Bacterial Characteristics of Importance for Recurrent Urinary Tract Infections Caused by *Escherichia coli*

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1. LIST OF PAPERS

The thesis is based on the following papers

Paper I:
Ejrnaes K., D. Sandvang, B. Lundgren, S. Ferry, S. Holm, T. Monsen, R. Lundholm, and N. Frimodt-Moller. 2006. Pulsed-Field Gel Electrophoresis Typing of *Escherichia coli* Strains from Samples Collected before and after Pivmecillinam or Placebo Treatment of Uncomplicated Community-Acquired Urinary Tract Infection in Women. J. Clin. Microbiol. 44:1776-1781.

Paper II:
Ejrnaes K, A. Reisner, B. Lundgren, S. Ferry, T. Monsen, S. Holm, E.L. Zechner, N. Frimodt-Moller. Characteristics of *Escherichia coli* Causing Recurrent Urinary Tract Infections: Phylogenetic Groups, Biofilm Formation, Antimicrobial Resistance and Plasmid Profiles. Submitted.

Paper III:
Ejrnaes K, M. Stegger, A. Reisner, S. Ferry, T. Monsen, S. Holm, B. Lundgren, N. Frimodt-Moller. Virulence Factors of Importance for Developing Relapse of Urinary Tract Infection with *Escherichia coli.*
Submitted.

2. SUMMARY

Urinary tract infections (UTIs) are among the most common bacterial infectious diseases encountered in clinical practice and account for significant morbidity and high medical costs. *Escherichia coli* is the most predominant pathogen causing 80-90% of community-acquired UTIs and 30-50% of nosocomially-acquired UTIs. Recurrent UTIs (RUTIs) are reported in 25% of women within 6 months of an acute UTI episode and pose a major problem. The aim of the present thesis was to look for bacterial characteristics of importance for recurrence of UTI caused by *E. coli*. The thesis is based on three papers.

The study is based on *E. coli* from 236 Swedish women with community-acquired symptomatic lower UTI from a large study of 1162 patients treated with one of three different dosing regimens of pivmecillinam or placebo. The women were evaluated clinically and bacteriologically at the initial visit and at two scheduled follow-up visits.

According to pulsed-field gel electrophoresis (PFGE) and culture results all primary infecting *E. coli* (initial isolates, pretherapy) were assigned into whether the initial infection was followed by cure, persistence, reinfection or relapse during follow-up. The prevalence of virulence factor genes (VFGs), phylogenetic groups, biofilm formation, plasmids and resistance to antimicrobials among primary infecting *E. coli* causing persistence or relapse at the follow-up visits were compared with the prevalence of these among *E. coli* followed by cure or reinfection.

Previous studies of RUTI using phenotypically based typing methods or less specific DNA based typing methods have concluded, that RUTIs are mainly attributable to reinfection with new strains. However, applying PFGE showed that 77% of RUTIs were caused by a relapse with the primary infecting *E. coli* (Paper I). This may support the recent observation that *E. coli* can invade and replicate within the murine bladder forming biofilm-like intracellular bacterial communities (IBCs) and establish quiescent intracellular reservoirs that may represent stable reservoirs for RUTIs. The IBC pathogenic cycle has not been studied in humans; however, recently exfoliated IBCs were detected in urine from women with acute uncomplicated cystitis supporting the presence of the IBC pathway and occurrence of an intracellular bacterial niche in some women with UTI.

Based on a triplex PCR *E. coli* can be divided into four main phylogenetic groups (A, B1, B2 and D). Phylogenetic group B2 was the most predominant group among the primary infecting *E. coli* followed by group D, A and B1. The majority of the tested 29

VFGs were associated with phylogenetic group B2, whereas only a few VFGs were more broadly distributed among the phylogenetic groups (Paper III). Primary infecting *E. coli* causing persistence or relapse of the infection were associated with phylogenetic group B2, whereas primary infecting *E. coli* followed by cure or reinfection were associated with group D (Paper II).

Phylogenetic group B2 was associated with susceptibility to many of the tested antimicrobials, whereas group A was associated with resistance to many of these antimicrobials and multidrug resistant (MDR) strains, and group D with MDR strains. Phylogenetic group A and D were associated with carriage of IncH and IncI plasmids, respectively. Resistance patterns or plasmid profiles of the primary infecting *E. coli* were not associated with outcome during follow-up (cure, persistence, reinfection or relapse) (Paper II).

Resistance to ampicillin, sulfamethizole, streptomycin and tetracycline was associated with a lower prevalence of some VFGs (*sfa/focDE*, *agn43bCFT073*, *chuA*, *iroN*, *cnf1*, *hlyD*, *ibeA*, *malX*, *usp*) and higher prevalence of other VFGs (*afa/draBC*, $agn43a_{CFT073}$, *iha*, *iutA*, *sat*) but the aggregate VFG score did not differ among the resistant and susceptible strains of these antimicrobials (Paper III).

Primary infecting *E. coli* causing persistence or relapse showed to have a higher biofilm formation capacity in vitro than those being followed by cure or reinfection (Paper II). This indicates that biofilm may be an important determinant for developing RUTI and may support the observation of IBCs.

Primary infecting *E. coli* causing relapse or persistence had a higher aggregate VFG score and higher prevalence of hemolysis and of many of the VFGs than those followed by cure or reinfection. The VFGs associated with persistence or relapse included: adhesins (*sfa/focDE*, *papAH*), a biofilm related factor (*agn43*), iron-uptake systems (*chuA*, *fyuA, iroN*), protectins (*kpsM II*, *kpsMII K2*), toxins (*cnf1*, *hlyD*), a marker of a pathogenicity-associated island from CFT073 (*malX*), and a bacteriocin-like factor (*usp*). No specific combination of VFGs could predict persistence or relapse (Paper III).

A regimen of three days pivmecillinam therapy for primary infecting *E. coli* positive for at least one of a number of traits (phylogenetic group B2, *sfa/focDE*, *papAH*, *agn43*, *chuA*, *fyuA*, *iroN*, *kpsM II*, *kpsM II K2*, *traT*, *cnf1*, *hlyD*, *ibeA*, *malX*, *usp* and being hemolytic) gave a significantly higher prevalence of persistence or relapse as opposed to primary infecting *E. coli* subjected to three days therapy with absence of these traits or primary infecting *E. coli* subjected to seven days therapy irrespective of these traits (Paper III).

In conclusion, our results may support the hypothesis of an intracellular reservoir of *E. coli* in the bladder. The recognition of uropathogenic *E. coli* as a potential intracellular pathogen challenges our current treatment regimens of UTI and argues for the development of new antimicrobials or treatment regimens/strategies. No distinct virulence profile could predict RUTI. However, we found VFGs associated with persistence or relapse that may be potential targets for prevention and treatment of UTI. Furthermore we identified potential markers that may be used to select a more differentiated and optimal treatment. Future studies must explore the function of these VFGs and other putative and novel VFGs in relation to persistence or relapse of UTI and their possible role in IBC formation. Defining the repertoire and mechanism of VFGs could facilitate the development of new diagnostic tools, regimens and drugs for prevention and treatment of RUTI.

3. INTRODUCTION AND BACKGROUND

Urinary tract infection (UTI) is a broad term that describes microbial colonization of the urine and infection of the structures of the urinary tract – kidney, renal pelvis, ureters, bladder, and urethra, as well as adjacent structures such as the perinephric fascia, prostate, and epididymis (99). UTI is usually categorized by infection site and can additionally be classified according to whether it is uncomplicated (occurring in the normal urinary tract of immunocompetent individuals, usually young healthy non-pregnant women) or complicated (occurring in individuals of all ages and sexes that are immunocompromised or have genitourinary tracts with structural or functional abnormalities, including urethral catheterization) (60,99,117).

EPIDEMIOLOGY AND COSTS.

UTIs are among the most common bacterial infectious diseases encountered in clinical practice and the overall annual incidence of UTI in USA has been estimated to be 12% among women and 3% among men (35). The incidence of UTI is influenced by gender and age, with UTI being most common among females in all age groups (28,35). Sexually active women aged 20 to 40 years and postmenopausal women older than 60 years are the two populations at greatest risk for UTI (35). The main focus for the following introduction and discussion will be UTI in women.

The incidence in women increases with age and has a peak in the twenties (28,35). The annual incidence of symptomatic UTI requiring prescription of medicine among women aged 18 and older has been estimated to be around 11% and by the age of 24, one third of women have been estimated to have had at least one physician-diagnosed UTI treated with a prescription medication (34). The incidence of symptomatic UTI among young sexually active women has been found to be 0.5-0.7 pr. person-year (61). The lifetime risk of symptomatic UTI among women has been found to be 60% (34).

Pyelonephritis is a less common type of symptomatic UTI than cystitis. A population-based epidemiological analysis showed an annual rate among outpatients of 12-13 per 10.000 population and among inpatients of 3-4 cases per 10.000 population (16).

The prevalence of asymptomatic bacteriuria (ABU) in healthy women has been shown to increase with age, from around 1% in females aged 5 to 14 years to more than 20% in women at least 80 years of age living in the community (129).

Recurrent UTI (RUTIs) are reported in 16-25% of women within 6 months of an UTI episode and in 40-50% of women within one year of an UTI episode despite antimicrobial treatment and that the women are healthy and generally have anatomically normal urinary tracts. RUTIs are thus common and pose a major problem (28,32,40,67,91).

The financial implications of UTI are quite high, predominantly as a result of the high incidence of UTI. The direct costs include the cost of outpatient doctor visits, antimicrobial prescription and hospital expenses as well as nonmedical cost associated with sick days and morbidity. The indirect cost of lost output should also be considered (33). In USA the overall expenditures for treatment of UTIs in 2000, excluding outpatient prescription drugs, were estimated to be around US $ 2.5 billion (45).

CRITERIA FOR UTI AND CLINICAL PRESENTATION

A UTI is defined as a significant number of pathogenic organisms in the urinary system. The limit for significant bacteriuria is

dependent upon presence/absence of symptoms, bacteria category, number of species isolated, method of specimen collection and gender (98). The European Urinalysis Guidelines have defined the limits for symptomatic UTI caused by *Escherichia coli* to be 10^3 CFU/ml (98).

UTI generally manifests itself in one of three clinical presentations: asymptomatic bacteriuria, cystitis and acute pyelonephritis.

Asymptomatic bacteriuria (ABU) is characterised by bacteria in the urine without clinical signs or symptoms of UTI in the host (129). The ABU strain thus exists in a commensal-like relationship with the host. The long-term complications of ABU are dependent on the population. For many groups ABU screening and treatment of ABU are not considered beneficial, and the colonizing organism has been reported to outcompete uropathogenic *E. coli* in the urine and prevent infections with other more virulent bacteria (65,129,154). For other groups including pregnant women and people undergoing traumatic genitourinary procedures ABU is associated with adverse outcomes, and screening and treatment is regarded beneficial (129).

