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

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Summary of Ph.D. Thesis

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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.

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. 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. 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.

1.1. The most important virulence factors of E. coli

Bacterial adherence to mucosal surfaces is considered to be a critical virulence factor. Uropathogenic *E.coli* are usually fimbriated and may express several adhesive surface organelles, such as P, type 1 and S fimbriae.

Flagella driven motility has 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.

A biofilm is a structured community of microorganisms encapsulated within a self-developed polymeric matrix adherent to a surface. In the urinary tract the formation of biofilm may protect bacteria against environmental stress, phagocytosis and antibiotics.

Lipopolysaccharides (LPS) can be found in the outer membrane of Gram-negative bacteria. LPS interact with other virulence factors and may also play a role in the protection against the human immune system.

Bacteria need iron ions to successfully colonize the urinary tract. *E. coli* strains express different types of siderophores, such as aerobactin or salmochelin that scavenge iron from the environment to overcome iron limitation in the host.

Many UPEC secrete toxins that facilitate infection by damaging host tissues or by disabling the host immune system. The α-hemolysin is a pore-forming toxin, which was shown to increase the clinical severity of urinary tract infections. Cytotoxic necrotizing factor 1 (CNF1) promotes apoptosis of bladder epithelial cells and counteracts phagocytic activity and chemotaxis of PMNs.

Toll/interleukin-1 receptor (TIR) domain containing proteins (Tcps) are soluble proteins secreted by virulent bacteria which inhibit Toll-like receptor (TLR) signaling. TcpC promotes bacterial survival by inhibiting the innate host response and specifically MyD88 dependent signaling pathways.

1.2. Deliberate establishment of asymptomatic bacteriuria

Asymptomatic bacteriuria (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. In 1989 Hansson and co-workers showed in pediatric populations that ABU may be protective against recurrent episodes of UTIs [4]. 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. Asymptomatic bacteriuria 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.

The idea of deliberately established bacteriuria of the lower urinary tract was born based on these observations. *Escherichia coli* 83972 was chosen for the purpose of colonization, which was originally isolated from a young girl with long term ABU [5]. *E. coli* 83972 is a non-fimbriated strain belonging to a non-pathogenic OKH serogroup (OR:K5:H-) and a phylogenetic lineage B2 of *E. coli*, indicating a close relatedness to the UPEC strains which cause symptomatic UTI. The strain has been fully sequenced and extensively studied. It carries the different virulence genes, but does not express them, and never has been shown

to express functional adhesion properties. It carries a 1.6 kb plasmid, which is stable, and can be used for strain identification.

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 [5]. Patients with long term asymptomatic bacteriuria develop symptomatic UTIs significantly less compared to controls [5] [6].

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.

2. Aims

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

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.

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

The technical details are beyond the scope of this summary. A detailed description of methods is available in the Thesis.

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

We investigated *E. coli* 83972 re-isolates causing symptomatic UTI episodes in patients colonized in a previously published human inoculation study by Sundén et al [6]. 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 study design, patient characteristics and clinical results are described in detail in the paper by Sundén et al [6].

3.1.1. Bacteria, cytokines and DNA Techniques

E. coli 83972 re-isolates were identified by polymerase chain reaction (PCR). Neutrophils were quantified in uncentrifuged urine using a hemocytometer chamber. Genomic DNA was analyzed by pulsed field gel electrophoresis. Phylogenetic classification of the re-isolates was done using a method described by Clemont et al [7].

3.1.2. Bacterial genotypes, phenotypic assays and gene expression profiling

Bacteria were genotyped for fitness- and virulence-associated genes of extraintestinal pathogenic *E. coli* (ExPEC) by PCR. Type 1 fimbrial expression was detected by mannose sensitive haemagglutination. P- and S/F1C fimbriae were detected by hemagglutination of defibrinated human and bovine erythrocytes, respectively. Hemolytic activity was detected on sheep blood agar

plates. The aerobactin siderophore was detected by the aerobactin cross-feeding bioassay [8].

Morphotype analysis on Congo red and Calcoflour plates was used to study curli fimbria and cellulose expression [9]. Biofilm formation was assessed in a microtiter plate assay modified after O'Toole and Kolter [10]. Isolation of LPS from the *E. coli* strains used in this study was performed as previously described by Grozdanov et al [11]. Motility was investigated using motility agar plates.

RNA preparation and microarray hybridisation was performed as previously reported [12].

3.1.3. In vitro cell experiments

The human kidney carcinoma A498 (ATCC HTB-44) and T24 bladder carcinoma cell lines were used for cell experiments. For adhesion studies 10^9 cells were exposed to 10^5 bacteria for 45 minutes. For host response experiment cells were exposed to the bacteria, supernatants were collected 6 and 24 hours after stimulation and the secreted cytokines were quantified.

3.1.4. Experimental animal infections

Female C3H/HeN mice were infected by intravesical inoculation with E. coli 83972 wt or re-isolates. 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.

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.

Bacterial genotypes and the presence of functional type 1 and P fimbriae, TcpC, curli and cellulose, as well as biofilm formation and hemolysis was determined as described previously.

4. Results

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

5 cases of symptomatic UTI episodes caused by the colonizing *E. coli* 83972 strains were documented. 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. Adhesion studies on uroepithelial cells ruled out the possibility that the re-isolates acquired new adhesins.

Increased motility compared to the wild type was the only shared feature we found in two of the re-isolates. 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.

To analyze the re-isolates capacity to induce host response, we performed in vitro host response experiments. The IL-6 and IL-8 secretion in A498 human kidney 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.

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.

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

247 women with microbiologically proven uncomplicated UTIs were included in the analysis (mean age 51 years, range 18-91) and their infecting *E. coli* strains were saved. 215 patients were diagnosed with acute cystitis only, while 32 patients (13%) also had upper urinary tract involvement. The infection was sporadic in 180 cases, while 67 women had recurrent infection.

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%).

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%). However, only 16% of all isolates formed biofilm, 15% of the cystitis group and 24% of the upper UTI group. There were no significant differences between the subgroups in the expression of cellulose and curli or biofilm formation.

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 cystitis group (41%), although the difference was not significant. 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%). 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.

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.

5. Discussion

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* strains. To detect if changes in bacterial virulence are responsible for the rare development of symptoms during asymptomatic *E. coli* 83972 bacteriuria 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 virulence-associated genotype and the expression of virulence factors was identical in the *E. coli* 83972 wild type and symptomatic re-isolates. We found increased motility in two of the re-isolates, however no common motility related expression pattern was detected during transcriptomic analysis. 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.

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.

Moreover, since there are 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.

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. The high frequency of type 1 fimbrial expression 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.

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.

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. 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. They found *TcpC* sequences to be present in about 40% of acute pyelonephritis isolates and 21% of cystitis isolates. 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. 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 and try to make risk assessments of the possible disease severity based on patient characteristics. 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.

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 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.

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.

Publications directly related to the Ph.D. thesis

I. **Köves B***, Salvador E*, Gronberg-Hernandez J, Zdziarski J, Wullt B, Svanborg C, Dobrindt U Rare emergence of symptoms during long-term asymptomatic *E. coli* 83972 carriage, without altered virulence factor repertoire. Journal of Urology 2014 Feb;191(2):519-28. **IF: 3.696**

* These two authors contributed equally to this work.

II. Norinder BS, **Köves B**, Yadav M, Brauner A, Svanborg C
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Publications related to the subject of the Ph.D. thesis

Full papers

III. Grönberg-Hernandez J, Ambite I, Ragnasdottir B, **Köves B**, Zdiarski J, Lutay N, Dobrindt U, Wullt B, Svanborg C
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