectively) (Fig. 1F). The results confirmed that *pap*+ uropathogenic strains express *TcpC* more often than *pap*− strains, but showed no significant association with acute cystitis.

3.6. Virulence, UTI history and host compromise

Medical conditions that compromise the host defense have previously been shown to influence the requirements for virulence in strains causing acute pyelonephritis [29]. The virulence factor profile was therefore compared between isolates from patients with diabetes/hypertension and those who were healthy except for the ongoing UTI episode. Furthermore, genetic predisposition has been shown to influence acute pyelonephritis susceptibility and the frequency of UTI in this group. Isolates from patients with sporadic infections were therefore compared to isolates from patients, who had a history of UTI (Fig. 1G and H). There was no significant difference in overall virulence profile related to these host variables. The frequency of fim+ and curli+ isolates was increased in patients with medical events compared to those with a history of UTI ($p < 0.05$).

3.7. Combined virulence profile

The *E. coli* isolates were assigned a virulence profile based on their expression of virulence factors (Fig. 2). The complete virulence profile, comprising the *fim*, *papG*/*prsG* and *TcpC* genotypes as well as curli was detected in 18% of the isolates; 15% of the cystitis only and 37% of the group with upper tract involvement ($p < 0.01$). 35% of the strains carried the *fim*, *papG*/*prsG* sequences and expressed curli and this combination was also more common in patients with upper tract involvement ($p = 0.001$). There was also a significant difference in the frequency of *fim*+ strains with curli expression between the two groups ($p < 0.05$). The results showed that strains with the combined virulence profile were significantly more common in patients with acute cystitis who had upper tract involvement than in patients with only lower tract symptoms.

4. Discussion

The molecular basis of acute cystitis has been extensively studied in cellular and experimental infection models [14,15,30]. Still, it remains unclear if a specific repertoire of virulence factors

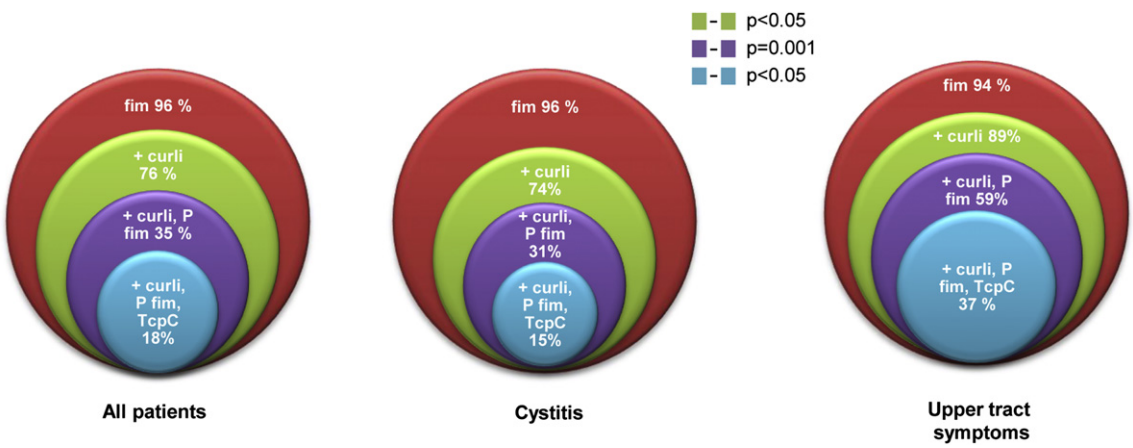

Fig. 2. Combined virulence repertoire including the *fim*, *tcpC*, *papG*/*prsG* sequences and curli formation in all patients, those with acute cystitis and upper tract symptoms, respectively. Strains with the combined virulence repertoire were more common in the subgroup of patients with acute cystitis and upper tract involvement compared to patients with acute cystitis alone ($p < 0.05$).

distinguishes acute cystitis strains from *E. coli* causing other forms of UTI. The present study examined *E. coli* isolates from 247 women with acute cystitis, using a combination of virulence genes commonly associated with acute pyelonephritis or cystitis. Type 1 fimbrial expression and *fim* sequences were common in the cystitis isolates, supporting their role in bladder infection. Curli, which have been proposed to improve biofilm formation, adhesion to host cells and internalization [31] were expressed by >70% of all isolates. In contrast, P fimbriae and TcpC were expressed by less than half of the cystitis strains, with *papG* being somewhat more common than *prsG*. A subgroup of strains expressed all the tested virulence factors (*fim*, *papG*, *prsG*, *TcpC* and curli) but such strains were not abundant in the acute cystitis group. Consistent with a role of these virulence factors in kidney infection, however, strains with the full virulence genotype were most common in patients with acute cystitis and upper tract involvement. The results suggest that Type 1 fimbrial expression is a unifying feature among acute cystitis strains, but provide no evidence that the virulence gene repertoire distinguishes strains causing acute cystitis from other uropathogens. In view of the variable virulence profile and high frequency of Type 1 fimbrial expression, we speculate that characteristic acute cystitis symptoms may be triggered Type 1 fimbrial interactions with the bladder mucosa. The symptoms reflect a different repertoire of host mediators than acute pyelonephritis possibly including bacterial tethering of neuronal circuits in the mucosal compartment.