Cystitis is characterised by dysuria, frequency and urgency of urination and sometimes suprapubic pain (29). Cystitis is associated with significant short-term morbidity, and it is considered to be a benign illness with minimal long-term sequelae. However, there is no large-scale prospective studies verifying this assumption (33).

Pyelonephritis is associated with flank pain, fever, nausea and vomiting and may occur in the absence of cystitis symptoms (179). Pyelonephritis may progress to bacteremia and is associated with a substantial morbidity, mortality, and long-term sequelae like renal scarring and impairment of renal function (37,147).

RUTI is a widely used term. However, there is little consensus regarding the definition of RUTI. Some have defined UTI as recurrent when there have been at least three episodes of UTI documented by urine culture in the last 12 months, whereas others have used other numbers and time intervals or a broader and less strict definition (32,67,163).

AETIOLOGY

By far the major cause of UTI is bacteria, but fungi and viruses may occasionally cause UTI. The majority of community-acquired, uncomplicated UTI is caused by *E. coli* (80-90%) or *Staphylococcus saprophyticus* (5-10%) whereas *Klebsiella*, *Enterobacter*, *Proteus* or *Enterococci* species infrequently cause infection outside hospitals (30,89). In contrast it is more diverse in nosocomially-acquired UTIs where these species and *Candida* species are more common and where *E. coli* accounts for only 35-50% (6,152).

E. coli is a very diverse bacterial species found naturally in the environment and in the intestine of all humans and many other animal species. The *E. coli* strains of significance for humans can be classified according to genetic and clinical criteria into three main groups: commensal *E. coli*, intestinal pathogenic *E. coli* and extraintestinal pathogenic *E. coli* (ExPEC) (90,158). The niche of commensal *E. coli* is the mucous layer of the colon and *E. coli* is a highly successful competitor at this site, comprising the most abundant facultative anaerobe of human intestinal microflora. Commensal *E. coli* are in general benign; they coexist with the human host with mutual benefits and do not cause disease. However, it may cause illness if the host is compromised immunologically or if the normal gastrointestinal barriers are breached (158). Intestinal pathogenic strains cause enteric/diarrhoeal diseases and six different categories have been well described: enteropathogenic *E. coli*, enterohaemorrhagic *E. coli*, enterotoxigenic *E. coli*, enteroaggregative *E. coli*, enteroinvasive *E. coli* and diffusely adherent *E. coli* (127). ExPEC have maintained the ability to exist in the gut without consequence but have the capacity to disseminate and colonize other host niches causing extra-intestinal diseases including neonatal meningitis, sepsis, nosocomial pneumonia, osteomyelitis, soft-tissue infections, wound infections and UTI (158). *E. coli* causing UTI has been denoted uropathogenic *E. coli* (UPEC). However, whereas the intestinal pathogens are characterised by specific virulence factors (VFs), UPEC remain less well defined and no single profile of urovirulence has been determined to date (10,114,158).

E. coli can be considered as having mainly a clonal genetic structure and phylogenetic analyses based upon multi locus enzyme electrophoresis (MLEE) have shown the existence of distinct phylogenetic groups within *E. coli* (19,57,131). Currently, there are four well-recognized phylogenetic groups and these have been designated A, B1, B2 and D. *E. coli* strains of the four phylogenetic groups differ in their phenotypic and genotypic characteristics and appear to have different ecological niches and propensity to cause disease. Commensal *E. coli* are mainly associated with phylogenetic groups A or B1 and intestinal pathogenic *E. coli* with phylogenetic groups A, B1 or D (137,158). In contrast, ExPEC including UPEC have shown to belong mainly to phylogenetic group B2 and, to a lesser extent, to group D (Paper I) (76,120,137,176).

Traditional classification of *E. coli* strains is based on the presence of certain O (somatic), K (capsular polysaccharide), and H (flagelar) antigens (134). The serotype of a strain refers to all three antigens, whereas the serogroup refers only to the O antigen type. The O antigen, which includes around 180 types, is a polysaccharide anchored in the outer core of the lipopolysaccharide component of the bacterial membrane (174). There is a high frequency of the antigens O1, O2, O4, O6, O7, O8, O16, O18, O25, O75 among UPEC and one study found that 3 serogroups O4, O6 and O75 accounted for 50% of the UPEC. Specific K and H antigens have less defined patterns (79,84,184).

PATHOGENESIS

Pathogenesis of uncomplicated UTI is complex and influenced by many host biological and behavioural factors, and by properties of the infecting uropathogens. Despite a considerable number of studies it remains poorly understood. Considering that *E. coli* is the dominant causative agent of UTI the following will focus on UTI with *E. coli*.

Most uropathogens originate in the rectal flora and enter the bladder via the urethra with an interim phase of periurethral and vaginal colonization, and sometimes they may reach the kidneys (9,171). The mechanism of ascension is uncertain, but motility mediated by flagella might be important (102,189). This faecal-perineal-urethral route of infection is supported by the finding of the causative *E. coli* in the woman's faecal flora at the time of a UTI episode (119,120,192). It has been debated whether the causative *E. coli* represents the most prevalent fecal clones within the host (the prevalence hypothesis) or instead represents a distinctive highly selected subset of the fecal *E. coli* population with enhanced virulence potential (the special-pathogenecity hypothesis) (119). A recent study suggested that these two hypotheses are not mutually exclusive, but instead they might contribute jointly to UTI pathogenesis with virulence factors and other group B2-associated characteristics possibly promoting intestinal dominance (120).

While it seems to be established that *E. coli* causing UTI originate from the fecal flora of the host, the external reservoir(s) from which these *E. coli* initially originated remains unclear. *E. coli* has been shown to be able to cause outbreaks of community-acquired UTI (110,112,133,139). Analysis of these outbreaks did not reveal any evidence that person-to-person transmission contributed to these outbreaks, and contaminated food- or water-borne sources have been suggested; however, the limited epidemiological data available prevented definitive conclusions regarding person-to-person transmission or an external source (110,112,133,139). Within-households, *E. coli* strain sharing has been shown to be widely prevalent, and it commonly occurs among pets, humans (non-sex partners as well as between sex partners) and between pets and humans (38,39,80). The underlying transmission route/source was not detected but person-to-person transmission between sexual partners and person-to-person transmission possibly by fecal-oral route for other contacts have been suggested. An external source like food or water has also been suggested although none of these transmission routes were proven (80). Several studies have suggested that the *E. coli* in human intestine may originate from contaminated food and this hypothesis of foodborne transmission has been supported by studies finding molecular similarities between retail meat products and human-source *E. coli* (53,69,83,111,144).

Haematogenous seeding of the urinary tract is uncommon, but occasionally UTI occurs following *Staphylococcus aureus* bacteremia or *Candida* sp. fungemia. The importance of lymphatic spread of uropathogens in the pathogenesis of UTI is unknown.

E. coli has traditionally been regarded as an extracellular pathogen; however, in the recent years UPEC have shown to be an opportunistic intracellular pathogen. *E. coli* have in murine models of cystitis been shown to utilize a multistep pathogenic cycle (IBC pathogenic pathway) during infection in which they progress through an intracellular niche within the bladder contributing to the establishment of acute UTI as well as the recurrence of UTIs (Figure 1) (87).

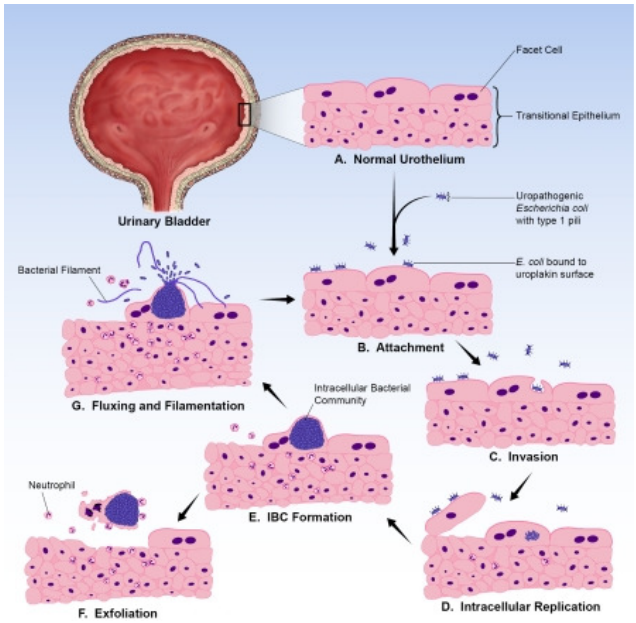

Figure 1
IBC pathogenic pathway observed in the murine cystitis model (155)

FimH, the adhesion of type 1 fimbriae, binds to α3 and β1 integrin of the urothelium of the bladder activating a signal cascade that stimulates the host plasma membrane to zipper around and envelop bound UPEC (24). Once internalized in the host bladder epithelial cells, UPEC are trafficked into membrane bound-acidic compartments that have many characteristics of late endosomes and lysosomes (25). The fate of UPEC within these compartments varies depending on the differentiation status of the host cells. Entering the cytosol of the facet cells, UPEC have been shown to rapidly multiply forming large inclusions called intracellular bacterial communities (IBCs) (former termed pods or bacterial factories) with biofilm-like properties e.g. regional expression of Antigen 43 and type 1 fimbriae, and polysaccharide rich matrix surrounding differentiated subpopulations of bacteria (1). IBCs exist only transiently before the *E. coli* dissociate and migrate out of the facet cells, many adopting a filamentous morphology (1,87,190). The filamentous UPEC avoid engulfment by neutrophils, thus allowing them to reinvade the urothelium and initiate subsequent rounds of IBC formation although with slower kinetics (88).

In response to infection facet cells exfoliate, furthermore the influx of immune cells also damage the urothelium and UPEC gain access to and invade the underlying immature bladder cells. Within these underlying cells UPEC-containing vacuoles are often enmeshed within a network of actin fibres and bacterial replication is limited and UPEC are proposed to establish quiescent intracellular reservoirs (QIRs), where UPEC can persist quiescently for long periods undetected from the host immune system and less susceptible to many antimicrobial treatments (66,92,125,126,162). It has been shown that QIRs can be activated by stimulation of the facet cell exfoliation, which activates cell differentiation and proliferation cascades in the underlying cells, leading to bacterial replication and subsequently new rounds of IBC formation, and thus relapse of infection (25,126). The QIRs may thus serve as a source for RUTIs in addition to the intestine and vagina. The IBC formation has been shown to occur with most UPEC isolates tested in a murine model and thus appears to be a general attribute of many UPEC isolates (43).

