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

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**Abstract**

Bacterial virulence factors influence the site and severity of urinary tract infections. While pylonephritis-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 infection; 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.

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1. Introduction

The severity of urinary tract infections (UTI) reflects the virulence and tissue specificity of the infecting strain. Acute pylonephritis 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 pylonephritis, 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 [1,2,5]. 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 slgA [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 [4,5,8–20] and three PapG adhesin variants have been identified [21]. The reported frequencies of P fimbriated strains vary among acute cystitis isolates as
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 fimbiae 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 fimbiae 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 (>10^5 cfu/mL) and were assigned a diagnosis of acute cystitis based on frequency, dysuria and/or suprapubic pain, a temperature <38.0 °C and no flank pain. Patients who also had flank pain and/or fever (>38.0 °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 ≥ 10 × 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 fimbiae are encoded by the pap operon [27]. The pap genotype was determined by DNA–DNA hybridization with probes specific for the 5' (HindIII) and 3' (SmaI) fragments of the pap operon and derived from the pap gene cluster [22]. The papG adhesin isotypes were defined by PCR, using primer pairs that matched unique regions of the papGIA2, prosG96 sequences [28]. Whole bacterial cells provided template DNA and primers did not cross-amplify other papG sequences, as shown by the recombinant strains containing a single known copy of papGIA2 or prosG96. The P fimbriated E. coli IA2 and E. coli J96, and the pap positive recombinants E. coli HB101 (papGIA2) and E. coli HB101 (prosG96) 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 fimH genotype was defined by PCR, using primer pairs that matched unique regions of the adhesin sequences [28].

2.5. Bacterial phenotypes

Type 1 fimbral 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 fimbrinated [15].

The P-fimbrial phenotype was defined by P blood group-dependent hemagglutination [22]. P-fimbrial expression was defined by agglutination of P1 (receptor positive) but not P (receptor negative) erythrocytes. Class II strains agglutinated A1P1, OP1 but not A1P erythrocytes and Class III agglutinated only A1P1 and not OP1 erythrocytes. Strains, which agglutinated A1P 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 ≥10^8 cfu/mL (98%); the remaining had 10^6 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
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.

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 papG257 sequences, recognize all P blood group determinants. Class III G adhesins, encoded by the prsG996 sequences, recognize P blood group determinants with a terminal blood group A residue [22,27]. Class I P fimbriae (papG996) are uncommon in clinical isolates.
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 papGIA2 adhesin sequences were present in 24% and prsGJ96 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 fimbrae (papG+) were more common (77%) than Class III fimbrae (prsG+) (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 fimbrae were papG+ (80%) and isolates expressing Class III P fimbrae were prsG+ (96%), 30% of the strains agglutinating A1p
with acute cystitis alone (p < 0.01). There is a higher frequency of Class II P- erythrocytes. There is a higher frequency of Class II P- 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).](image-url)
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 were expressed by Type 1 strains, with *papG* being somewhat more common than *prsc*. 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. However, 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 uropithelial 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 following 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 lose 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 TNFx [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 A1.

<table>
<thead>
<tr>
<th>Table A1</th>
<th>Signs and symptoms of acute cystitis at the time of diagnosis.</th>
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<tbody>
<tr>
<td>Symptoms</td>
<td>Patients No. (%)</td>
</tr>
<tr>
<td>Lower tract symptoms only</td>
<td></td>
</tr>
<tr>
<td>Frequency and dysuria</td>
<td>92 (37)</td>
</tr>
<tr>
<td>Frequency and suprapubic pain</td>
<td>71 (29)</td>
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<tr>
<td>Frequency or suprapubic pain</td>
<td>39 (16)</td>
</tr>
<tr>
<td>Frequency, suprapubic pain or dysuria pain</td>
<td>13 (5)</td>
</tr>
<tr>
<td>Additional upper tract symptoms</td>
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<tr>
<td>Flank pain and/or fever</td>
<td>32 (13.4)</td>
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</tbody>
</table>

Total No. of patients 247

References