Type 1 fimbriae are ubiquitously expressed by uropathogenic *E. coli* as well as other Gram-negative bacteria. Due to this high frequency, their role as independent virulence factors has been debated [15]. Recently, strains causing asymptomatic bacteriuria have been shown to carry *fim* deletions, suggesting that an intact *fim* gene cluster may be counterproductive and that a loss of functional type 1 fimbriae promotes bacterial adaptation to long-term bacterial carriage in the urinary tract. The high *fim* frequency in the present study is consistent with a contribution of Type 1 fimbriae to acute cystitis pathogenesis, either during the colonization phase or by enhancing inflammation and symptoms [9,14,15,30,32]. Furthermore, type 1 fimbriae are major virulence factors in the murine cystitis model, where they act by promoting bacterial attachment and by triggering a partially TLR4 dependent innate immune response [33]. FimH has also been shown to suppress NFkB-dependent transcription of pro-inflammatory genes [34,35] and Type 1 fimbriae have been proposed to enhance *E. coli* uptake into specialized dome cells in the bladder mucosa and promote intracellular bacterial proliferation, thus creating persistent infection and resistance to antibiotic therapy [36,37]. Binding of the FimH adhesin to uroplakin complexes on the uroepithelial surface mediates bacterial entry into uroepithelial cells [32,38] through elevated cAMP levels [34]. In addition, Type 1 fimbriae may be involved in eliciting apoptosis in uroepithelial cells [35]. In mucosal mast cells, FimH binding to the CD48 receptor has been proposed to direct bacterial uptake.

Human inoculation studies have provided somewhat contradictory results, regarding Type 1 fimbriae and their contribution to UTI. The prototype ABU strain *E. coli* 83972 fails to express Type 1 fimbriae and gives rise to a weak host response. After transformation of this strain with the *fim* gene cluster followed by human inoculation, the Type 1 fimbriated strain did not trigger a higher innate immune response than the wild type strain and there was no difference in the establishment of bacteriuria, suggesting that Type 1 fimbriae might function differently in the human and murine urinary tracts [15]. In addition to *fim* sequence variation, virulence for the urinary tract is modified by controlled variation in Type 1 fimbrial expression [30,39,40]. In a clinical study of *E. coli* O1K1H7 and acute pyelonephritis in children, disease severity was augmented when the infecting strain expressed both Type 1 and P fimbriae compared to infections caused by the same strain, but having lost Type 1 fimbrial expression [40]. This difference was also observed *in vivo*, where reconstitution with functional *fim* sequences restored virulence in the murine model [30] consistent with Type 1 fimbriae contributing to kidney infection. In the present study, Type 1 fimbrial expression was maintained in the large majority of the strains, suggesting that acute cystitis strains do not loose Type 1 fimbrial expression through phase variation or mutation during the acute phase of infection, consistent with a functional role for these fimbriae in acute cystitis.

The efficiency of the bacterial virulence factors in causing UTI depends on the immune status of the host. Innate immunity controls many aspects of the host response to acute UTI and variation on the efficiency of this response has been shown to affect the degree of tissue damage and the clearance of infection [41]. As a consequence, host genetic variants that modify the innate immune response have been associated with different forms of UTI [42,43]. In patients with recurrent UTI, which mostly denotes cystitis, several genetic screens have proposed gene associations, including promoter polymorphisms in LTA and TNFα [44], in the coding regions of TLR1, TLR4 and TLR5 [45]. The functional importance of these genetic variants in cystitis is not well understood, however. Several genetic markers of acute pyelonephritis have been established but have shown no association with acute cystitis. Low expression of the chemokine receptor CXCR1 is associated with APN susceptibility and *CXCR1* gene polymorphisms are common in pyelonephritis prone individuals [46]. Other genetic markers of pyelonephritis susceptibility include IRF 3 polymorphisms [43]. These genetic studies emphasize the difference in pathogenesis and genetic control as well as the symptoms typical of acute pyelonephritis and cystitis. Finally, in ABU, genes like TLR4 may be mutated and promoter polymorphisms have been associated with reduced TLR4 expression and ABU but not with acute cystitis [42]. In future studies, it may be relevant to match bacterial properties against the host immune repertoire, to better understand the pathogenesis of acute cystitis.

It is interesting to speculate that acute cystitis strains may share as yet undefined virulence factors that specifically enhance the attack on the bladder mucosa. The cystitis strains are genetically diverse, however, and it appears less likely that strains of very different clonal origin would share a new, disease-defining cystitis-specific virulence factor. The clinical presentation of disease might instead be determined by the host response pathways, which are activated by the different acute cystitis strains. Innate immunity is crucial for the antimicrobial defense of the urinary tract, and TLR4 dependent signaling pathways have been shown to influence the susceptibility to acute pyelonephritis and asymptomatic bacteriuria. It remains possible that distinct innate response circuits may distinguish cystitis prone patients from patients prone to other forms of UTI. In this case, strains with different virulence profiles may converge on similar host signaling pathways creating the characteristic acute cystitis symptoms. The relevant pathways and host response dynamics need to be further explored.

Conflicts of interest

None of the authors has a conflict of interest related to this study.

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Appendix

See Table A.1.

Table A.1
Signs and symptoms of acute cystitis at the time of diagnosis.

Symptoms	Patients No. (%)
Lower tract symptoms only	
Frequency and dysuria	92 (37)
Frequency, dysuria and suprapubic pain	71 (29)
Frequency or dysuria or suprapubic pain	39 (16)
Frequency, suprapubic pain or dysuria, suprapubic pain	13 (5)
Additional upper tract symptoms	
Flank pain and/or fever	32 (13.4)
Total No. of patients	247

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