The IBC pathogenic cycle has not been studied in humans. However, an older study showing bacteria being cultured form bladder tissue of women with RUTIs during periods with absence of bacteriuria may support the presence of the IBC pathogenic cycle in humans (23). Recently exfoliated IBCs and filamentous bacteria were detected in urine from women with acute uncomplicated cystitis, which supports the presence of the IBC pathway and occurrence of an intracellular bacterial niche in some women with UTI (Figure 2). The study did not show whether intracellular bacteria persist and contribute to recurrence of UTIs as they appear to do in mice (155).

Recently it was shown that *Klebsiella pneumonia* may progress through the IBC pathway although with fewer IBCs than UPEC, a difference that may be related in part to expression of type 1 fimbriae (156). This study did not investigate the ability to form long-lasting QIRs (156). This suggests that the IBC pathogenic pathway may not be specific for UPEC, but may occur with other uropathogens. Other bacteria have also been proposed to establish long-lived intracellular reservoirs that may contribute to the chronic and recurrent nature of a number of bacterial infections. An example of this is *Streptococcus pyogenes* (Group A) causing recurrent tonsillophyngitis and *Streptococcus pneumonia* causing otitis media with effusion (14,140).

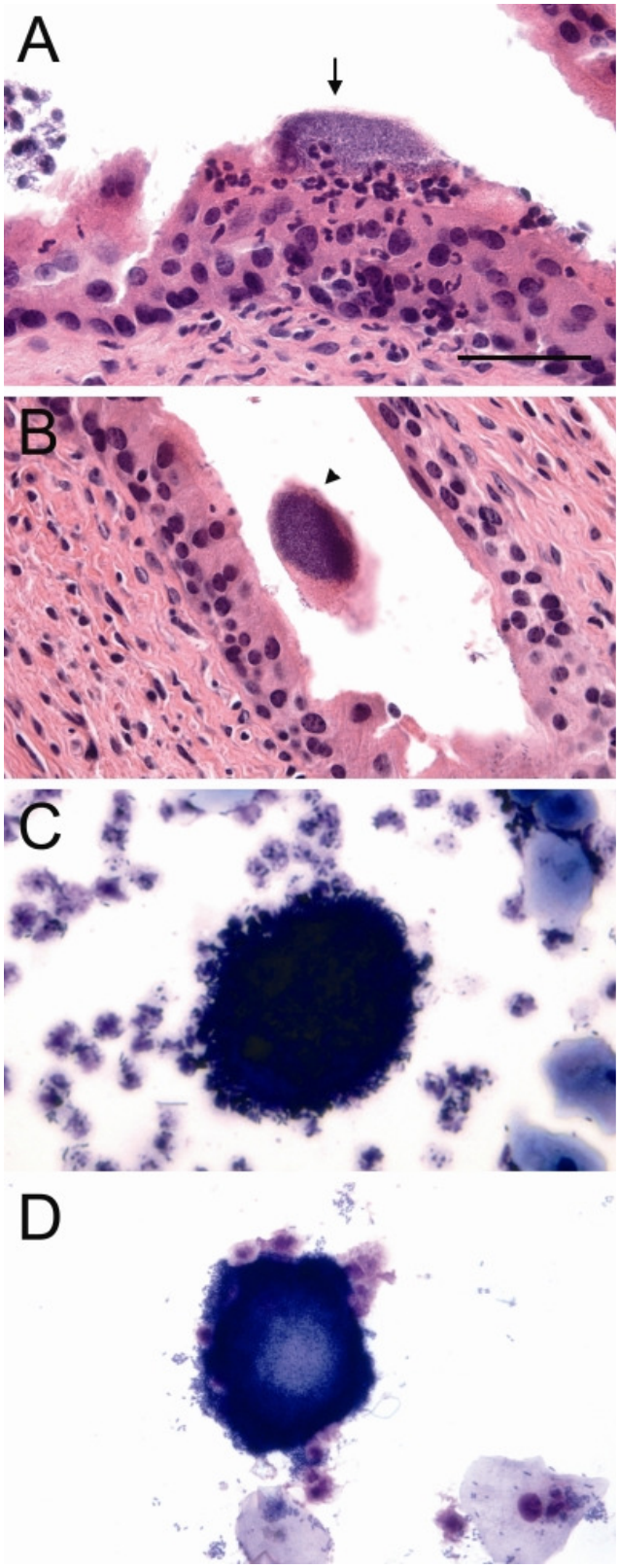

Figure 2
Mouse trail and comparison of human and mouse urine (155). A: IBC (arrow) in the murine bladder. B: IBC (arrow) exfoliated into the lumen of the murine bladder. C: Urine from mice containing IBC. D: IBC in the humane urine similar in morphology and size to those seen in the urine from mice.

HOST FACTORS

Several host factors have been associated with increased risk of UTI. These host factors can be divided into biological and behavioural factors.

Sexual intercourse, diaphragm and spermicide use have been shown to predispose to UTI (61). Recent use of antimicrobial agents has been associated with an acute UTI episode. The exact mechanism is unclear, but it has been suggested that antimicrobials disrupt the vaginal or periurethral flora enabling colonization by a more uropathogenic organism (36,55).

It has been shown that a family history of UTI in the mother and a history of childhood onset of cystitis are associated with RUTI indicating that inherited factors could predispose to UTI, although it could also reflect shared environmental factors or behaviours (163). Some studies have shown that non-secretors of histocompatibility blood group antigens are significantly more susceptible than secretors to be colonized by P fimbriated UPEC supporting an association between genetic factors and RUTI (93). Women with RUTI have been found to have an increased susceptibility to vaginal colonization with uropathogens compared to women without RUTI and the vaginal colonization with Gram-negative bacteria showed to be heavier and to last longer than in women without a history of RUTI (59). A recent study found RUTI to be associated with compromised vaginal immune response and an aberrant vaginal microbiota with lack of lactobacilli (95). Anatomic abnormalities of the urinary tract or a compromised host is associated with increased susceptibility to UTI (35).

HOST INFLAMMATORY RESPONSE TO UPEC

Once inside the urinary tract UPEC elicit the host immune response. Attachment of UPEC to the urothelium is inhibited by the flow of urine, its low pH and high osmolarity and a number of soluble factors like lactoferrin, lipocalin, Tamm-Horsfall protein and secretory IgA (185). UPEC avoiding this first line defence attach and invade the urothelium which activates different toll-like receptors inducing an array of defence mechanisms: a release of antimicrobial molecules like cathelicidin or defensins (alfa and beta defensins), a release of interleukin 6 and 8 which attract additional immunocompetent cells like neutrophils and dendritic cells to eliminate the invading UPEC, inhibition of cytoskeletal rearrangements preventing further invasion, and activation of apoptotic pathways within facet cells leading to exfoliation (185).

VIRULENCE FACTORS OF UPEC

Virulence refers to the degree of pathogenicity of an organism, or in other words the relative ability of a pathogen to cause disease. Virulence factors (VFs) are specific properties that enable organisms to overcome host defenses and cause disease (72). A molecular version of Koch's postulates has been devised by Falkow in an attempt to provide a definition for the term VF (27). This new version has three criteria. First, the potential VF should be found in all pathogenic strains of a species but be absent from their non-pathogenic relatives. Second, specific inactivation of the relevant gene(s) should attenuate virulence in an appropriate animal model. Third, subsequent reintroduction of the gene should restore virulence in the animal model. However, recent advantages within genomics, including the finding that features previously thought to be pathogen associated or restricted to pathogens were identified in the commensal genome, question the uncritical use of these criteria and might argue for these to be re-evaluated (135).

Table 1
A selection of Virulence Factors of Uropathogenic *E. coli*

Functional Category	Name	Abbreviation	Gene	Function of the Virulence Factor	References
Adhesin	Dr binding adhesin		*afa/draBC*	Adhesin, ass. with cystitis and pyelonephritis, invasion of urothelium	72,123
	Blood group M fimbria		*bmaE*	Adhesin	72
	Type 1 fimbria		*fimH*	Adhesin, mediates binding to urothelium and invasion, role in IBC formation	1,124, 190
	F1C fimbria		*focG*	Adhesin	123
	G fimbria		*gafD*	Adhesin	72, 85
	Iron-regulated gene A homologue adhesion		*iha*	Adhesin, siderophore function	82
	P fimbria		*papAH*	Adhesin, mediate binding to urothelium, ass. with pyelonephritis	104, 123
	S fimbria/F1C fimbria		*sfa/focDE*	Adhesin, ass. with cystitis and pyelonephritis	113, 123
Biofilm related	Antigen 43	Ag43	*agn43*	Adhesin, autotransporter, aggregation, biofilm related	1, 181, 183
	Antigen 43, allele a CFT073		$agn43a_{CFT073}$	Adhesin, autotransporter, aggregation, biofilm related	183
	Antigen 43, allele b CFT073		$agn43b_{CFT073}$	Adhesin, autotransporter, aggregation, biofilm related	183
	Antigen 43, allele K12		$Agn43_{K12}$	Adhesin, autotransporter, aggregation, biofilm related	161, 183
Iron uptake	Heme receptor		*chuA*	Uptake of hemin	180
	Yersiniabactin siderophore receptor		*fyuA*	Uptake of ferric iron	54
	Salmochelin siderophore receptor		*iroN*	Uptake of ferric iron	54, 82
	Iron-regulated element		*ireA*	Uptake of ferric iron	54, 157
	Aerobactin siderophore receptor		*iutA*	Uptake of ferric iron	54, 180
Protectins	Increased serum survival	Iss	*iss*	Outer membrane protein, resistance to serum bactericidal activity	72, 85
	Group II capsule		*kpsM II*	Protect against phagocytosis, opsonisation and lysis	72, 143
	Group II capsule, incl K2		*kpsM II K2*	Protect against phagocytosis. Opsonisation and lysis	72, 143
	Group III capsule		*kpsMT III*	Protect against phagocytosis. Opsonisation and lysis	72
	Serum resistance	TraT	*traT*	Outer membrane protein, resistance to serum bactericidal activity	72, 85
Toxins	Cytotoxic necrotizing factor 1	CNF	*cnf1*	Cytoskeleton reorganization, modulation of signalling pathways	105, 151
	Cytolethal distending toxin	CDT	*cdtB*	Create abnormalities in host cell function or morphology, cell cycle arrest or lysis	73, 85
	Alpha hemolysin	HlyA	*hlyD*	Cell lysis, modulation of host signal pathways, tissue injury, exfoliation of urothelium	72, 167, 187
	Secreted autotransporter toxin	SAT	*sat*	Create abnormalities in host cell function or morphology, cell cycle arrest or lysis	48, 49
Miscellaneous	Invasion of brain endothelium		*ibeA*	Neonatal meningitis, invasion of endothelium	64, 85
	Pathogenicity-associated island marker of CFT073		*malX*	Encoding different VF, marker of PAIs	85
	Uropathogenic specific protein	USP	*usp*	Unknown, suggested bacteriocin function	191

The genomic sequence of some UTI isolates (pyelonephritis isolates CFT073 and 536, cystitis isolates UTI89 and F11) has been recently been published (10,12,145,186). However, despite many similarities among UPEC isolates, genomic features that are specifically unique to UPEC have not yet been identified, and there are considerable differences in the repertoire and expression levels of VFs among UPEC (Paper III) (10,145).

Epidemiological studies and in vivo experimental animal studies of diverse properties of UPEC have suggested the existence of a diverse array of VFs that enable UPEC to overcome host defenses and establish infection in the urinary tract contributing to virulence of UPEC. These VFs can be grouped by functional category e.g. adhesins, toxins, iron acquisition systems and protectins (Table 1) (73). Experimental and epidemiological data have shown that no single VF is sufficient for UPEC to cause disease. Rather, a timely and stepwise expression of multiple, potentially redundant factors working in concert contributes to the successful establishment of a UTI (73).

Genes encoding these VFs have been shown to be located on the chromosome or plasmids. Some virulence factor genes (VFGs) may be exclusively chromosomal e.g. *pap* and *hly* (encoding P fimbriae and hemolysin, respectively), exclusively or principally plasmid-associated e.g. *iss* and *traT* (coding for outer membrane proteins associated with serum survival), or occurring in either location e.g. *afa/dra* (coding for Dr antigen-specific adhesin) (73). VFs may thus be transmitted vertically as well as horizontally.

Horizontal transfer may also occur with pathogenicity islands (PAIs). PAIs are large (>30 kb), unstable regions of chromosomally located DNA, that can be inserted or deleted from the genome. They contain bacterial virulence genes (e.g. genes for P fimbriae, S fimbriae, cytotoxic necrotizing factor 1, yersiniabactin) and can be characterized by: often having a G+C content different from the rest of the genome; frequently being associated with tRNA; often containing mobile genetic elements such as insertion sequences, transposons, integrases and origins of plasmid replication (42,50,51,132).

Adherence is thought to contribute to virulence by promoting colonization and by facilitating bacterial interactions between UPEC and host cells and matrix elements. UPEC produce a great variety of adhesins. The majority is fimbrial but some are amorphous fibers or capsule-like. Fimbrial adhesins are assembled from multiple subunits and the majority of fimbriae are heteropolymers (many non adhesive structural subunits and one adhesin molecule at the tip) and few are monopolymers (72). Broadly adhesins of UPEC can be categorized into mannose-sensitive and mannose-resistant adhesins and the latter subdivided further based on their receptor specificity and other characteristics (73). UPEC genomes can carry many fimbrial gene clusters, the majority of which are not well characterized, making the contribution of each fimbria type to UPEC virulence difficult to discern (10,186). Cross-talk among fimbriae operons within a bacterial cell, likely triggered by environmental cues, can result in a switch in expression from one fimbriae type to another, a process known as phase variation (58). Common adhesins in UPEC are type 1 fimbriae, P fimbriae, S fimbriae, F1C fimbriae, Dr adhesins, afimbrial adhesins, and the iron-regulated gene A homologue adhesins (76,120). Of these, type 1 fimbriae and P fimbriae are the most intensively studied fimbriae.

Iron is an essential factor for many cellular processes of UPEC; however, iron is a limiting micronutrient in the urinary tract (2). Consequently, UPEC have evolved multiple strategies for acquiring iron from the host. These include primarily siderophore-siderophore receptor systems but also utilization of heme. Siderophore systems recognized in UPEC include the enterobactin, salmochelin, yersiniabactin, and aerobactin siderophore systems (54). The siderophores are secreted low molecular weight molecules that have a high affinity for ferric (Fe3+) iron, which is insoluble and toxic as a free cation (146). Bacteria retrieve iron-bound siderophores through receptors that facilitate the transport of siderophore-iron complexes through the bacterial membrane and into the cytosol where the iron is released (146).

UPEC secrete a number of toxins, including alpha-hemolysin, cytotoxic necrotizing factor 1, cytolethal distending toxin and secreted vacuolating autotransporter toxin (76). These proteins have been demonstrated to variously modulate host signal pathways and cause abnormalities in host cell function or morphology, cell cycle arrest or cellular lysis (56,167,187).

UPEC exhibit a number of defenses against host antibacterial systems. Among these are capsule polysaccharides, which may interfere with phagocytosis and protect against complement-mediated opsonization or lysis and thus contribute to virulence (72). Group 2 and 3 capsule polysaccharides have been associated with UPEC and have been speculated to be important in UTI pathogenesis (76,120). Outer membrane proteins TraT and Iss have been shown to confer serum resistance by interfering with complement-mediated killing and have been associated with UPEC (72).

The recent advantages within molecular techniques including whole-genome sequencing and comparison of different pathogenic and commensal strains has revealed a great number of uncharacterized genes that may be potentially important VFs in UTI (107,145).

E. coli 83972 is a prototype ABU strain and has been widely studied. Studies of this strain has revealed that it harbors many UPEC associated genes encoding many of the above described VFs, e.g. type 1 fimbriae, P fimbriae, S fimbriae and F1C-fimbriae, alfa-hemolysin and multiple iron acquisition systems. Except for iron acquisition systems, the genes encoding all of these VFs have been found to be nonfunctional and in various states of genomic decay, suggesting that ABU strains may descend from more toxic and inflammatory UPEC isolates (96,97,153). Most ABU strains are found to adhere poorly to epithelial cells; however, those that adhere strongly have been shown to be unable to stimulate the epithelial cell immune response, and poor immune activation has been suggested to be a mechanism whereby ABU strains establish bacteriuria (108).

BIOFILM

In most ecological niches, bacterial interactions with a surface promote novel behaviors of planktonic cells leading to the development of structured and heterogeneous, matrix-encased bacterial communities known as biofilms (20). Biofilm bacteria demonstrate coordinated behavior with the formation of complex three-dimensional structures and functionally heterogeneous bacterial communities with differences in expression of surface molecules, antimicrobial resistance, nutrient utilization and VFs (20,52). Biofilm infections are important because they are characterized by increased tolerance to stress, antimicrobials and host immunological defenses (20). Biofilm is recognized as causing or exacerbating a number of infections including periodontitis, device-related infections, cystic fibrosis pneumonia, recurrent tonsillitis, and chronic otitis media (15,52). As described earlier biofilm has recently been suggested to play a possible role in the pathogenesis of RUTI, and it has been speculated to have a role in persistent colonization e.g. during ABU (Paper II) (1,108).

Several molecular and genetic studies concerning mechanisms involved in *E. coli* biofilm formation, and especially *E. coli* K12 biofilm formation, have led to identification of some factors of importance for biofilm formation. Flagella have been shown to contribute to the initial phase of approaching the surface although studies have shown it is not an absolute requirement for biofilm formation (3). In the further step of biofilm formation, attachment of bacteria to the surface has shown to include a number of fimbriae including type 1 fimbriae, curli fimbriae and conjugative pili (3). These fimbriae may also be involved in biofilm maturation; however, outer membrane proteins e.g. Antigen 43 (Ag43) have been shown to be involved in the bacterial interadhesion and biofilm architecture (17,161). The biofilm matrix is a complex milieu embedding the biofilm bacteria and determining mature biofilm architecture. It is essentially composed of water, but includes also proteins, nucleic acids, lipids/phospholipids, absorbed nutrients, metabolites, and exopolysaccaride polymers e.g. cellulose and colanic acid (3).

TREATMENT OF UTI

The resolution of symptoms and the sterilization of the urine is the aim for treating uncomplicated UTI. The spontaneous cure rate of symptoms and bacteriuria of lower UTI has been shown to be around 25% after 5-7 weeks, and around 80% of lower UTIs

have been shown to clear spontaneously within five months if left untreated (30,109). However, antimicrobials have shown to be superior to placebo regarding clinical and microbiological success in adult women with microbiologically confirmed acute uncomplicated cystitis (26,31).

Several factors should be considered when selecting drugs for the empiric treatment of uncomplicated lower UTI including: the antimicrobial spectrum of the common uropathogenic agents including the local prevalence of resistance among uropathogens, pharmacokinetics favoring infrequent dosing intervals, drug active at urinary pH values, drug excreted in the active form of drug by glomerular filtration into the urine, the duration of adequate levels of drug achieved in the urine (and renal tissue if pyelonephritis), effect of the drug on the fecal and vaginal flora; the potential for undesirable side effects, the cost of the treatment regimen, and public health concerns about resistance (41,60,66,128).

Sulfamethoxazol-trimethoprim (co-trimoxazole), flouroquinolones, beta-lactams, nitrofurantoin and fosfomycin are the most common antimicrobial agents used in the therapy of UTI. The trend toward increasing antimicrobial resistance among UPEC in many countries e.g. to beta-lactams and trimethoprim-sulfamethoxazole among uropathogens complicates the treatment of UTI and questions the treatment guidelines (47).

In Denmark the first line official recommended drug for the empiric treatment of uncomplicated lower UTI is currently pivmecillinam or sulfamethizole, given as a short duration regimen of three days therapy for both drugs (101). Sulfamethizole and mecillinam do not seem to have an effect on the IBCs in the murine models (92,162). Resistance towards sulfamethizole in *E. coli* from UTIs from primary health care in Denmark has been reported to be increasing and the level of resistance was around 38% in 2007 (18). The increasing resistance to sulfamethizole has raised concerns about its role as a first-line agent for UTI treatment. Resistance to mecillinam does not seem to increase and has been reported to be around 4% (18).

4. OBJECTIVE OF THE THESIS

As stated in the introduction and background *E. coli* is the major cause of community-acquired uncomplicated UTI in women and recurrence of UTIs occurs frequently and poses a major problem. However, despite the clinical significance of RUTIs caused by *E. coli* several questions remain regarding the pathogenesis, treatment and prevention of RUTI.

We aimed to study whether recurrence of UTI is caused by a relapse with the preceding *E. coli* or caused by a reinfection with a new *E. coli*. Furthermore we aimed to study bacterial characteristics of *E. coli* associated with recurrence of UTI in order to identify factors of importance for developing RUTI, especially to identify factors which may be used to predict a risk of RUTI and guide the handling of the patient at time of initial diagnosis. Finally, we aimed to study if *E. coli* causing recurrence are associated with bacterial characteristic proposed to be involved in the recent described theory of UPEC being an opportunistic intracellular pathogen utilizing a pathogenic cycle involving IBC.

5. STUDY POPULATION

The *E. coli* collection in this thesis is based on a subgroup of *E. coli* from a large prospective multicenter, randomized, double-blind, placebo-controlled comparative study of different dosing regimens for pivmecillinam (piv-amdinocillin) for community-acquired symptomatic lower UTI in women conducted in Umeå, Sweden 1995-1997. A total of 1162 women were randomized to one of three dosing regimens of pivmecillinam or placebo and were evaluated clinically and bacteriologically at the initial visit (day 1) and at two scheduled follow-up visits (day 8-10 and day 35-49) (Figure 3) (30,31).

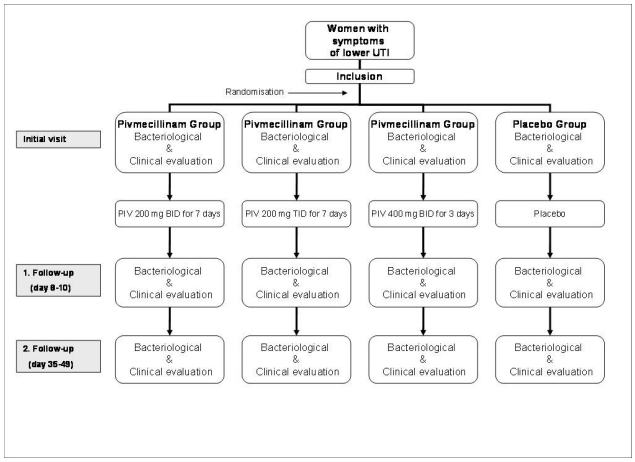

Figure 3
The *E. coli* collection was selected from a parent study. A placebo-controlled comparative study of three different dosing regimens for pivmecillinam for community-acquired symptomatic lower UTI.

For the present studies *E. coli* were selected from the all three pivmecillinam groups and the placebo group in the parent study (Figure 4):

- Primary infecting *E. coli* from women having significantly *E. coli* bacteriuria at one or both follow-up visits. The corresponding *E. coli* isolates from the follow-up visits of these women.
- Primary infecting *E. coli* from patients having a negative culture at both follow-up visits.

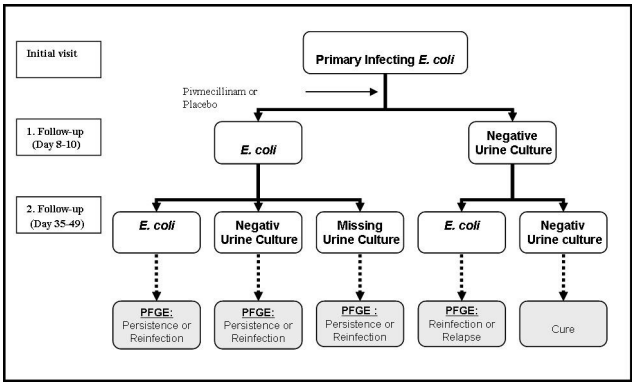

Figure 4
E. coli isolates selected for this thesis from the placebo group and the pivmecillinam group. According to culture results and PFGE all primary infecting *E. coli* were assigned into whether the initial infection was followed by cure, reinfection , persistence or relapse.

Table 2
Distribution of the Primary Infecting *E. coli* (n=236) according to the Course of Infection and Treatment Group

Culture result			Course of infection[a]		Pivmecillinam Treatment Groups n=155			Placebo Group n=81
Initial visit	1st Follow-up[b]	2nd Follow-up		n	400 mg BID[d] for 3 days	200 mg BID[d] for 7 days	200 mg TID[d] for 7 days	
E. coli	Negative	Negative	Cure	86	17	20	20	29
E. coli	Negative	Missing	Cure	0	0	0	0	0
E. coli	Same *E. coli*[e]	Same *E. coli*	Persistence	32	7	4	4	17
E. coli	Same *E. coli*	Negative	Persistence	22	3	1	1	17
E. coli	Same *E. coli*	Missing	Persistence	28	10	0	2	16
E. coli	Negative	Same *E. coli*	Relapse	47	17	16	13	1
E. coli	Negative	New *E. coli*[f]	Reinfection	15	4	5	5	1
E. coli	New *E. coli*	New *E. coli*	Reinfection	2	1	0	1	0
E. coli	New *E. coli*	Negative	Reinfection	2	1	0	1	0
E. coli	New *E. coli*	Missing	Reinfection	2	2	0	0	0
Total				236	62	46	47	81

[a] Course of infection according to PFGE results (22)
[b] 8-10 days post inclusion
[c] 35-49 days post inclusion
[d] BID, twice a day; TID three times a day
[e] Same *E. coli* as the primary infecting *E. coli* at inclusion according to PFGE (22)
[f] *E. coli* different from the primary infecting *E. coli* at inclusion according to PFGE (22)

In total 243 primary infecting *E. coli* along with corresponding *E. coli* from the two follow-up visits from 243 women in the parent study were selected for the present studies.

The 156 primary infecting *E. coli* from women having significantly E. coli bacteriuria at one or both follow-up visits and the corresponding *E. coli* from follow-up were subjected to pulsed-field gel electrophoresis (PFGE) typing. According to PFGE, primary infecting *E. coli* were assigned into whether the primary infecting *E. coli* was followed by persistence, relapse or reinfection (Paper I) (Figure 4).

A total of 236 primary infecting *E. coli*, representing *E. coli* that caused persistence or relapse during follow-up or *E. coli* that were followed by cure or reinfection during the follow-up were subjected to further characterization (Paper II and Paper III) (7 strains out of 243 were excluded: two were resistant to mecillinam; and four could not be assigned into whether the initial infection was followed by relapse, reinfection, or persistence; one showed mixed infection). Persistence, relapse, reinfection and cure were defined as depicted in table 2.

6. RESULTS AND DISCUSSION

RECURRENCE OF UTI – RELAPSE OR REINFECTION

For many years recurrences of UTI have been believed to be attributed mainly to a reinfection with a new strain and not a relapse with the same strain as in the preceding infection. This perception was primarily based on studies using phenotypically based typing methods as serotyping and biochemical typing (4,8,68,100,115,141,188). The shortcomings of phenotypically based typing methods have led to the development of typing methods based on the microbial genotype or DNA sequence which give better typeablity, reproducibility, and higher differentiation power (182). Studies applying newer techniques like ribotyping, dot blot hybridization patterns did also find RUTI primarily to be caused by a reinfection with a new strain (36,67,91). However, studies applying PFGE questioned the traditional prevailing perception of RUTI being caused by new strains and not the primary infecting strain and argue for RUTI being primarily caused by relapse (Paper I) (40,70,159).

In the pivmecillinam treatment group we found that 77% of the UTIs occurring at the second follow-up visit after a negative culture at first follow-up visit were due to a relapse with an *E. coli* identical to the primary infecting *E. coli*, and only 23% were due to a reinfection with a new strain. A total of 80% of those having *E. coli* at first follow-up and an *E. coli*, a negative urine culture or a missing culture at the second follow-up visit showed to have persistence with a strain identical to the primary infecting strain, and 15% had a UTI due to reinfection with at new strain. In the placebo group the majority had UTI at the first follow-up visit and an *E. coli*, a negative culture or a missing culture at second follow-up visit. PFGE showed that 96% of these UTIs were due to persistence with a strain identical to the primary infecting strain (Figure 4) (Paper I).

The few other studies that have applied PFGE all showed that the precentage of RUTI caused by a relapse was higher than the RUTI being caused by a reinfection, albeit with a lower percentage than found in our study (40,70,159). One of these studies was conducted in children. The two others differed from our study in many aspects e.g. a follow-up period of 6 months, and the populations studied were smaller.

We would expect that typing methods with a higher typeability and differentiation power used in recent studies would result in lower rates of same-strain recurrence, but this was not the case. However, it is difficult to compare the studies as they differ with respect to other factors than typing methods: study population, treatment regimens, urine sample collection method, criteria for significant bacteriuria, length of follow-up time etc. One recent study using serotyping of *E. coli* from women with RUTI showed that recurrence of the same serogroup within the first four months accounted for 50-81% of recurrences, whereas later recurrences with a different serogroup were more frequent than recurrence with the same serogroup (184).

Outbreaks of community-acquired UTI with a specific UPEC clone have been observed (110,133,139). However, PFGE re-

vealed that no clones were dominating our study population, and clonal dominance, which could be a result of a particular endemic UPEC strain in the community, could thus not explain the high frequency of relapses among the RUTIs in our study (Paper I). Multi locus sequence typing (MLST) is a useful and widely used genotyping tool. However, we chose to use PFGE since MLST does not seem to have a superior discriminatory ability for UPEC strains as opposed to PFGE typing (178).

The microbiological success rates for the parent study showed that pivmecillinam was an effective drug. However, it is not possible to exclude treatment failure as a possible explanation for finding UTI at follow-up caused by a relapse with the primary infecting *E. coli* (31). In the parent study the first follow-up visit were supposed to take place on day 10. The design of the study may however have permitted some of these first follow-up visits to have taken place on day 8, making it possible that a smaller fraction of the relapses in the pivmecillinam arms of seven days might represent suppressed persistence, and thus incorrectly inflated the rate of relapse with the same strain.

As with initial UTIs, it is widely thought that recurrence occurs through ascension and inoculation of the bladder lumen by a UPEC strain from the periurethral or fecal flora (120,192). However, it has been found that *E. coli* in the fecal flora may be decreased in number after antimicrobial treatment including treatment with pivmecillinam, and the fecal flora may therefore not constitute a stable reservoir (118,130,142,162,175). Furthermore application of antimicrobial ointment to the periurethral area did not significantly reduce the risk for RUTI (11). These observations, together with our finding that a high percentage of RUTIs is caused by the same strain as in the preceding UTI, argue for an alternative reservoir for *E. coli* causing RUTIs. This potential reservoir could be the recently discovered intracellularly located UPEC in the QIR in the murine bladder (87,126,162). Although not shown directly in the human bladder, the recent finding of IBCs and filamentous *E. coli* in the urine of women with cystitis supports the presence of this pathogenic cycle and the presence of the bladder as a reservoir for RUTI in humans (155). However, it was not shown whether intracellular bacteria persist and form QIRs, and contribute to RUTI. An older study showed that bacteria could be cultured from bladder tissue of women with recurrent UITs during periods with absence of bacteriuria. Currently the clinical implications of this very important and interesting finding of IBCs and filamentous *E. coli* in the urine of women with UTI are unknown and further studies are required.

PHYLOGENETIC GROUPS AND RUTI

The existence of distinct phylogenetic-groups within *E. coli* is well-acknowledged and currently there are four well-recognized phylogenetic groups (19,57,131). Several genetic methods have been developed to detect these groups including MLEE and ribotyping (19,57). Since of both these typing methods are complex and time-consuming we chose to use a simple and rapid triplex PCR based on the presence/absence of two genes (*chuA* and *yjaA*) and an anonymous DNA fragment (TSPE4.C2) (13). Using few genotypic features to discriminate between phylogenetic groups requires that the genes are not deleted from a phylogenetic group or horizontally transferred between phylogenetic groups, and that recombination in the genes is very rare. Whether the triplex method fulfills these criteria is not directly stated in article of Clermont et al. However, the method has been widely accepted and is frequently used (13).

The triplex PCR has recently been reevaluated and new interpretive criteria have been proposed. No studies to date have applied these criteria but we chose to use these (44). Applying these new criteria created a group of non-typeable (6%), reduced the number of *E. coli* belonging to phylogenetic group A, and finally caused a few *E. coli* to change phylogenetic group from D to B2 compared to the phylogenetic grouping created by the former criteria.

We found that phylogenetic group B2 was the predominant phylogenetic group among the primary infecting *E. coli*, followed by D, A and B1 (Paper II). The majority of studies concerning the phylogenetic grouping among UPEC have reported a similar distribution; however, one study reported a predominance of phylogenetic group A among UPEC strains in Russia (46,76,120,176). The distribution of phylogenetic groups among commensal *E. coli* has been shown to vary among geographically distinct human populations (21,165). The same is probably not true for UPEC, and the reported predominance of phylogenetic group A among UPEC might be explained by a study population with a greater number of clinical compromising conditions (21,46).

The primary infecting *E. coli* causing persistence or relapse at follow-up interestingly showed to be associated with phylogenetic group B2, whereas those followed by cure or reinfection was associated with phylogenetic group D (Paper II). This has not been reported before. One recent study of 15 children with recurrence showed an association between phylogenetic group B2 and recurrence in general but no association was found when limiting to recurrences with the same strain. However, the relevance of this study in relation to RUTI in women can be questioned since it describes RUTI in children of whom some also had compromising medical conditions (75). Our observation might be explained by our finding that the majority of VFGs were more prevalent among the B2 phylogenetic group than among the non-B2 phylogenetic group, as reflected in a higher aggregate VFG score among the B2 than among non-B2 isolates (Paper III).

The association between phylogenetic groups and course of infection was only seen in the pivmecillinam group and not in the placebo group. This might reflect the smaller size of the placebo group limiting the possibility of detecting differences in the distribution of phylogenetic groups. Alternatively it possible that mecillinam is better at preventing *E. coli* of phylogenetic A, B1 and D from causing persistence or relapse whereas it is less successful in preventing *E. coli* of phylogenetic group B2 from causing persistence or relapse.

E. COLI BIOFILM FORMATION AND RUTI

The finding of biofilm-like IBCs in the murine bladder and in the urine of women with UTI could indicate a possible role for biofilm formation in the pathogenesis of RUTI (1,155). A recent study of 43 women showed biofilm formation *in vitro* to be more frequent among *E. coli* causing relapse as opposed to *E. coli* associated with reinfection (169). In paper II it was shown that biofilm formation capacity *in vitro* was significantly higher in primary infecting *E. coli* causing persistence or relapse than in those being followed by cure or reinfection. It is unknown whether the genetic determinants that contribute to biofilm formation on abiotic surfaces in vitro also contribute to intracellular biofilm formation *in vivo*. Although extrapolation from biofilm formation i*n vitro* to a possible biofilm formation intracelluarly in the bladder is difficult, the results indicate that biofilm might play a role in RUTI and may support the presence of the IBC pathogenic pathway in humans. However, the observed differences in our study were

relatively small so further studies are required before biofilm formations *in vitro* can be used as a test to predict RUTI and to select a specific therapeutic approach.

The capacity to form biofilm showed to be significantly higher in *E. coli* belonging to phylogenetic group B2 than in *E. coli* belonging to phylogenetic group A or D (Paper II). This might reflect that phylogenetic group B2 harbors significantly more VFs than group A and D, and some of these VFs may be related to biofilm formation (Paper III) (81,120,121)

Biofilm formation capacity is strongly dependent on the applied growth medium and different *E. coli* respond differently to changing the growth medium (Paper II) (149). This may complicate the interpretations of biofilm results and comparisons between studies and may explain why we did not find the described differences in biofilm formation capacity expressed in all three used types of media.

Development of model systems for studying biofilm formation in vitro in UPEC under conditions mimicking the urinary tract has proven difficult (149). It would however improve future studies if such well-functioning model systems could be developed. Analysing biofilm in IBCs or under conditions mimicking IBC conditions would also be interesting.

VIRULENCE FACTORS OF E. COLI AND RUTI

A wide variety of VFs have been associated epidemiologically or experimentally (in vivo) with UPEC. We selected a broad array of 29 VFs, which from the literature could be suspected to play a potential role in the recurrence of UTI, and analyzed for the presence of genes coding for parts of these VFs. The prevalence of VFGs among the primary infecting *E. coli* was ranging form 0-98 %, and the median of the aggregate VFG score was found to be 13 (Paper III). The prevalence of these VFGs seems to correlate well with other studies of cystitis (76,78,81,120).

Paper III shows that the primary infecting *E. coli* causing persistence or relapse exhibited a significantly higher prevalence of many of the individual VFGs analyzed (adhesins (*sfa/focDE*, *papAH*), a biofilm related factor (*agn43*), iron uptake systems (*chuA*, *fyuA, iroN*), protectins (*kpsM II*, *kpsM II K2*), toxins (*hlyD* ,*cnf1*), a marker of pathogenicity island (*malX*), and a bacteriocin-like factor (*usp*)) compared to those followed by cure and reinfection. This was also reflected in a significantly higher aggregate VFG score among the primary infecting *E. coli* causing persistence or relapse than among primary infecting *E. coli* followed by cure or reinfection.

The observed correlation between VFGs and *E. coli* causing persistence or relapse was only observed in the pivmecillinam group and not in the placebo group. This might reflect the smaller size of the placebo group. Alternatively pivmecillinam was better at preventing *E. coli* without certain VFs from causing persistence or relapse, whereas it was less successful in preventing *E. coli* with these VFs from causing persistence or relapse thereby uncovering a difference in virulence that was not present during the natural course of UTI (placebo therapy).

Only few studies have looked at the prevalence of VFGs of UPEC in relation to RUTI in women and none of these have had a combination of a large study population and a broad spectrum of VFGs comparable to ours (40,78,169,172). One of the studies did not show any correlation between VF's and RUTI whereas the other studies taken together showed P fimbria, Dr-binding adhesin, iron-regulated gene A homologue adhesin, aerobactin siderophore receptor and yersiniabactin siderophore receptor to be associated to RUTI (40,78,169,172). Of these VFGs only P fimbriae and yersiniabactin siderophore receptor were found to be associated with persistence or relapse in our study. A recent study of RUTI in 15 children showed P fimbria, S fimbria, F1C fimbria, salmochelin siderophore receptor, and cytotoxic necrotizing factor 1 to be associated with recurrence of the same strain (75). We found the same VFGs to be associated with persistence or relapse in our study; however, the relevance of this study in relation to RUTI in women can be questioned since it describes RUTI in children of whom some also had compromising medical conditions (75).

Fimbriae and RUTI

P fimbriae have been associated with pyelonephritis and appear to have a role in mediating adherence to urothelial cells *in vivo* and in establishing inflammatory response during renal colonization (104,123). S fimbria and F1C fimbria have been associated with UTI but their functions are less well described (72,113,123). Johnson et al found *papA* and *papG* allele II to be associated with multiple same strain recurrences of UTI; however, this was based on only three cases of multiple same strain recurrences (78). Our results suggest that fimbriae like P, S and F1C fimbriae may play a role in the development of persistence or relapse of UTI (Paper III).

Type 1 fimbriae are encoded by the *fim* gene cluster. *FimH* encodes the adhesin at the tip of type 1 fimbriae, and the key receptor for this has been shown to be uroplakin 1a, which is abundantly expressed on the bladder (72). However, it may also bind to other host proteins e.g. α3 and β1 integrin subunits expressed on bladder (24). Although highly prevalent in commensal *E. coli* as well as in UPEC, type 1 fimbriae are considered to be one of the most important VFs involved in the establishment of a UTI mediating extracellular binding to the host urothelium and invasion (124). The invasion has been shown to be mediated by α3 and β1 integrins (24). Type 1 fimbriae have also been shown to be expressed in the IBCs (1). Recently, type 1 fimbriae was reported to be necessary for intracellular aggregation into IBCs, and the inability of UPEC to express type 1 fimbriae postinvasion showed to attenuate virulence in the murine UTI model (190). Although type 1 fimbriae may contribute to IBC formation and thus possibly the pathogenesis of RUTI, we did not find a correlation between type 1 fimbriae and persistence or relapse, since almost all *E. coli* contained *fimH* and expressed type 1 fimbriae phenotypically (Paper III).

Autotransporter Antigen 43 and RUTI

Ag43 an autotransporter has been shown to be associated with cell aggregation and biofilm formation in *E. coli* K12 (161,183). The role of Ag43 and the allelic variants of Ag43 have not been studied in clinical isolates; however, we found the presence of two allelic variants ($agn43a_{CFT073}$ and $agn43b_{CFT073}$) of the UPEC strain CFT073 to correlate with biofilm formation *in vitro* in the primary infecting *E. coli*, indicating a role for Ag43 in UPEC biofilm formation (Paper III). Ag43 has shown to be expressed by UPEC within IBC in the murine bladder suggesting that Ag43 may be involved in both abiotic biofilm development and biofilm like formation in living tissue (1). Recently it was shown that Ag43a (a variant of Ag43 in the UPEC strain CFT073) promotes long-term persistence in a mouse model. These findings together with our observation of *agn43* to be associated with persistence or relapse indicate that Ag43 may be important in the UPEC disease pathogenesis regarding RUTI.

Iron acquisition and RUTI

Genes involved in iron acquisition have shown to be associated with UPEC and a recent study using a quantitative metabolomic approach comparing coincident urinary and rectal *E. coli* from patients with recurrent UTI revealed that urinary *E. coli* exhibited significantly higher production of yersiniabactin and salmochelin, even among genotype-positive strains (54). One study of women with RUTI has found *fyuA* to be associated with relapse of UTI (169). A recent study in mice showed that components of ferric iron acquisition systems (e.g. *chuA* and *iroN*) are expressed at significantly higher levels in IBCs compared with the intestine and that *chuA* mutants produce significantly smaller IBCs compared with the wild type (148). This indicating that heme and siderophore associated iron play a key role in the development of IBCs (148). Another recent study, using an *in vitro* model supposed to mimic IBC formation using human immortalized urothelial cells, found *iroN* to be upregulated under intracellular conditions (5). Our results agree with these studies and indicate that iron uptake systems like heme, salmochelin and yersiniabactin siderophores might be important factors for development of persistence or relapse (Paper III).

Toxins and RUTI

The toxin alfa-hemolysin (HlyA) is able to lyse erythrocytes and nucleated host cells, a process that may facilitate the crossing of mucosal barriers, damage effector immune cells and release host nutrients and iron stores (72). It has been shown to evoke extensive shedding of the urothelium and induce hemorrhage in the bladder during the early stages of cystitis (167). At sublytic concentration it has been shown to modulate host signaling pathways and to trigger bladder cell apoptosis, exfoliation, and shedding of the uroepithelium (166,187). Bladder exfoliation could be speculated to facilitate the bacterial dissemination throughout the multiple layers of bladder epithelium and perhaps thus promote establishment of QIRs that may be reservoirs for RUTI. *HlyA* has also been shown to be expressed at higher levels in the IBCs than in the intestine, an observation that may also support the idea of HlyA playing a role in RUTI (148). In line with this study is a recent study that found *hlyA* to be upregulated under intracellular conditions in an *in vitro* model of IBC formation (5). Altogether these findings may indicate that HlyA has a role in the pathogenesis of RUTI, which is further supported by our observations showing *hlyD* and phenotypic expression of hemolysin to be associated with persistence or relapse (Paper III).

HlyA is often co-expressed with cytotoxic necrotizing factor 1 (CNF1), a toxin which activates the Rho GTPases leading to cytoskeleton reorganization, induction of membrane ruffling and modulation of inflammatory signaling pathways (105). CNF1 has been found to be associated with UPEC; however, the specific function is unclear and partly conflicting results have been published. A recent study suggested that CNF1 may promote bacterial attachment to and invasion of uroepithelial cells and induce an inflammatory response *in vitro*, and another study found CNF1 to promote UTI in a murine model (56,151). In contrast, one study did not find the mutant strain to be attenuated in a murine model (71). The capability of CNF1 to cause cytoskeleton reorganization could when unregulated be speculated to disrupt actin network and thus facilitate the intracellular proliferation of UPEC in QIR that has been described to be restricted by actin structures (25). One study found *cnf1* to be upregulated under intracellular conditions in an *in vitro* model of IBC formation and this agrees with our study finding *cnf1* to be associated with persistence or relapse (Paper III) (5).

Other virulence factors and RUTI

Recently *kpsD*, which is part of the operon coding for group 2 and 3 capsular polysaccharides, was found to be upregulated under intracelluar conditions in a in vitro model for IBCs indicating a possible role for these in IBCs formation (5). This observation agrees with our finding showing genes encoding group 2 capsular polysaccharides to be associated with persistence or relapse (Paper III).

Uropathogenic-specific protein (USP) proposed to be a bacteriocin has shown to enhance infectivity of *E. coli* in a murine model of UTI, but the exact role is unclear (191). However, our results indicate that it may play a role in persistence or relapse of UTI (Paper III).

VFs of UPEC may be encoded on PAIs. Recently it was shown that in addition to encoding virulence genes, pathogenicity islands may contribute to the overall fitness of CFT073 (106). We found *malX* coding for a pathogenicity island of CFT073 to be associated with persistence or relapse (Paper III). This could reflect the presence of known virulence genes like *hlyD*, but the association could also be mediated through novel VFs (107).

Flagellum-mediated motility has in ascending murine models of UTI been shown to facilitate the ascension of UPEC from the bladder to the kidneys and the dissemination within the host (102). Furthermore flagella has been shown to contribute to the fitness of UPEC during urinary tract colonization in a murine model (103,189). Recently it was shown that flagella may be involved in the entry of UPEC into renal collecting duct cells (138). *fliC* has in a recently described *in vitro* model supposed to mimic IBCs been shown to be upregulated under intracellular condition indicating a possible role for flagella in IBC formation. We found no association between flagella and RUTI, as all primary infecting *E. coli* showed motility in semisolid agar (Paper III).

VIRULENCE FACTOR PROFILE OF *E. COLI* AND RUTI

To visualize the distribution of VFGs and detect if certain VFGs or combinations of VFGs were associated with RUTI we made a cluster analysis. Primary infecting *E. coli* causing persistence or relapse and primary infecting *E. coli* that were followed by cure or reinfection did not segregate in separate clusters with a clear distinction between these two groups. However, in general primary infecting *E. coli* causing persistence or relapse were more prevalent than those followed by cure or reinfection among clusters characterised by a broad array of VFGs and consisting of strains belonging to phylogenetic group B2. In contrast, primary infecting *E. coli* followed by cure or reinfection were more prevalent than those causing persistence or relapse among clusters consisting of strains from phylogenetic group A, B1, D and the non-typeable group and characterized by the presence of *fimH* and very few scattered VFGs that differed among the strains, or the presence of *fimH* and *agn43/agn43K12* and very few scattered VF genes that differed among the strains (Paper III).

To conclude from the cluster analysis, we could not detect a specific combination of VFGs that could predict persistence or relapse, or cure or reinfection. Despite belonging to the same cluster and thus sharing a similar virulence profile, the *E. coli* could give rise to persistence or relapse as well as cure or reinfection. The outcome could thus not be explained entirely by the presence or absence of the VFGs examined, indicating that other factors must be in play. Other factors to consider could be VFs not

included in our analysis. A few clusters were characterized only by fimH and a few scattered VFGs that differed among the strains, indicating that we need to look at other putative VFs or search for new VFs. The recent technological advances have facilitated the search for novel virulence determinants that may play a role in the UTI pathogenesis. Studies comparing the genomes of UPEC strains like CFT073, 536, UTI 89 and F11, and comparing these with commensal strains have suggested the presence of a great number of UPEC specific hypothetical genes (107,145). Finally, the impact of the host, which could be biological or behavioral, is another factor to consider (35). Secretor status has been associated with RUTI and recently, abnormal vaginal immunological profiles and vaginal microbiota were found in women prone to UTI (94,164). Our study was not designed to examine the contribution of host factors. However, we found that the women experiencing persistence or relapse were significantly older than the women experiencing cure or reinfection, which could indicate that host factors may play a role.

It is important to notice that we only determined the presence of the VFGs, and apart from two genes (*fimH* and *hlyD*) we have no information as to whether these VFGs were actually expressed and fully functioning. We cannot exclude that the examined VFGs of the primary infecting *E. coli* actually could predict persistence or relapse and cure or reinfection if they had been subjected to an analysis based on the expression and functioning of these VFs.

TREATMENT LENGTH OF PIVMECILLINAM THERAPY AND THE RATE OF PERSISTENCE OR RELAPSE

The rate of recurrence at the second follow-up caused by relapse with the primary infecting *E. coli* in the pivmecillinam treatment group was found to be 81% (17/21) for the 400mg twice a day (BID) for three days, 76% (16/21) for the 200mg BID for seven days, and 72% (13/18) for the 200mg three times a day (TID) for seven days. The differences between these rates for relapse for the different dosing regimens of pivmecillinam were not significant. The observed linear trend for relapse among the different treatment groups, indicating a possible association between relapse rate and duration of therapy in combination with size of dose, was not significant (Paper I). However, when stratifying for phylogenetic groups and VFGs the treatment length of pivmecillinam seemed to influence the rate of persistence or relapse. A stratified analysis of persistence or relapse and cure or reinfection versus treatment duration and phylogenetic groups and VFGs for the pivmecillinam treatment group showed that a regimen of three days therapy for strains being positive for at least one of number of traits (phylogenetic group B2, *sfa/focDE*, *papAH*, *agn43*, *chuA, fyuA*, *iroN, kpsM II*, *kpsM II K2*, *cnf1*, *hlyD*, *ibeA*, *malX, usp*, and being hemolytic) gave a significantly higher prevalence of persistence or relapse as opposed to *E. coli* given three days therapy with absence of these traits or *E. coli* given seven days therapy irrespective these traits (Paper III).

The results concerning treatment length agree with a meta-analysis of duration of therapy of lower UTI recommending 5-7 days therapy also for other beta-lactam antimicrobials. It is further supported by a recent Cochrane review of duration of antibacterial treatment for uncomplicated UTI in women comparing the efficacy and safety of three-day antimicrobial therapy to multi-day therapy (five days or longer) on relief of symptoms and bacteriuria at short-term and long-term follow-up. It showed that for symptomatic failure rates, no difference between three-day and 5-10 day antimicrobial regimen was seen at short-term or long-term follow-up. In contrast, comparison of the bacteriological failure rates showed that three-day therapy was less effective than 5-10 day therapy for the short term and long-term follow-up. The advantages in long-term therapy in terms of bacteriological success appeared to be independent of the antimicrobials chosen for UTI treatment, including quinolones (116)

In terms of the IBCs pathogenic cycle, where none of the antimicrobials used for UTI have proven efficient in eradicating the IBCs, it is not evident how an extended treatment length could be beneficial. It could be speculated that a longer duration of antimicrobials in the urine to combat the circles of resurgence of UPEC from bladder might be an advantage. An extended length treatment regimen for treating uncomplicated UTI in women may however increase the risks of emergence of antimicrobial-resistant strains as well as increasing the prevalence of adverse effects (116). This approach should probably be reserved for a selected group of women with UTI. Results like ours could help identifying potential markers in UPEC that could be used in selecting a more differentiated and optimal treatment duration for uncomplicated UTI.

PHYLOGENETIC GROUPS AND ANTIMICROBIAL RESISTANCE

Susceptibility to ampicillin, chloramphenicol, streptomycin, sulfamethizole, trimethoprim and tetracycline was significantly associated with phylogenetic group B2, whereas resistance to these antimicrobials except for streptomycin was significantly associated with group A. Multidrug resistant (MDR, resistant to ≥ 3 antimicrobials) *E. coli* were significantly associated with group A and D (Paper II). These findings are in line with previous studies showing resistance to antimicrobials to be associated with non-B2 phylogenetic groups in UPEC, but our study is the first to show this in an area with low human-antimicrobial-consumption and low resistance rates (63,77,122).

Several theories regarding the association between phylogenetic groups and resistance phenotype have been put forward. One theory proposed the possibility of a greater exposure to selection pressure among non-B2 strains, which could occur in animal farms or another environmental source (74,173). Studies of meat products and wastewater sources have shown that antimicrobial drug-resistant *E. coli* may originate from these sources (53,83,111,160). Another theory addressed is a differential capability to acquire or retain antimicrobial resistance among B2 and non B2 strains. However, no studies have been able to show this; one study concluded that B2 and non-B2 strains exhibited a similar tendency to acquire and/or retain mobile genetic elements associated with resistance, and another study showed no greater ease of transition to resistance among non-B2 than among B2 strains (74,150).

Analyzing for the prevalence of eight plasmid families showed the presence of four plasmid families (IncF, IncI, IncH1 and IncP) among the primary infecting *E. coli*. The prevalence of these was in accordance with other studies and confirmed that IncF plasmids were by far the most prevalent (86,150). IncF was equally distributed among the phylogenetic groups, which is in contrast to a previous observation based on the ECOR strain collection showing an underrepresentation of IncF plasmids among strains of phylogenetic group A and B2 (7). However, the ECOR collection consists of *E. coli* from various sources and not exclusively from UTI. Interestingly, IncH1 and IncI were associated with phylogenetic group A and D, respectively, and *E. coli* belonging to phylogenetic group B2 showed a tendency towards having a lower aggregate content of plasmids than non-B2 *E. coli* (Paper II).

These findings could be suggested to contribute to the observed correlation between antimicrobial resistance and phylogenetic groups. Unfortunately our results are based on a relatively low number of strains and this interesting observation requires further confirmation.

Antimicrobial resistance and virulence factors

A number of studies have shown that quinolone and flouroquinolone-resistant UPEC typically appear to be less virulent than their susceptible counterparts, according to both clinical behavior and VF content (62,122,173). This reduction in virulence genes among resistant strains has also been reported for strains resistant to trimethoprim/sulfamehoxazole, chloramphnicol, and tetracycline (122,173). We found resistance to tetracycline, ampicillin, sulfamethizole and streptomycin to be associated with a lower prevalence of a number of VFGs (*sfa/focDE*, *agn43bCFT073*, *chuA*, *iroN, cnf1*, *hlyD, ibeA*, *malX*, *usp*) and a higher prevalence of other VFGs (*afa/draBC*, *agnaCFT073, iha*, *iutA*, *sat*). The aggregate VFG scores did not differ among the resistant and susceptible *E. coli* for these antimicrobials, but the individual VFGs showed the same associations with antimicrobial resistance or susceptibility as reported in the former studies (62,122,173).

The mechanism behind the association between resistance and reduced virulence is unclear. Several mechanisms behind these findings have been proposed and these studies have been focused on the association with quinolones and flourquinolones. A quinolone-induced loss of pathogenicity islands has been proposed and studies have both supported and contradicted this hypothesis (74,168). It has also been proposed that acquisition of quinolone and fluoroquinolone resistance may require a particular genetic background not strictly correlated with phylogenetic groups (136,177). Finally, it has also been has been suggested that resistant *E. coli* may derive from external reservoirs (an animal or environmental source) (74,173).

In line with the association between VFGs and antimicrobial susceptibility we found *E. coli* susceptible to sulfamethizole, tetracyclin or to all tested antimicrobials to have a significantly higher biofilm score than those resistant to sulfamethizole, tetracyclin or those not classified as fully susceptible (Paper II). This agrees with a recent study showing that biofilm forming *E. coli* are less resistant to nalidixic acids than those negative for biofilm formation in *E. coli* from cystitis, pyelonephritis and acute prostatitis (170). No studies have shown a correlation between biofilm formation and resistance to other antimicrobials.

METHODOLOGICAL CONSIDERATIONS

Some specific methodological considerations have been described in the preceding sections. However, a few general methodological considerations can be made to the studies.

We studied the association between a broad array of VFs and the course of infection, phylogenetic groups and antimicrobial resistance and thus made multiple comparisons between these factors. Applying multiple comparisons implies a risk for creating type 1 errors.

In the analyses we pooled the primary infecting *E. coli* causing persistence and relapse when analyzing data. This implies a risk for not detecting differences that could have been seen if the two groups where analyzed separately. However, considering the numbers of included *E. coli* and to avoid comparing many different groups we chose to combine the groups.

7. CONCLUSION

The main findings of this thesis are:

- Recurrence of UTI was mainly caused by a relapse with the preceding infecting *E. coli*.
- Persistence and relapse of UTI was associated with *E. coli* belonging to phylogenetic group B2, whereas cure and reinfection was associated with *E. coli* belonging to phylogenetic group D.
- The biofilm formation capacity was higher among *E. coli* strains causing persistence or relapse than among *E. coli* being followed by cure or reinfection.
- Relapse and persistence of UTI was associated with *E. coli* having a higher aggregate virulence score and higher prevalence of hemolysis and of many VFGs than *E. coli* followed by cure or reinfecton. These VFGs included adhesins (*sfa/focDE*, *papAH*), a biofilm related factor (*agn43*), iron uptake systems (*chuA*, *fyuA*, *iroN),* protectins (*kpsM II*, *kpsM II K2*), toxins (*cnf1*, *hlyD*), a marker of pathogenicity island (*malX*), and a bacteriocin-like factor (*usp*).
- No specific profile of VFGs could predict persistence or relapse of UTI.
- A regimen of three days pivmecillinam for *E. coli* belonging to phylogenetic group B2 and/or being positive for a least one of a number of VFGs gave a significantly higher prevalence of persistence or relapse as opposed to *E. coli* given three days therapy with absence of these traits or *E. coli* given seven days therapy irrespective of these traits.
- The majority of the VFGs were associated with phylogenetic group B2, whereas only few VFGs were more broadly distributed among the phylogenetic groups.
- Phylogenetic group B2 was associated with susceptibility to many of the tested antimicrobials, whereas phylogenetic group A was associated with resistance to many of these antimicrobials and with MDR strains.
- Plasmid distribution among the phylogenetic groups may contribute to the observed association between phylogenetic groups and antimicrobial resistance.
- Resistance to ampicillin, sulfamethizole, streptomycin and tetracyclin was associated with a lower prevalence of some VFGs and a higher prevalence of other VFGs but the aggregate VFG score did not differ among the susceptible and resistant strains.

E. coli causing persistence or relapse of UTI were associated with phylogenetic group B2, which showed to be associated with a high number of VFGs and with susceptibility to antimicrobials. Our finding of RUTI being mainly caused by relapse with the preceding *E. coli*, of biofilm formation capacity *in vitro* being associated with persistence or relapse, and of the biofilm related gene *agn43* being associated with persistence or relapse of UTI may support the recent proposed theory of an IBC pathogenic pathway of UTI and RUTI in women. Some of the VFGs found to be associated with persistence and relapse may have a potential role in the IBC pathogenic pathway. The VFGs associated with persistence or relapse could be potential target for prevention and treatment of UTI. Unfortunately, we were not able to detect a combination of VFGs that could be used to predict the outcome of infection. Other factors not considered may thus be important for the course of infection. However, we identified a number of traits that could be potential markers used to select a more differentiated and optimal treatment for a selected population of

women with symptomatic lower UTI in order to reduce the risk of persistence and relapses of UTI.

8. FUTURE PERSPECTIVES

The finding of IBCs and filamentous *E. coli* in the urine of women with UTI indicates that the IBC pathogenic pathway may exist in humans. However, it was not detected whether these observed IBCs persist and contribute to RUTI. Future studies are needed to address this issue. Examining bladder biopsies from women with UTI would be the optimal study; however this may prove difficult to conduct. Alternatively, it would be interesting to follow women with UTI over time to see if there is an association between presence of IBCs and filamentous bacteria in the urine and treatment response and relapse.

The recognition of UPEC as potential opportunistic intracellular pathogens rather than strictly extracellular organisms challenges our perception of the pathogenesis of UTI/RUTI and our treatment strategies. Our study identified certain VFGs that may be important for the development of persistence or relapse of UTI. To further explore the relevance of these findings it could be interesting see if these VFGs are expressed in the IBCs in a murine model and to study their role in the IBC pathogenic pathway in a murine model. Since we were not able to detect any specific combinations of VFGs that could predict persistence or relapse it could be interesting to investigate other putative VFs and novel UPEC related VFs preferentially among phylogenetic group B2 showing association with persistence and relapse.

Another extensive area to address is the contribution of the host. Identifying the host receptors and mechanisms of adherence and invasion of host cells might lead to therapeutic targets. Another field that could be addressed is the host defense including the expression of defensins.

Since none of the often used antimicrobials have proven efficient in eradicating IBCs it would be interesting to study new potential drugs or maybe combinations of drugs in a murine UTI model. This could be an antimicrobial acting in conjunction with another therapeutic adjuvant e.g. an antimicrobial combined with an other drug that could induce epithelial exfoliation thereby expelling bacteria from intracellular locations (126). It could also be interesting to pursue our finding of the possible influence of treatment length on the relapse rate, when statifying for VFs and phylogenetic groups. These studies could initially be done in a murine UTI model and maybe later extended to humans using the approach of looking for IBCs and filamentous bacteria in the urine as done by Rosen et al (155).

Finally another interesting field for future studies could be to find potential markers of *E. coli* from the urine of women with symptomatic lower UTI that could predict a risk of proceeding to bacteriamia and sepsis.

We have tried to answer some questions regarding the *E. coli* causing RUTI. However, many questions regarding the pathogenesis and treatment remain. Pursuing some of the above raised ideas could facilitate the development of diagnostic tools that can be used in selecting the optimal therapeutic approach at the time of initial diagnosis, and facilitate the development of new drugs and regimens for prevention and treatment of RUTI.

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10. ABBREVIATIONS

ABU	Asymptomatic bacteriuria
BID	Twice a day
CFU	Colony-forming unit
ExPEC	Extraintestinal pathogenic Escherichia coli
IBC	Intracellular bacterial community
MDR	Multidrug resistant
MLST	Multi locus sequence typing
MLEE	Multi locus enzyme electrophoresis
PAIs	Pathogenicity islands
PFGE	Pulsed-field gel electrophoresis
RUTI	Recurrent urinary tract infection
TID	Three times a day
UPEC	Uropathogenic Escherichia coli
UTI	Urinary tract infection
VF	Virulence factor
VFG	Virulence factor gene
QIR	Quiescent intracellular reservoir

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